University of Veterinary Medicine, Budapest
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BEHAVIOUR, MORPHOLOGY AND ECOLOGICAL CONSTRAINS IN BUTTERFLIES

PhD thesis
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Summary

There is a complex network between insect pollinators and the plants they visit. These connections are shaped by co-evolutionary processes. The key to these relationships is that plants offer rewards (generally nectar and pollen as food) for their visitors in exchange for fertilisation (pollination). Lepidopterans can be important pollinators: more than 140 thousand butterfly and moth species are assumed flower visitors, potentially pollinator species. Butterflies use their highly specialised mouthpart, the proboscis, to suck up nectar. The evolution of the complex plant-pollinator relationships is driven partially through the proboscis – corolla length ratio.

Our primary goals were to investigate floral traits influencing foraging behaviour in Clouded Apollo butterflies, and the role of proboscis length in flower choice.

First, we investigated if the Clouded Apollo butterflies' nectar plant choice was influenced by the importance of insect pollination, amount of nectar reward, flower abundance, colour, structure and the year of the observation. We found that the main driver of flower visitation was the abundance of plant species, whereas colour and structure had minor effects. Species composition, and the relative abundances of insect-pollinated plant species differed in two nearby habitats, where we conducted the field studies, and these resulted in slightly different results.

Second, we reviewed available methods estimating proboscis length in Lepidoptera. We found a vast range of techniques for preparing and measuring proboscis length. In many papers, proboscis length was measured in dead specimens, whereas in a few studies in live butterflies, while several papers did not clarify if measurements were taken on dead or live specimens. We found that the reviewed articles had not disclosed detailed descriptions of the applied procedures. We provided recommendations on reporting methodology including description of the preparation and measurement procedures as well descriptive statistics. Then, we developed methods to measure proboscis length in live butterflies and corolla lengths *in situ* for long-corolla forbs. These methods are non-invasive, both preventing the loss of the measured individuals and through this, bias in nectar plant availability due to measurements. We found these methods repeatable, sufficiently accurate and easy to apply to a relatively large sample.

Third, we found individual and annual variation in Clouded Apollo proboscis length and the corolla lengths of its most visited nectar resources during five consecutive years. We found that individual proboscis length might be related to nectar plant choice in natural circumstances in a flower visitor species not specialised to a single nectar plant. However this relationship was not consistent across years and plant species.

Taken together, Clouded Apollos' nectar plant choice is influenced by multiple traits. The connection between nectar plant choice, lepidopteran and floral traits may change

considerably from year to year, indicating that long term studies are mandatory to describe and understand visit patterns.

Összefoglaló

A növényeket és rovar beporzóikat bonyolult kapcsolatrendszerek fogják össze. Az egyes kapcsolatokat koevolúciós folyamatok formálják. Ezen kapcsolatok mozgatórugója, hogy a növények valamilyen jutalmat (általában nektárt és pollent, mint élelmet) kínálnak látogatóinknak megporzásukért cserébe. A lepkék fontos beporzók lehetnek: több mint 140 ezer lepkefajról feltételezhető, hogy virágokat látogatnak és potenciálisan be is porozzák azokat. A lepkék erősen specializálódott szájszervüket, a pödörnyelvüket használják a virágok nektárjának felszívására. A növény-beporzó kapcsolatok evolúciója részben a viráglátogatók nyelvhosszának és a látogatott virágok kehelyhosszának arányán keresztül alakul.

Elsődleges célunk a kis Apolló-lepkék táplálkozási viselkedését befolyásoló növényi tulajdonságok vizsgálata volt, valamint a pödörnyelv szerepének értékelése a nektárnövény-választásban.

Először azt vizsgáltuk, hogyan befolyásolja a kis Apollók nektárnövény-választását a rovar beporzás jelentősége, a nektár mennyisége, a virág színe, típusa, illetve növényfaj gyakorisága, valamint a megfigyelés éve. Azt találtuk, hogy a virággyakoriságnak jelentős szerepe volt a választásban, míg a színnek és típusnak kisebb. A növények fajösszetétele, valamint relatív gyakoriságuk különbözött a két vizsgált helyszínen, ami kissé eltérő eredményhez vezetett.

Másodjára áttekintettük az elérhető pödörnyelv-mérési módszereket. Sokféle preparálási és mérési módot találtunk. Sok vizsgálat elpusztult egyedek pödörnyelvét mérte, néhány élő példányokét, számos közlemény nem tisztázta a mért állatok állapotát. Az átnézett cikkek sok esetben nem közöltek részletes leírást az alkalmazott módszerekről. Ajánlásokat fogalmaztunk meg az alkalmazott mérési módszerek és a leíró statisztikák közlésével kapcsolatban. Kifejlesztettük saját módszereinket, amellyel élő lepkék pödörnyelvét, illetve *in situ* kehelyhosszokat lehet mérni. E módszerek nem invazívak, így elkerülhető a mért egyedek pusztulása és a nektárnövény-kínálat mérések miatt bekövetkező változása. Mindkét módszer megfelelően pontos és könnyen kivitelezhető nagy minták esetén is, ismételhetőek, és élő példányok mérésére alkalmasak.

Harmadjára egyedi és éves változatosságot találtunk a kis Apollók nyelvhosszában, valamint a leggyakrabban látogatott növényfajok kehelyhosszúságában, öt egymást követő év során. Azt találtuk, hogy az egyedi pödörnyelvhossz természetes körülmények között összefüggésben lehet a nektárnövény-választással egy olyan lepkefaj esetében, amelyik nem specializálódott egy adott nektárnövényfajra, de ez a kapcsolat nem volt állandó évek és növényfajok között.

Összefoglalva, a kis Apolló-lepkék nektárnövény-választását egyszerre több tulajdonság befolyásolja. A kapcsolat, a nektárnövény-választás, a növény- és a

lepkejellegek évről-évre változhatnak, jelezve, hogy hosszútávú vizsgálatok szükségesek mind a mintázatok leíráshoz, mind a megértésükhöz.

Note to the reader

Chapter One is the translation of an article published in Hungarian, Chapters Two and Three were published in English. Upon the reviewers recommendations, we slightly modified the texts of the original publications in this thesis. However, most of the original texts are presented here verbatim, although we used standard formatting throughout the thesis, e.g. numbering of the figures and tables, scientific names, listing.

Yellow highlight means modification from the text submitted to the first review.

General introduction

Plant-pollinator relationships are diverse networks, and they play a particularly important role in terrestrial ecosystems. The key to these relationships is that plants offer rewards (generally food) for their visitors in exchange for fertilisation (pollination) (Alexandersson and Johnson, 2002; Filella et al., 2013).

The evolution of these complex relationships is driven partially through the proboscis – corolla length ratio (Liang et al., 2021; Martins and Johnson, 2007; Pauw et al., 2009). Darwin had a hypothesis on specialised plant-pollinator co-evolution: the orchid *Angraecum sesquipedalia* with an extremely long corolla should have had a pollinator with a proboscis exceeding corolla length. Darwin predicted it had to be a sphingid moth, identified decades later as *Xanthopan praedicta* (referred to as *Xanthopan morganii praedicta* by Arditti et al., 2012). However, when both parties are generalists, the explanation of their relationships may not be that simple. Indeed, many flower visitors are supposed to be generalists: they visit many flowering plant species when foraging, and they may pollinate their flowers. Most animal-pollinated plants are also generalists, since it is risky to entrust fertilisation on a single pollinator species (Willmer, 2011). Although specialised pollinators' morphological adaptation to their nectar sources through co-evolutionary processes is well-studied (e.g. (Bauder et al., 2015, 2011; Wasserthal, 1997)), to our best knowledge, similar investigations for generalist pollinators are missing. Variation in plants' characteristics – traits that bait their pollinators (e.g. longer or shorter corollas) – leads to resource-partitioning among pollinators (Johnson, 1986; Rodríguez-Gironés and Santamaría, 2007), a potential driver of the diversity of plant-pollinator relationships.

Pollinators' flower choice is determined by (i) floral traits such as nectar composition, flower structure and colour, the quantity of the available nectar, etc. (ii) pollinator traits, like the perception of the floral signals (e.g. visual ability, preference of scents (Filella et al., 2013; Ômura and Honda, 2005)) and other characteristics (such as learning ability (Arbulo et al., 2011; Broadhead & Raguso 2021; Dixit et al., 2020; Goulson, 1999; Goyret et al 2008; Goyret & Raguso 2006; Inoue and Yokoyama, 2006; May, 1992; Patiny, 2014) and (iii) the interaction between the two parties: e.g. phenological match, the abundance of the interacting species, the ratio of the pollinators' mouthpart and the flowers' corolla length (Agosta and Janzen, 2005; Alexandersson et al., 2002).

Importance of feeding and variability of feeding patterns

Out of the of cca. 352 thousand plant species, 87.5% are pollinated partially or wholly by animals (Ollerton, 2021; Ollerton et al., 2011) and 75% of the main crop species are animal-pollinated (Ollerton, 2021). Even in the marine plant *Thalassia testudinum*, although of terrestrial origin, pollen transfer by marine invertebrates is important in the absence of water-flow (van Tussenbroek et al., 2016). Three hundred and fifty thousand animal species are flower visitors and potentially pollinators, including cockroaches (Xiong et al., 2020), bugs, beetles, hymenopterans (ants, wasps, bees), flies, butterflies, birds and bats, (Ollerton, 2021; Willmer, 2011). Feeding has a vital role in the survival and reproductive success, ultimately in fitness (Stephens et al., 2007). The quantity and the quality of the consumed food influence reproductive success (May, 1992; Molleman et al., 2008). The nutrition preferred by flower visitors is mostly the flowers' pollen and nectar. Abiotic pollination requires pollen grains, but changes (e.g. moderate wind speeds) during the early phases of flowering-plant evolution favoured biotic pollination (Willmer, 2011). The benefits of biotic pollination over abiotic pollination are that it works in smaller isolated populations, pollen dispersal efficiency is increased, and self-fertilisation can be avoided (Willmer, 2011). Pollen is the male gametophyte, its major role is fertilisation. Biotic-pollinated plants offered pollen firstly as reward for their pollinator visitors (Willmer, 2011). Pollen grains contain proteins, starch, lipids, minerals, vitamins and water (Halmágyi and Keresztesi, 1991; Nicolson et al., 2007; Willmer, 2011). However, the plants' interest, fertilisation contrasts with the consumption of the male gametophyte. Pollen-only flowers have to protect some amount of their pollen stock from being eaten by the pollinator or have to produce two different kinds of anthers (Willmer, 2011). Later on in plant evolution, offering nectar became more widespread among species rather than offering pollen. The nectar is an aqueous solution of various sugars (Dreisig, 1995), and it contains small amounts of amino acids, fats and antioxidants (Alm et al., 1990; Baker and Baker, 1983). Plants need to be visited sequentially by the same visitor in order to get fertilised: they advertise themselves to their visitors (e.g. visual and olfactory signals), and they offer reward for pollination (e.g. nectar or pollen) (Willmer, 2011). Flowers with shorter nectar tube lengths contain smaller amount of nectar than longer ones (Lázaro et al., 2015) and shorter corolla is also associated with less sugar content (i.e. as mg sugar/flower) (Carvalheiro et al., 2014), thus their flower visitors have to visit more flowers to gain enough energy.

Most studies on insect pollinators are focused on bees due to their enormous economic role in agriculture. However, other insects, such as butterflies may also be important pollinators (Conner et al., 1995; Johnson and Bond, 1994; Ollerton, 2021; Wardhaugh, 2015). More than 140 thousand butterfly and moth species are assumed flower visitors, potentially pollinator species (Ollerton, 2021). The majority of adult butterflies consume nectar, but several species also feed on fruit, different kinds of plant saps, mud, animals' excretion and cadavers, blood and eye fluid of larger mammals (Erhardt and Mevi-Schütz, 2009; Hilgartner et al., 2007; Stang et al., 2009; Wardhaugh, 2015): from those resources they try to gain energy, amino acids and minerals. Different species prefer different flowers and nectars and choose among the available sources (Bąkowski and Boroń, 2005; Erhardt et al., 2009; Thomas and Schultz, 2016) to increase feeding efficiency (Corbet, 2000;

Goulson, 1999), ultimately, their fitness. Higher nectar sugar content enhanced the longevity of butterflies and attained higher fecundity (Hill, 1989), and the nectars' amino acid content increased male (Cahenzli and Erhardt, 2013) and female butterflies' fecundity (Mevi-Schütz and Erhardt, 2005). Furthermore, lepidopteran life-history is related to the range of the imago diet: monophagus caterpillars were found mostly in single-brooded species, the imago being relatively long-lived and the larvae exploiting species of food plants of undisturbed habitats (Hodgson, 1993). In contrast, species feeding on a wide range of plant species as larvae were also nectar feeding on a wide range of plant species as adults (Altermatt and Pearse, 2011).

Proboscis length

The ancestral lepidopteran proboscis can be traced back to the pair of small galeae, which is preserved in a few biting-chewing moth families (Krenn, 2019, 2010). However, mainly consuming nectars resulted in a highly specialised mouthpart in adult butterflies: the two galeae formed an elongated tube, the proboscis (Krenn, 2010), which is used for imbibing their liquid nutrition (May, 1992; Willmer, 2011). The dorsal part of the proboscis tip is functioning as a nanosponge, resulting in a strong capillary force on the fluids (Lee et al., 2014; Monaenkova et al., 2012). Fluid uptake is supported by the sucking pump in the head (Kornev et al., 2017; Krenn, 2010). Energy dissipation is found to be associated with the viscous drag of liquid moving through the proboscis or through the moving pump plunger, depending on the pump size and proboscis length ratio (Kornev et al., 2017).

The length of the proboscis is important, since butterflies are considered unable to suck up nectar with a proboscis shorter than the length of the corolla (Corbet, 2000; May, 1992; Pauw et al., 2009). Nectar can usually be found at the bottom of the corolla tube. Butterflies with long proboscis can feed both from deep and shallow flowers, but butterflies with short proboscis can only reach nectars hidden in shallower flowers (Corbet, 2000; Rodríguez-Gironés et al., 2007; Rodríguez-Gironés and Santamaría, 2006). With time, nectar may accumulate in the corolla, an opportunity for a short-proboscis insect to imbibe nectar (Vlašánková et al., 2017). A longer proboscis is beneficial because flowers with deeper corollas contain more nectar (Gómez et al., 2008; Inouye, 1980; Lázaro et al., 2015; Rodríguez-Gironés et al., 2007). However, short proboscis may also have an advantage: it is capable to imbibe more concentrated nectar than a longer proboscis (Kim et al., 2011; Willmer, 2011). Shallower flowers usually include more concentrated nectar, because of water loss by evaporation, thus the nectar’s energy-density and viscosity are higher than those found in longer corollas. The more concentrated, and in consequence, the more viscous the nectar is, the stronger force is required to suck it up with a long and narrow tube, and the longer the tube, the stronger the force needed (Kim et al., 2011).

There is selection on proboscis length: flowers with elongated corolla are increasing the chance of more effective pollination by a specialist flower visitor with a matching long mouthpart (Bauder et al., 2011; Rodríguez-Gironés et al., 2006). Multiple aspects of butterfly proboscis-corolla length ratio and the effectiveness of nectar feeding and pollination is summarised in Table 0. According to their proboscis lengths, pollinators may partition their resources (flowers with different corolla lengths). This can reduce competition for similar resources among different pollinators (Inouye, 1978). Flower visitors can be nectar thieves or robbers by eating the floral reward without pollination via piercing holes or using existing holes in the corolla, or crawling inside the wide nectar tubes with their tiny bodies (Carvalheiro et al., 2014), or foraging with a much longer tongue than the flowers' depth, thus avoiding contact with the anthers or the stigma (Fox et al., 2015).

Table 0 Advantages and disadvantages of long and short proboscis and corolla.

	Long corolla	Short corolla
Long proboscis	• Effective way of feeding (plus more nectar) and pollination (Gómez et al., 2008; Inouye, 1980; Lázaro et al., 2015; Rodríguez-Gironés et al., 2007). • Specialised, effective plant-pollinator relationship (Bauder et al., 2011; Ollerton, 2021; Rodríguez-Gironés et al., 2006; Stang et al., 2009). • Specialised relationships are optimal in stable a environment (Nilsson et al., 1985), otherwise there is a risk of losing that given nectar source or pollinator. • Longer corolla may decrease the evaporation of nectars (Witt et al., 2013). The more diluted nectar is easier to such up with longer proboscis (Kim et al., 2011). • Feeding efficiency from longer corolla is higher with long proboscis, than with shorter proboscis (Ranta, 1984; Ranta and Lundberg, 1980). • Longer handling time is required (i.e. harvesting less nectar per unit time) (Kunte, 2007).	• The more concentrated nectar is more difficult to imbibe because of higher viscosity (Kim et al., 2011). • Chance of nectar consumption without pollination (Erhardt et al., 2009; Fox et al., 2015).
Short proboscis	• Possible e.g. in cases of piercing or using a hole in the corolla in order to reach the nectar (Carvalheiro et al., 2014; Willmer, 2011). • Tiny insects may live within the flowers (Needham, 1948). • Actual corolla length may be an overestimate of the distance to nectar source (McCanna, 2004; Vlašánková et al., 2017). • Flowers with long corolla often act as nectar reservoirs and may accumulate high volumes of nectar, consequently, nectar levels maybe high in the corolla tube (Vlašánková et al., 2017).	• Easy way of feeding and get pollinated (Corbet, 2000; Rodríguez-Gironés et al., 2007, 2006). • Shorter corolla does not impede the evaporation of nectars (Witt et al., 2013), which result more concentrated, hence more energy dense nectar (Kim et al., 2011; Willmer, 2011). • Generalist flower visitors are not the most effective pollinators (Larsson, 2005). • Feeding efficiency from short corolla is higher with short proboscis, than with longer proboscis (Plowright and Plowright, 1997; Ranta, 1984; Ranta et al., 1980).

Proboscis length is influenced by many other factors besides the relationship with the corolla length, nectar concentration and the selection on these, described above. For example, the limited quantity and/or poor quality of larval nutrition may induce smaller adult body size, and a smaller adult may lose the ability to use specific resources (Boggs and Freeman, 2005).

In natural lepidopteran populations, individual-level variance in proboscis length is hardly investigated. Studying intraspecific variation may result in fine-scale, detailed data-sets in which novel patterns may be noticed, and it may also yield further insights on the mechanisms of resource use (Araújo et al., 2010). Studying mouthpart at the individual level alongside the observed flower visitation could reveal further details on individual resource use.

Major goals

Our main goal was to investigate the role of the proboscis length of a butterfly species in flower choice among the available nectar-yielding plant species.

Szigeti et al., 2018 found differences in flower visitation ratios and flower abundances among years and within flight periods in a Clouded Apollo butterfly population. Variability in flower abundance may impact the butterflies' flower choice: they choose among the available resources, and their nectar plant species choice is plastic (Szigeti, 2018). Vajna et al., (2020b; Chapter One) is about which floral traits (e.g. colour, structure, amount of nectar reward) determine the butterflies' choice of available nectar plant species. In this study, we listed the visited flower species, and analysed the floral traits influencing the butterflies' choices.

Besides the floral traits studied in Vajna et al., (2020b; Chapter One), corolla and proboscis length ratio may also influence the butterflies' choice and this can be one of the reasons why individuals of the same butterfly population may use different resources (Szigeti, 2018). Can the individual differences in proboscis length explain this difference? Since gauging proboscis length on live butterflies is not a daily routine, first we reviewed in Vajna et al., (2020a; Chapter Two) the literature on how lepideptorologists measured proboscis length. Then we provided a short description of our own measurement technique in Szigeti et al., (2020; Chapter Three).

We investigated how the proboscis length of an individual is related to foraging behaviour in Szigeti et al., (2020; Chapter Three). Specifically, we analysed how proboscis length was related to corolla length of the most visited nectar species in 2015. Proboscis length was measured at the individual level, whereas corolla length of a given species is its population level trait: in case of a flower visitation we know the proboscis length of the given

Clouded Apollo individual, but we did not know the corolla length of the observed flower, only its population distribution for that plant species.

As data accumulated, we extended these analyses to the following years with an updated corolla length measurement protocol, presented in Chapter Four. Multi-year studies of a natural population may provide a deeper insight into the natural processes in a changing environment than observations in just a single year (Lindenmayer et al., 2011; Werner et al., 2020). This is beneficial to understanding ecological processes and essential for species protection, habitat restoration and conservation in general (Clutton-Brock and Sheldon, 2010; Lindenmayer et al., 2012; Werner et al., 2020). In Chapter Four, we analysed the variability of the relationship between proboscis and corolla length during five consecutive years, as well as how proboscis length affected visitation of the most frequently visited flower species, with different ranges of corolla length.

The Clouded Apollo butterfly

To achieve these goals, we used the Clouded Apollo butterfly (*Parnassius mnemosyne* (Linnaeus, 1758); Lepidoptera: Papilionidae) as a model species. The Clouded Apollo is an ideal model for several reasons: (i) they spend much of their time on feeding (Konvička and Kuras, 1999; Szigeti, 2018; Vojnits and Ács, 2000), (ii) feeding adults can be easily observed (iii) their resight probability can be high in a suitable habitat, where they can be locally abundant (Konvička et al., 1999; Kuussaari et al., 2016), thus individual behaviour can be repeatedly observed, and (iv) the sexes can be easily distinguished in the field (Weiss, 1999).

Clouded Apollos occur from Europe to Central Asia in several habitats. In Hungary, its habitats are mostly colline and mountainous areas, where open grasslands are close to forests (Bálint et al., 2006; Ronkay, 1997). The size and number of their populations are decreasing in Europe (Kuussaari et al., 2007; Luoto et al., 2001; Settele et al., 2008; Weiss, 1999), and both its southern and northern European distribution borders shifted polewards during the second half of the 20th century (Parmesan et al., 1999). These reasons justify the legitimacy of its protection: Clouded Apollos are protected by the Bern Convention, and are listed in the IUCN Red List (van Swaay et al., 2010).

The eggs are overwintering (Bergström, 2005), in the early spring the caterpillars feed on *Corydalis* spp. (in Hungary *C. solida* and *C. cava* (Pecsenye, 2017; Vojnits et al., 2000)). Weather affects the speed of larval development (Välimäki and Itämies, 2005; Warren et al., 2001) and also the larval food plants' quantity and quality (Carroll et al., 2001; Pfeifer et al., 2006). Fitness is strongly influenced by environmental impacts in the larval stage, i.e. a caterpillar with poor food intake will probably develop to a smaller imago, compared to a well-fed conspecific (Boggs et al., 2005). The adults are emerging in late April, and their flight period lasts until early June to July in Hungary (Bálint et al., 2006; Ronkay, 1997). They have one generation per year.

Chapter One: Flower choice in Clouded Apollo butterflies (*Parnassius mnemosyne*)

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Introduction

Feeding determines the animals' survival and reproductive success (Stephens et al., 2007). The availability of food resources, its variability in time and space shapes its consumers' behaviour, survival, population size, and in consequence, the composition of the communities (Curtis et al., 2015; Steffan-Dewenter and Tscharntke, 1999; Stephens et al., 2007). Animals choose among food resources according to their nutritional needs and possibilities. Although several hypotheses explain the relationships between sources and foraging (Goulson, 1999; Pyke et al., 1977; Stephens et al., 2007; Wilson, 1998), several aspects of foraging are unknown, e.g. how animals choose among available resources. It is important to investigate which floral traits pollinators choose to understand plant-pollinator relationships. Flowering plants lure animals, e.g. bees, geckos, hummingbirds and bats (Willmer, 2011), to get fertilised, i.e. pollinated. The bait is food, e.g. pollen or nectar offered to the pollinators. Plant-pollinator relationships are essential because more than a quarter-million plant species' reproduction depends on animals, and this task is carried out by 130–300 thousand flower-visiting species (Willmer, 2011). It is often difficult to collect detailed data from most flower-visiting insects' feeding behaviour. Although some butterflies can be easily observed, and these could be appropriate models for resource-use studies providing good-quality data (Lebeau et al., 2016).

Caterpillars of butterflies and moths (Lepidoptera) are herbivorous; they can be mono-, oligo- and polyphagous (Schoonhoven et al., 2005). Single-brooded species inhabiting undisturbed habitats, with long-living imagines tend to be monophagous as larvae (Hodgson, 1993), while a polyphagous larval diet was associated with a wide range of nectar plants visited by the adults (Altermatt et al., 2011). The imagines of most species consume floral nectar, and they can be important pollinators of the visited nectar sources (Conner et al., 1995; Johnson et al., 1994; Wardhaugh, 2015), e.g. for Caryophyllaceae (Bloch et al., 2006; Jennersten, 1988), or other, economically important plant species (Abrol, 2012). Various nutrients acquired in the larval or the adult stage are rich in proteins, carbohydrates and minerals (Erhardt et al., 2009). The quality and the quantity of these nutrients may affect the butterflies' reproductive success and survival (Boggs, 1997; Cahenzli et al., 2013; Lebeau et al., 2016; Mevi-Schütz et al., 2005; O'Brien et al., 2004).

Adult butterflies require water, carbohydrates, amino acids, sterols, vitamins and minerals (Erhardt et al., 2009). The nectar produced by flowers contains water, and in different amounts, sugars (mainly sucrose, glucose and fructose), and in smaller quantities amino acids, fats, alkaloids, and antioxidants (e.g. ascorbic acid) (Abrol, 2012; Baker et al., 1983; Nicolson et al., 2007). The butterflies' reproductive success and survival may be influenced by the nectars' amino acid content (Cahenzli et al., 2013; Hill, 1989; Mevi-Schütz et al., 2005). The taste of the nectar is determined by its sugar and amino acid ratio; butterfly-pollinated flowers are mostly rich in sucrose (Baker et al., 1983; Erhardt, 1991; Erhardt et al., 2009). With increasing sugar concentration, the nectar's energy content is increasing, as well as its viscosity. With increasing viscosity, imbibing nectar becomes more difficult (Kim et al., 2011). the optimal nectar-sugar concentration is 20–45% for butterflies (Kim et al., 2011; Willmer, 2011). The composition and quantity of the produced nectar are different among plant species (Gilbert et al., 1991; Hicks et al., 2016; Nicolson et al., 2007; Willmer, 2011), it can be genetically determined, and various factors may influence it (Baker et al., 1983; Farkas et al., 2012; Nicolson et al., 2007). Although pollen contains protein, carbohydrates, water, oils, mineral salts and vitamins (Halmágyi et al., 1991; Nicolson et al., 2007; Willmer, 2011), it is not a significant food resource for butterflies. The ancestral chewing mouthpart developed into an elongated closed tube, the proboscis, the common mouthpart of modern butterflies (Glossata; (Krenn, 2010)). The proboscis is probably unfit for taking up granular materials, such as pollen (Erhardt et al., 2009; O'Brien et al., 2003). Only a few existing pollen-feeding butterfly species are known, like the Micropterigidae moths, bearing chewing mouthparts (Krenn, 2010).

Besides or instead of nectar, the imagines of several butterfly species feed on other food resources providing nutrients that can not be found or not in the necessary amounts in nectars; some species may also live in habitats, where nectar resources are scarce (Settele et al., 2008). Some species get sugar or ferments from plant sap (Knopp and Krenn, 2003; Ômura et al., 2008), others get ethanol and acetic acid from rotting fruit (Ômura et al., 2008), amino acids and nitrogen from excrements and carcasses (O'Brien et al., 2003), proteins and potassium from blood (Plotkin and Goddard, 2013), proteins and salts from eye fluids (Hilgartner et al., 2007; Plotkin et al., 2013), nitrogen (O'Brien et al., 2003) and dissolved minerals from puddle and mud (Erhardt et al., 2009; Hilgartner et al., 2007; Krenn, 2010; Krenn et al., 2001; Stang et al., 2009). Some other species live from nutrients accumulated in the larval stage and they do not feed as adults (Boggs et al., 2005; Erhardt et al., 2009; May, 1992; Willmer, 2011).

Butterflies choose from the nectar-source supply (Bąkowski et al., 2005; Erhardt et al., 2009; Thomas et al., 2016). They are capable adjusting to the dynamically changing resources (Blackiston et al., 2011; Hantson and Baz, 2011; Kandori and Ohsaki, 1996), and within short time periods, they feed on the same nectar plant species in a row (Erhardt et al., 2009; Goulson and Cory, 1993; Lewis, 1989). The choice is essential for the necessary nutrition intake, and the consecutive visits of the same nectar plant species can enhance the flower visitors' feeding efficiency. With recognising the resource and practising, the time to find the nectary is decreased (Goulson, 1999). The interest of nectar-producing, insect-pollinated plant species is repeated visitation since it ensures pollination (Andersson, 2003; Willmer, 2011), and in turn, seed-production. Consequently, pollinators influence nectar plant availability of the following years and the population sizes of their resources (Elzinga et al., 2007; Kunin, 1997; Mahoro, 2002; Nicolson et al., 2007). Species richness of plant communities influence the diversity of butterfly communities through food supply (Kitahara et al., 2008; Kubo et al., 2009; Wallisdevries et al., 2012).

The stability of the natural and agricultural ecosystems is endangered by the worldwide decline in the number of pollinators (Burkle et al., 2013; Potts et al., 2010). This decline is probably impacted by decreasing flower abundance and plant species richness caused by the current landscape use, habitat loss and fragmentation (Potts et al., 2010; Wallisdevries et al., 2012). Due to the tight connection between insect pollinators and their nectar plants, small changes (e.g. the number of the pollinator and/or plant individuals or the time-shift between flowering and pollinator presence) may significantly affect entire communities. The decline of the number of pollinators results in decreased pollination; fewer seeds are produced, thus, fewer plants develop in the following years (Hegland et al., 2009). If flowering and the pollinators' active periods are mismatched, pollination would fail, inducing lower reproductive success of the plants as well as malnutrition in pollinators (Hegland et al., 2009). Therefore, plant-pollinator relationships are considered vulnerable (Kearns and Inouye, 1993; Nilsson et al., 2013; Potts et al., 2010). Since several hundreds of thousands of plants' survival depend on the proper operation of plant-pollinator relationships and networks (Willmer, 2011), for conducting effective conservation strategies, it is essential to have detailed information on the status of protected species, their occurrences, population sizes, vulnerability, and ecological traits determining these (Dicks et al., 2013; New et al., 1995; Simberloff, 1998; Sutherland, 2000). In contrast, there is hardly any information on most species, including most protected insects' ecological requirements (New, 2012). Few studies investigate nectar plant choice based on field observations (Jennersten, 1984; Thomas et al., 2016) and the dynamically changing relationships between flower visitation and flower availability (Bąkowski et al., 2005; Pratt and Wiesenborn, 2009; Stefanescu, 1997; Szigeti et al., 2018). For bringing appropriate conservation strategies, detailed information about adult butterfly resource use would be necessary (Dennis, 2010; Dicks et al., 2013; New, 2012; Thomas et al., 2016). Studies on butterfly foraging behaviour may help us understand important plant-pollinator relationships, founding conservation strategies, and model systems; it may help us understand significant agricultural processes, such as pollination (Abrol, 2012; Albrecht et al., 2007).

Here we investigate (i) which nectar plant species are visited by Clouded Apollo butterflies *Parnassius mnemosyne* (Linnaeus, 1758; Lepidoptera: Papilionidae), and (ii) what floral traits influence their choice. Adults spend much time on feeding (Konvička et al., 1999; Szigeti, 2018; Vojnits et al., 2000). Feeding can be observed easily, and in suitable habitats, resight probability may be high (Konvička et al., 1999; Kuussaari et al., 2016), and the sexes are easy to distinguish (Weiss, 1999). Thus Clouded Apollos are ideal subjects to investigate population- and individual-level nectar plant choice and foraging behaviour.

Clouded Apollos are found in habitats where larval host plants occur together with open, sunny meadows rich in nectar plants (Konvička et al., 2006; Kuusemets et al., 2005; van Helsdingen et al., 1996; van Swaay et al., 2010; Weiss, 1999). In Hungary, this species is frequent in hills and mountains, and flies between late April and early June (Ronkay, 1997). The caterpillars feed only on *Corydalis* species. In Hungary, these are *C. cava,* and *C. solida* (Meglécz et al., 1997). Imagines visit different nectar sources in different habitats (Konvička et al., 2006, 2001; Kudrna and Seufert, 1991; Lara Ruiz, 2011; Pecsenye, 2017; van Helsdingen et al., 1996; Vojnits et al., 2000).

Clouded Apollos are protected under the Bern Convention and included in the Hungarian (KöM, 2001) and the European Red List (van Swaay et al., 2010). The number of populations, as well as population size, is decreasing (Cini et al., 2020; Kuussaari et al., 2007; Settele et al., 2008; Weiss, 1999). One driver of this decrease may be the replacement of deciduous forests, rich in geophytes, thus favourable to Clouded Apollos, to coniferous woods (Felton et al., 2010; Konvička et al., 1999). The southern and northern border of its European distribution range shifted polewards in the second half of the 20th century, possibly due to climate change (Parmesan et al., 1999). These unfavourable tendencies are likely to continue, habitats loss has been predicted (Schweiger et al., 2012; Wilson and Maclean, 2011), and the species might be extinct in Hungary in a few decades (Settele et al., 2008). Its efficient conservation requires information on its ecological needs, including foraging behaviour.

Methods

Location and period

We carried out field work at two meadows in the Visegrádi-hegység: at Leány-kúti rét (47°44’23.20”N, 19°03’33.42”E, 300 m a.s.l., 0.6 hectares) between 2009 and 2013 (5 years) and at Hegyesd (47°45’22.62”N, 19°02’49.54”E, 295 m a.s.l., 0.5 hectares) from 2014 to 2015 (2 years), from late April to early June. These two habitats are approximately 2 km from each other, separated by a closed forest. We did not observe any migration between these two habitats, although in open habitats, 3 km individual movements were recorded with mark-recapture (Á. Kőrösi, pers. comm.). The entire population is appropriate for detailed mark-resight studies with a few observers due to the small size of the habitat.

We monitored the Clouded Apollos every day except rainy and very cold days, when the butterflies were inactive (~ 12% of the flight period), approximately from 8 am. to 5 pm. at Leány-kúti rét, and from 9 am. at 6 pm. at Hegyesd. The differences are due to different exposure: in the afternoon, Leány-kúti rét was earlier in the shade when the butterflies became inactive.

Clouded Apollo sampling

We sampled butterflies with mark-resight. We monitored the entire meadow several times per day at a slow pace. Sampling started a few days before the expected flight period. We used constant, narrow paths during sampling to minimize trampling. We recorded all Clouded Apollos. We captured the unmarked specimens and marked them individually: they received a number (with a black permanent marker) on the ventral side of their hind wings as well as three colour dots (with edding® paint markers) on the apex of their front wings' ventral sides, where the wing is transparent, so the colour code is visible from both the ventral and dorsal sides. The marks wear out rarely during the butterflies' lifetime, thus the specimen can be identified without further capture with binoculars. We did not observe any modification in the butterflies' behaviour due to marking. When we observed an individual feeding, we recorded the colour code, sex, the time of the observation, and the visited nectar plant species.

Flower abundance sampling

We estimated flower abundance with scanning (Szigeti et al., 2016a) at Leány-kúti rét every 3 days (median: 3, range: 2–6 dependent on the weather), at Hegyesd also in every 3 days (median: 3, range: 1–5). Sampling was started on the second day of the flight period the latest and was finished at the earliest two days before the flight period ended. During the approximately one-hour long sampling, we walked through the entire meadow and listed all the flowering, insect-pollinated plant species; we estimated their abundance. Abundance categories were estimated for all forbs only for the opened, non-withered flowers for the entire meadow: 0: extremely rare, 1: rare, 2: more or less rare, 3: more or less frequent, 4: frequent, 5: extremely frequent. Abundance of a given species' flowers was estimated in relation to all the flowers in the habitat investigated. We tried to handle these as approximately equal-distanced categories. Sampling was carried out by JK and VS.

Floral traits

We collected floral traits from the BiolFlor database (Klotz et al., 2002), we assumed to be important from the Clouded Apollos' perspective, in order to understand their choice among nectar plant species observed during 7 years. These were (i) the importance of insect pollination (categorical: possible, rare, rule, often, always, unknown); (ii) the amount of nectar reward (categorical: none, little, present, plenty, unknown); (iii) flower structure (Kugler's flower type, categorical: lip flower, pollen and wind flowers, disk flower, stalk disk flower, flag blossom, flower head, other (we listed here 3 original categories, which includes 9 species, (Kugler, 1970)); (iv) flower colour (categorical: blue, brown, green, red, pink, purple [we used "purple" for flowers, which are described in the database as "violet" (N = 2)]), white, yellow. When we did not find information about a species, we used the traits that seemed the most likely or most frequent from similar species of that genus.

Data analysis

We were interested in which flower traits are related to flower visitation frequencies for each plant species to investigate which traits influence flower choice. We summed annual flower visits, and calculated flower visitation percentages for each forb. Individuals were observed several times, and we summarized the observations annually, not weighing with individuals. We calculated annual flower abundance medians per plant species. We used flower abundances as factors for the analyses.

Our response variable was the $\log_{10}(x + 0.1)$-transformed flower visitation ratio per plant species. Potential explanatory variables were flower abundance, year, the importance of insect pollination, amount of nectar reward, Kugler's flower structure, flower colour. We analysed the data of the two habitats separately. We selected important explanatory variables using random forests (Strobl et al., 2007). Then we used the most important explanatory variables in decision trees. Decision trees graphically visualise hierarchical relationships between the response and the explanatory variables (De'ath and Fabricius, 2000). The limitation of this method is that it can not take into account that several individuals are repeatedly present in the data set – as is our case. Our work is exploratory analysis; this should be taken into account by interpreting the results.

We made all analyses and the figures in the R 3.6.3 statistical environment (R Core Team, 2018). We used the „party 1.3-4" library for random forest and decision tree analyses (Hothorn et al., 2006).

Results

At Leány-kúti rét, we observed 524 individuals' 2676 flower visits in 5 years. At Hegyesd, 234 individuals' 2552 visits were observed in 2 years. Clouded Apollos chose among the available nectar plants: at Leány-kúti rét, we did not observe visits on 36 species among the 71 insect-pollinated forbs present, in case of 18 species, the visitation per species was lower than 1%, while butterflies visited 17 species in at least 1% or more in any year (list of species: Table A1.1). At Hegyesd, Clouded Apollos avoided 44 species out of 75, they fed less often than 1% on 24 species, and on 7 species we observed them in 1% or more of the visits (list of species: Table A1.1). Visitation ratios per flight periods changed a lot across plant species (min–max: 0–60.4% [Leány-kúti rét], 0–73.6% [Hegyesd]). Cumulative visitation ratios of the 4 most visited species were 76.4–84.8% at Leány-kúti rét, and 89.2–92.8 at Hegyesd (Table A1.1). Seven forbs were among the 4 most visited in different years at Leány-kúti rét: *Buglossoides purpurocaerulea* (we refer to it with its synonym *Aegonychon purpurea-coeruleum* in the original publication [World Flora Online, formerly: The Plant List]), *Dianthus giganteiformis subsp. pontederae*, *Fragaria viridis*, *Polygala comosa*, *Silene viscaria*, *Thymus odoratissimus, Trifolium montanum*. 5 species were included in the 4 annually most visited species at Hegyesd: *Ajuga genevensis, B. purpurocaerulea, D. giganteiformis, S. viscaria*, *Vicia cracca*. The visitation rates of the most visited species varied 36.5–60.4% at Leány-kúti rét and 70.3–73.6% at Hegyesd; the most visited species of Leány-kúti rét were *D. giganteiformis* and *S. viscaria*, whereas at Hegyesd the most visited species was *D. giganteiformis* (Table A1.1). Clouded Apollos visited less species at Hegyesd than at Leány-kúti rét (Table A1.2).

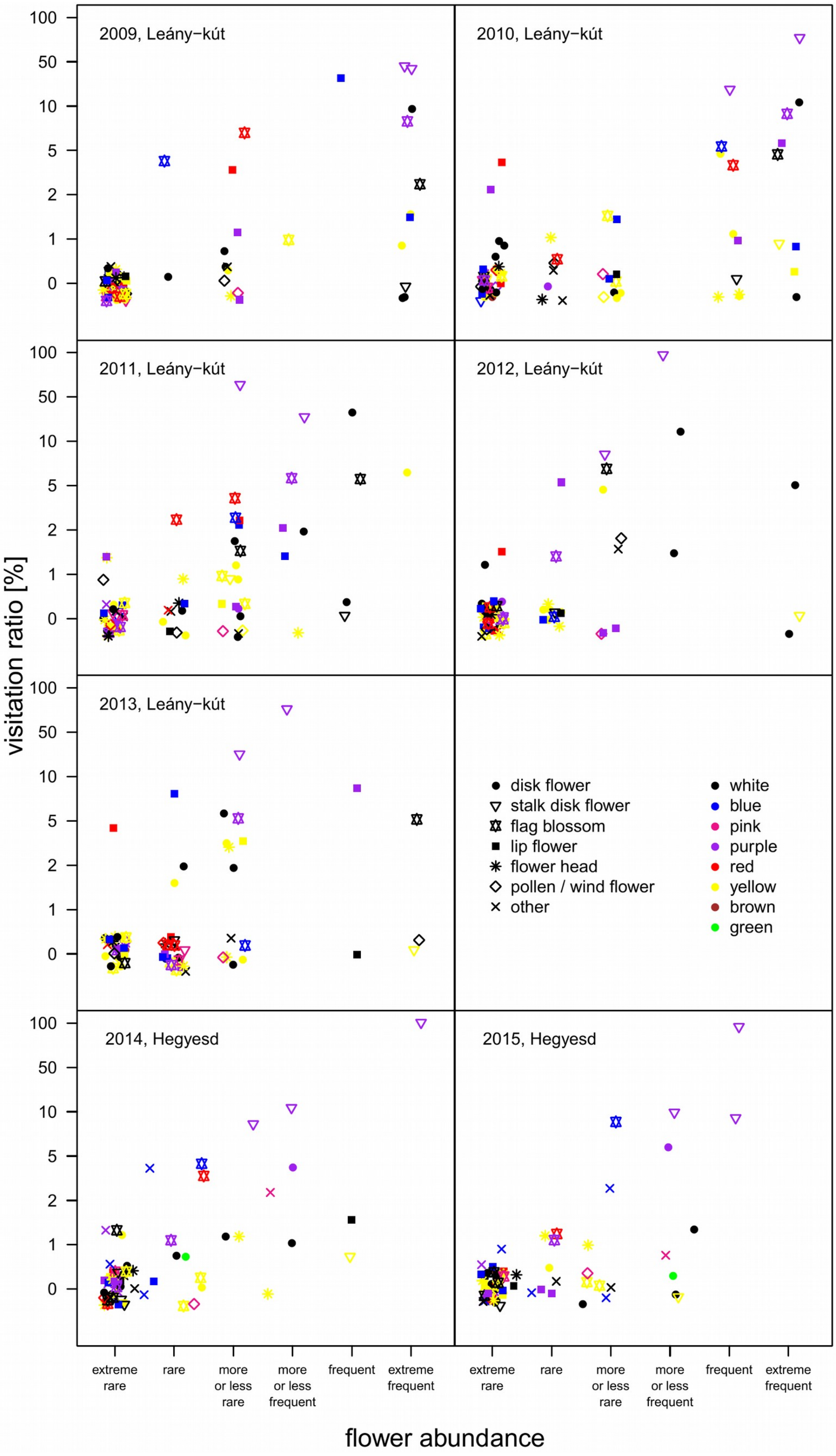

Figure 1.1 Clouded Apollo butterflies' annual flower visit ratios (%) and flower abundances at the two study sites. Each symbol represents a flower species. We jittered symbols on both axes for better visibility. The y-axis is log-10-scaled.

We found large variability between years and habitats in the occurrence of flowers and visitation ratios according to flower colours and flower structures (Figure 1.1). Nectar plant choice was influenced by flower abundance and flower colour in both meadows. Furthermore, at Leány-kúti rét flower structure and the importance of insect pollination also influenced visit rates (Figure 1.2). The hierarchy of the explanatory variables shows that Clouded Apollos visited nectar species that were not very rare, were purple or red at Leány-kúti rét. In contrast, at Hegyesd, blue, purple and red flowers were visited more often, than flowers with other colours. In the case of the species that were not very rare, the colour of the flower influenced choice significantly (Figure 1.3–4). At Leány-kúti rét, the not very rare purple or red flowers were visited more often if they had flag bossom or stalk disk flowers (Figure 1.3). Extremely rare species were visited more often if the importance of insect pollination was exclusive (BiolFlor category: “always”). Nectar plant choice at Hegyesd was not influenced by either flower structure, or the importance of insect pollination. We did not find association between the amount of nectar reward and floral choice and we did not find differences among years (Figure 1.3–4).

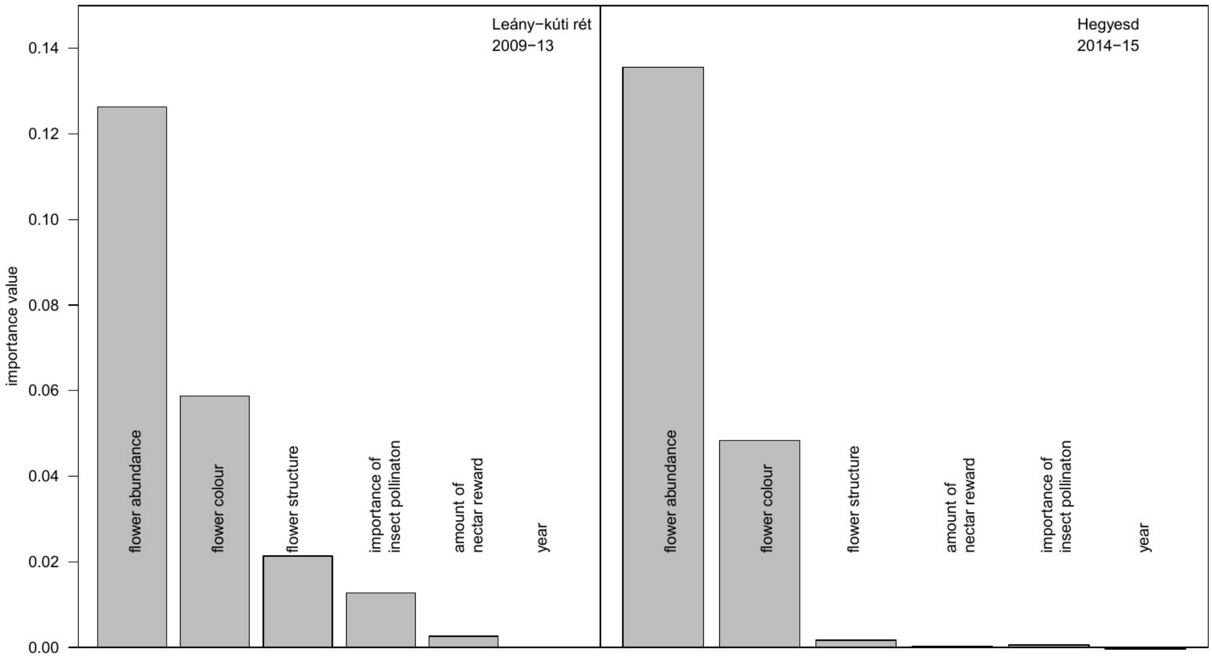

Figure 1.2 Explanatory variables’ importance in flower visit ratios according to the random forests, pooled for years at the two study sites.

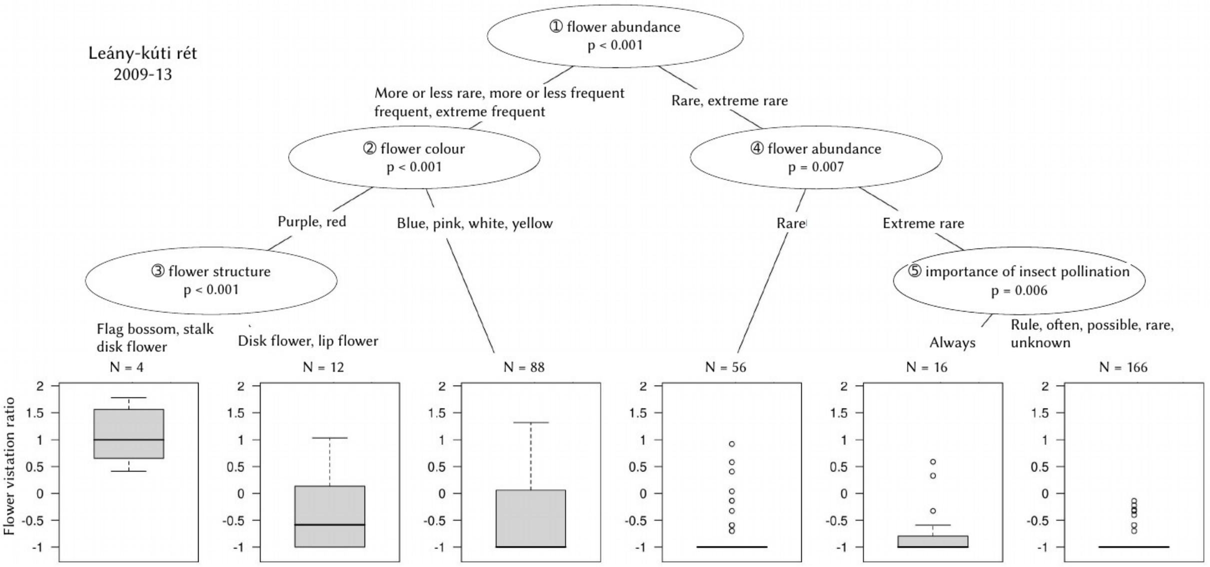

Figure 1.3 Floral traits influencing visit ratios at Leány-kúti rét, years pooled. The box-plots' y-axes are log-10-scaled.

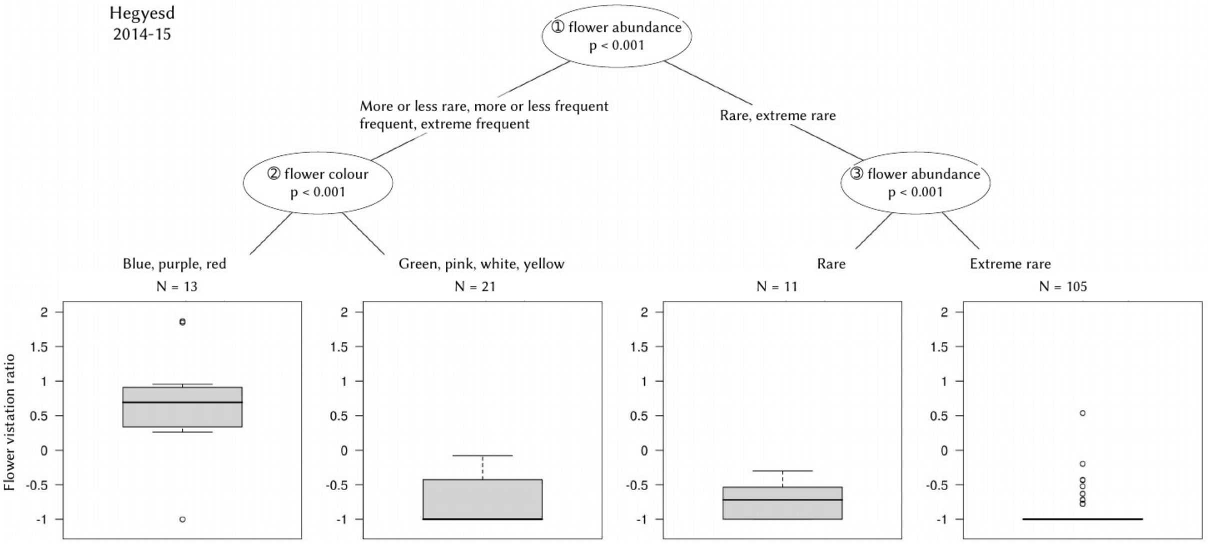

Figure 1.4 Floral traits influencing visit ratios at Hegyesd, years pooled. The box-plots' y-axes are log-10-scaled.

Discussion

We found 71 (Leány-kúti rét) and 75 (Hegyesd) insect-pollinated plant species during the 1–1.5 months of the Clouded Apollos' flight period. This is similar to the Central European meadows' species richness found in other studies (Binkenstein et al., 2013; Hejcman et al., 2013). Clouded Apollos visited fewer species at Hegyesd than at Leány-kúti rét, presumably because of the exceptionally high abundance of *D. giganteiformis* at Hegyesd. We observed more than 70% of the visits on this species. No species were visited at a similarly high ratio at Leány-kúti rét, where we observed more than 80% of the annual visits on 4 species combined, and none of these four species was visited at much higher rates than the other three. These patterns suggest strong diet choices, and the difference between the two meadows implies that choice depends on environmental factors. The most influential trait among all nectar plant traits on visitation was flower abundance, suggesting environmental dependence (Table A1.3). Flower abundance was the most influential trait on visits in both meadows, also implying environmental dependence.

Clouded Apollos' diet choice was influenced mainly by flower abundance. Flower colour also impacted choice, although colour was not found to be influencing visits on the extremely rare species (Figure 1.3–4). At Leány-kúti rét, the structure of red and purple flowers also influenced floral choice. In case of the extremely rare species, the importance of insect pollination was also influential (Figure 1.3). Neither flower structure nor the importance of insect pollination influenced visit rate at Hegyesd (Figure 1.4). Probably the high abundance of *D. giganteiformis* and its spatial homogeneity resulted in more than 70% visitation ratio; the other frequently visited species had also long, purple corollas that may cover the effect of floral structure and the importance of insect pollination. The importance of insect pollination is a proxy for the necessity of insects in fertilising a given plant, if obligatory, the plant needs to attract its flower visitors by colour, shape, scent, etc.; *see* pollination syndromes (Ollerton, 2021; Willmer, 2011), whereas if facultative, the plant probably will invest less into attractive traits. Although flower abundance was the most influencing trait on the Clouded Apollos' flower visits, a few abundant species, such as *Ornithogalum orthophyllum subsp. kochii* at Leány-kút rét were not visited at all, and the majority of the most visited species were only more or less abundant (Figure 1.1, Table A1.1). Clouded Apollos possibly visited most frequently the species the most beneficial in terms of net gain per energy investment and/or best quality nectar yields. A few abundant forbs were likely unsuitable nectar sources for Clouded Apollos.

Flower abundance is a rough estimate of the quantity of the available food because it provides information only on the number of available flowers rather than the quantity and quality of nectar. According to our analysis, nectar amount was not a statistically influencing variable (Figure 1.2). One possible reason is that we used a rough categorical variable extracted from a database instead of gauging values from the actual study site. We suppose that intensively sampled actual nectar amounts in the butterflies' habitat would associate with nectar plant choice (van Rijn and Wäckers, 2016), as better predictors of floral resource values compared to data retrieved from the literature, even if nectar amount and composition are influenced by several environmental factors (Baker et al., 1983; Farkas et al., 2012; Nicolson et al., 2007), possibly resulting in very high inter-specific variability (Witt et al., 1999). According to Szigeti (2018), among the frequently visited flower species except for *D. giganteiformis* and *S. viscaria*, a vast number of flowers contained no detectable nectar, suggesting that the butterflies tried to feed on flowers that contained no or just tiny amounts.

High-intensity on-the-spot nectar sampling would be mandatory to investigate this question (Szigeti, 2018).

The most visited species were blue, purple or red (Figure 1.3–4). Kudrna et al. (1991) found no colour preferences in Clouded Apollos, whereas van Swaay et al. (2010) observed that red and purple flowers, Vojnits et al. (2000), purple and mauve, Pecsenye (2017) that purple Lamiaceae had been frequently visited. Other butterfly species, such as the related *Parnassius smintheus* visited yellow (Matter et al., 2009), some Lycaenidae yellow and purple, other Lycaenidae mostly yellow flowers (Bąkowski et al., 2005; Pecsenye, 2017). The database's colour categories were based on human vision, and different sources used different colour names. The flowers' colours may depend on their habitat, so data from an international database is probably biased for a given habitat. For example, *Trifolium alpestre,* an abundant and visited species at Hegyesd, is red according to the Biolflor database, although we would describe it as purplish pink. Here we present a few examples for the colours with some species occurring at Leány-kúti rét and Hegyesd: white: *F. viridis, T. montanum,* blue*: A. genevensis, V. cracca,* pink*: T. odoratissimus,* purple*: B. purpurocaerulea, D. giganteiformis, S. viscaria,* red*: T. alpestre, T. pratense.* In contrast to humans, butterflies use the upper range of UV and the human-visible light range for visual perception (Arikawa, 2003), thus flower colours should be assessed according to the butterflies' visual abilities, at constant solar radiation with *in situ* spectrophotometric measurements. In contrast to the majority of the Apoidea (Peitsch et al., 1992), the vision of different butterfly species can be very different (Eguchi et al., 1982). To our best knowledge, the only species in the *Parnassius* genus is *P. glacialis* (Butler, 1866), in which vision has been investigated (Awata et al., 2010). This species is closely related to the Clouded Apollos (Michel et al., 2008; Omoto et al., 2009). Their vision might be similar, and may help drawing a more credible picture on how Clouded Apollos perceive colours of the visited flowers.

Nectar plant choice can not be fully explained only by flower abundance and colour. Several traits probably influence choice, and traits can be related to each other: structure and colour may not be independent: e.g. the blue-purple-red species have deep corollas and produce more nectar than the shallow (Galetto and Bernardello, 2004; Neumayer and Spaethe, 2007; Rodríguez-Gironés and Santamaría, 2004).

Similarly to other butterfly species (Baz, 2002; Hantson et al., 2011; Jennersten, 1984; Matter et al., 2009), Clouded Apollos visited different nectar plant species in different ratios. Changes in flower abundance in time and space could be important factors in shaping cross-population, individual-level, and annual flower visitation patterns, as well as within-habitat dietary differences among butterfly individuals within a single flight period (Szigeti et al., 2015). Adult diets may be different across habitats and populations. This is supported partially by our findings (Table A1.1): the two closely situated, similar-sized meadows' nectar sources differed from each other, as well as differences were found in the nectar sources in other publications (Konvička et al., 2006, 2001; Kudrna et al., 1991; Lara Ruiz, 2011; van Helsdingen et al., 1996; Vojnits et al., 2000). Many insect-pollinated plants of the two investigated habitats are the same; although their abundances are greatly different, e.g. *D. giganteiformis* was much less frequent at Leány-kúti rét than at Hegyesd. An individual-level tracking of the Clouded Apollos' feeding behaviour showed that handling times (the time period between landing on a flower and access to nectar) and feeding times on the flowers are similar across the most visited species (Gór, 2017). The profitabilities of these species (namely, the net nutrition and energy gain compared to the time and energy spent on finding the flower and imbibing its nectar) seems to be similar (Gór, 2017), although this study did not take into account that the probability of whether a flower contains nectar or not can be distinct in different species (Szigeti, 2018). Nor did it measure nectar quality and quantity that depend on various factors, e.g. age and sex of the flower, time of the day, weather and flower visitors activity prior to a focal visit (Erhardt et al., 2009; Kay et al., 1984). Similarly profitable species can be interchangeable sources for butterflies: it does not matter which one occurs during a given period and habitat, provided one is available. Based on the different nectar sources found in different Clouded Apollo habitats, the validity of our results is limited to the two meadows investigated. Expanding such studies to many more meadows with different climates would be necessary for a general conclusion.

For flower-visiting insects, it is worth choosing among the wide range of available supply, optimising their presence in time and space, and learning to handle different flowers, because the effect of diet on fitness is significant (Erhardt et al., 2009; Goulson, 1999; Stephens et al., 2007), thus individuals using their resources more effectively than others are in advantage. Butterflies have innate preferences, and are able to learn to recognise e.g. nectar composition (i.e. sugar and amino acid content), as well as flower colour and structure (Broadhead and Raguso, 2021; Goyret et al., 2008; Goyret and Raguso, 2006), that inform them on how to feed efficiently. Laboratory flower preference experiments manipulating floral abundance of multiple nectar species would be worthy complements to our studies in understanding the role of learning, as well as floral traits on choice.

Populations of Clouded Apollos are decreasing in Europe, except Estonia, where their distribution area increased (Liivamägi et al., 2013). It can be extinct from Hungary due to the changes in the landscape and the climate (Parmesan et al., 1999; Settele et al., 2008). The plant-pollinator relationships are particularly exposed to human impact, thus they are highly vulnerable (Burkle et al., 2013; Nilsson et al., 2013; Potts et al., 2010). We witnessed this vulnerability during fieldwork carried out in two small habitats. In one of them, at Leány-kúti rét, the population drastically decreased by 2013; that was why we shifted our studies to Hegyesd afterwards. Besides the number of individuals, the ratio of the feeding individuals among all individuals observed also decreased. In contrast, the number of available and visited plant species, as well as flower abundance, did not decrease significantly during the five years. One reason for this decline may be forest overgrowth (Szigeti, 2018), proceeding with the shrinkage of patches in the forest edge where *Corydalis solida*, the local larval food plant grows, and the caterpillars and the pupae may develop (Välimäki et al., 2005). Changes in forest management involving shifts in tree species composition may be another reason for geophyte decline (Konvička et al., 1999). The larvae of Clouded Apollos are monophagous (Meglécz et al., 1997), their diet as imagines is narrow since they visit just a few species frequently. These explain its protected status (Bern Convention; (van Swaay et al., 2010)). The presence of larval food plants is considered essential for the occurrence of a butterfly species in a specific habitat. However, other resources, including nectar plants, are also indispensable (Erhardt et al., 2009), because both food from the larval and the adult stage together are necessary to fulfil diet requirements, including the variety of protein, carbohydrate, and mineral sources (Erhardt et al., 2009). For Clouded Apollo butterflies, favourable habitats are patchy with alternating blotches of open meadows including nectar source and basking place, with forest where larval food plants can be found and eggs can be laid (Liivamägi et al., 2013; Szigeti et al., 2015; Välimäki et al., 2005). Patchiness involves vegetation heterogeneity and can play an essential role in maintaining of the necessary nectar sources' diversity: if some forbs, e.g. due to specific climatic conditions in some years do not grow sufficiently for the butterflies, other similarly valued sources may replace them. It would be worth exploring the factors jeopardising Clouded Apollos, eliminating them in the future by developing management strategies that facilitate population persistence. Moreover, it would be beneficial to create a species conservation plan like the one conceived for *Euphydryas maturna* (Linneaus, 1758) (Varga, 2006). It is essential for founding the necessary protective strategies by incorporating in-depth ecological knowledge on the species (Bergström, 2005; Luoto et al., 2001). We think that besides protecting larval food plants and their habitats, equal attention should be paid to the protection of nectar sources when designing species and habitat protection plans: Clouded Apollos require blue, purple or red flowers with moderately deep (8–12 mm) corollas.

We showed that Clouded Apollos chose among the available nectar plant species: they visited a few frequently, others occasionally, while avoiding several species even if they were abundant. The most important floral traits influencing choice were abundance, flower colour and structure, albeit factors not investigated here may also be crucial.

In Vajna et al., (2020b; Chapter One), we investigated the effect of many floral traits (colour, structure, abundance, amount of nectar reward and importance of insect pollination) on the nectar plant species choice of Clouded Apollo butterflies. Nevertheless, other floral traits can also impact butterfly or, in general, insect foraging behaviour.

The floral scent is a strong determining trait for flower visitor insects' nectar source choice; the flowers use volatile chemical attractants in order to advertise themselves to their potential pollinators (Filella et al., 2013; Ollerton, 2021; Willmer, 2011). Different flower-visiting insects prefer different scent components; for example, butterflies prefer benzenoids and terpenoids (Willmer, 2011). *Vanessa indica* butterflies decide on the flower visits primarily on floral colour and secondary on its scent (Ômura et al., 2005). Tangle-veined flies *Nemetrinus* spp. show constancy for scent and colour of the visited plants (Gao et al., 2020). Scents are perceived with the olfactory sensillas of the antennae (Willmer, 2011). Bees are considered fast and precise in learning many scents (Willmer, 2011).

Insects can also detect the electric field of flowers: bumblebees are able to discriminate the various patterns of floral electric fields (Clarke et al., 2013) by their mechanosensory hair, while honey bees use their antennae for this (Clarke et al., 2017). The difference between the negativity of the flowers' electric field and the positivity of the flower visitors' body enhances pollen transfer (Clarke et al., 2013). Flowers' electric potential changes after being pollinated, which can be perceived by its visitors (Clarke et al., 2013).

Floral humidity is also a clue for pollinators since humidity is linked to the flowers' nectar volume, as water evaporates from the nectar (von Arx et al., 2012). The hawkmoth *Hyles lineata* uses humidity cues and shows preference for elevated floral humidity (von Arx et al., 2012). Humidity is detected by hygroreceptive sensilla in *Manduca sexta* (von Arx et al., 2012).

Flower visiting insects are ectotherms: they rely on the warmth of the environment. Flowers can provide them a relatively warm shelter from rain and wind (Willmer, 2011). A flower with a relatively long corolla or with a bowl-shaped structure can offer a warm microclimate (Ollerton, 2021; Willmer, 2011). Mosquitoes are able to detect temperature via receptors in their antennae (Chapman and de Boer, 1995).

Corolla length is an important floral trait, which can affect feeding behaviour: nectar is on the bottom of the corolla, and visitors have to have an adequately long mouthpart in order to reach the nectar (Corbet, 2000; Rodríguez-Gironés et al., 2007). Corolla and proboscis length relationships were studied concerning flower visitation and nectar plant choice (Atwater, 2013; Dohzono et al., 2011; Huang and Fenster, 2007; Inouye, 1980; Martins and Johnson, 2013; Meerabai, 2013; Miller-Struttmann et al., 2015; Sultana et al., 2017; Tiple et al., 2009).

We studied the role of butterfly mouthpart (proboscis) length in nectar-flower choice in relation to the corolla length of the nectar plant species. One of the best nectar providers is Sticky Catchfly *Silene viscaria,* the nectar plant species with the longer corolla Clouded Apollos visited in the two studied meadows. Although abundant throughout the years we investigated diet choice at Leány-kúti rét (2009–13), this species was among the most frequently visited in some years and scarcely visited in others (Szigeti et al., 2018). One potential explanation for this pattern could be the proboscis length – corolla length mismatch if corolla and proboscis length ratios vary across years. Before testing this hypothesis, we reviewed available methods estimating proboscis length in Lepidoptera in Chapter Two.

Chapter Two: Measuring proboscis length in Lepidoptera: a review

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The final publication is available at Spinger: https://link.springer.com/article/10.1007/s00435-020-00507-z
We erroneously cited Zenker et al. (2011) in the following sentence of the original publication: “In several cases the type of the statistics, i.e., if a value was the mean or the median or a single value was not provided (Zenker et al. 2011; Meerabai 2012).”. Here we omitted this reference from the text, otherwise the verbatim copy of the original. A correction to our article has been published here: https://link.springer.com/article/10.1007/s00435-020-00514-0

Introduction

Animals’ mouthpart morphologies relate to their diet: variation in mouthpart shape and size across similarly feeding species determines which species exploits which part of a diet range if potential food is heterogeneous and mouthpart morphology associates with differences in exploitation efficiency. Morphological variance within foraging guilds may result in specialization and niche segregation (Conant, 1988; Freed et al., 1987; Pratt, 2005). Similarly, within-population variation results in differences in food exploitation and may also induce character displacement, and ultimately, speciation (Grant and Grant, 2003, 2014). Evidence for these effects was found in a wide range of taxa (fishes: (Sampaio et al., 2013); amphibians: (Amanat Behbahani et al., 2014); lizards: (Brecko et al., 2008); birds: (Herrel et al., 2005). In insects, the mouthparts’ structural adaptation to diet is striking e.g. when comparing blood- vs. nectar-feeding flies (Karolyi et al., 2014), or fruit-piercing moths vs. nectar-feeding Lepidoptera (Ramkumar et al., 2010; Srivastava and Bogawat, 1969).

In insect pollinators, mouthpart length in conjunction with corolla length variation may impact the choice between flowers and influence feeding efficiency, resource partitioning, and pollination. Hence, to understand interactions between plants and their pollinators, investigating morphological variation in nectarivorous insect mouthparts is essential (Borrell and Krenn, 2006; Harder, 1985; Haverkamp et al., 2016; Inouye, 1980; Johnson, 1986; Krenn et al., 2005; Pauw et al., 2009; Stang et al., 2009; Szigeti et al., 2020). Kearns et al., (1993) suggested that the most interesting measurable traits among morphological characters in flower-visiting insects is mouthpart length. On the one hand, nectarivores with short mouthparts are excluded from deep flowers due to size incompatibility. On the other hand, species with long mouthparts may be excluded from shallow flowers, due to high nectar viscosity, since viscous liquids require more strength to imbibe through longer tubes (Haverkamp et al., 2016; Johnson, 1986; Kim et al., 2011).

Nevertheless, generally used methodology on how to measure mouthpart length in pollinators is not available, especially for measuring live specimens, and recommendations for reliable measurements are still scarce (e.g. (Harder, 1982) for bees; similarly, widely accepted methods for investigating terrestrial arthropod morphology were missing (Moretti et al., 2017)). In contrast, several papers include well-detailed protocols that may be used as a sound basis of a general methodology (*see* e.g. (Bauder et al., 2014; Cariveau et al., 2016; Düster et al., 2018; Krenn et al., 2001). According to Kearns et al. (1993), proboscis length measurement seems to be relatively easy in insect pollinators. In fact there are a variety of procedures available. These require different amounts of research investment, likely yielding different results.

Documenting and measuring different anatomical traits, such as size and shape have been important parts of natural history ever since its early students (*see* e.g. (Aristotle, n.d.; Swainson and Richardson, 1831). Behavioural biologists, ecologists and taxonomists use a broad spectrum of morphological methods, they investigate organisms from different perspectives, thus morphometrics is not a coherent discipline (Daly, 1985; Wipfler et al., 2016). The number of studies quantitatively investigating anatomical shapes is still increasing and morphometric methods will probably remain important techniques in the near future (Adams and Otárola-Castillo, 2013; Wipfler et al., 2016). Recently, a vast range of studies applied morphometric methods, e.g. for classifying taxa (e.g. (Csősz and Fisher, 2015; Görföl et al., 2014; Peruzzi and Passalacqua, 2008); revealing morphological changes at different circumstances (e.g. (Kaliontzopoulou et al., 2010; Langerhans et al., 2004); looking for relationships between body size and reproductive success (e.g. (Berger et al., 2008; De León et al., 2012; Réale et al., 2009); investigating tiny surface structures for understanding functional mechanisms (Wipfler et al., 2016; Xue et al., 2016); and developing new measurements or analysis techniques (Adams et al., 2013; Bánszegi et al., 2014; Csősz et al., 2015; Stec et al., 2016). The range of devices and methods has been rapidly increasing (Muñoz-Muñoz and Perpiñán, 2010).

Data quality is a central concern for researchers (Garamszegi et al., 2009), and is determined by the characteristics of the measurements applied, including the practice and experience of the person performing the measurements. Although comprehensive norms and rules for precise measurement in morphometrics exist (Moretti et al., 2017; Muñoz-Muñoz et al., 2010; Stec et al., 2016; Van Hook et al., 2012; Walther and Moore, 2014), Ioannidis, (2018) suggest that a large part of the studies lack high measurement accuracy. The quality of morphological data depends on *preparation and measurement* techniques (Arnqvist and Mårtensson, 1998), and the followings are worth considering before taking measurements. First, different treatments and techniques during *specimen preparation* likely yield different results e.g. dried specimens lose their water content, thus their flexibility, and may become contracted to some degree compared to fresh individuals (Kearns et al., 1993; Knapp, 2012;

Moretti et al., 2017; Muñoz-Muñoz et al., 2010; Van Hook et al., 2012; Von Schiller and Solimini, 2005). Second, the morphological *landmarks* should be undoubtedly homologous for all measured individuals and/or species. Landmarks should be easily detectable and measurable, and similar across studies, in order to acquire repeatable measurements (Daly, 1985; Kouchi et al., 1999; Van Hook et al., 2012; Zelditch et al., 2004). However, accurate landmark definition is challenging in many cases, e.g. when the measured structures are flexible (Moretti et al., 2017; Muñoz-Muñoz et al., 2010; Yezerinac et al., 1992). Third, the quality of the results depends on measurement *resolution, accuracy and precision* (i.e. device quality and adequacy) (Harris and Smith, 2009; Ulijaszek and Kerr, 1999; Walther et al., 2014; Wolak et al., 2012; Zelditch et al., 2004). The potential error of the device and the skills of the measuring person limit measurement repeatability (Blackwell et al., 2006; García-Barros, 2015; Kearns et al., 1993; Van Hook et al., 2012; Zelditch et al., 2004). Furthermore, as measurement error increases, the chance to fail to detect biologically relevant differences among the investigated groups is also increasing (Yezerinac et al., 1992). Fourth, the power of analyses depends on *sample size* (Batterham and Atkinson, 2005; Cardini and Elton, 2007; Stec et al., 2016; Van Hook et al., 2012). Researchers' choice of sample size depends on the aim of the study, the population variability in the target variables, the effect size of interest and the confidence level needed (Cardini et al., 2015; Moretti et al., 2017; Van Hook et al., 2012). Sample size may be constrained by limited sampling opportunities or the number of available specimens, as well as by ethical issues. If researchers sample only a small part of a population, the potential error of measurement will increase considerably, even in case of random sampling, and in field ecology, true random sampling is nearly impossible. Fifth, if scientists are working with living organisms, they should take into account *ethical considerations* (Costello et al., 2016; Farnsworth and Rosovsky, 1993; Kugler, 1970). In small natural populations, removing specimens for measurements may alter population structure, thus collecting sufficient data to estimate population distribution using dead specimens may severely harm the population or is simply not feasible (Joint Committee for the Conservation of British Invertebrates, 2002). These not only constrain sample size but make some desired measurements to be avoided and the development of new measurement protocols mandatory (Moretti et al., 2017).

Our aim was to review and reveal the available proboscis length measurement methodologies for butterflies and moths (Lepidoptera). Glossatan Lepidoptera have long proboscides, specialised mouthparts evolved as an adaptation to imbibe floral nectar as a primary food resource at the adult stage in most species (Bauder et al., 2011; Erhardt et al., 2009; Krenn, 2019, 2000). Nectar consumption affects lifespan and fecundity (Cahenzli et al., 2013; O'Brien et al., 2004), and butterflies may choose the most rewarding among the available nectar plant species. This may ultimately result in resource partitioning and evolution (Erhardt et al., 2009; Thomas et al., 2016). Some species consume other resources, such as pollen, fruit and plant sap, mud and excrement, whereas several species do not feed as an imago (Erhardt et al., 2009). Lepidopteran proboscis is an ideal study organ to address plant-pollinator morphological compatibilities, since its length may be highly variable within a single population (Szigeti et al., 2020) and is an important predictor of resource-use (Bauder et al., 2011; Krenn, 2000). Here we present a methodological review on proboscis length measurements and we hope it can facilitate further mouthpart studies. Our focus is on how the authors performed measurements of lepidopteran proboscides, how accurate the measurements were, and how were these constrained by sampling effort. We also highlight challenges in measuring proboscis length, and we provide recommendations for future sampling, taking into account the five important points for appropriate measurements listed above.

Methods

Data sources

To review studies measuring proboscis length in Lepidoptera, we searched for research papers upon three groups of search terms: (i) “funct*”, “length”, “morpho*”, “size”; (ii) “galea”, “mouthpart”, “mouth-part”, “proboscis”, “tongue”; and (iii) “butterfly”, “lepidoptera”, “moth”. We used “and” operators between groups, “or” operator between keywords within groups and “*” denotes wildcards. We used the databases ISI Web of Science and Scopus, accessed on 2020-06-04. We found 420 papers and we selected 114, those presenting their own measurements of the total length of lepidopteran proboscis. We found 6 further papers by browsing the Internet and 15 from other articles’ reference lists. We included only research articles, we did not use books, book sections, or theses. All-together, we used 135 research articles, 126 were in English, 5 German, 2 French, 1 Portuguese and 1 Spanish (*see* references of the reviewed studies: List A2.1).

Extracted variables

We categorised the reviewed studies according to (i) the aim of the proboscis length measurement, (ii) the method of specimen preparation, and (iii) the method of proboscis length measurement (*see* raw data: Table A2.1).

If the title and the abstract were available in English, we counted the number of the important keywords (“galea”, “mouthpart”, “mouth-part”, “proboscis”, “tongue”) in both, then we calculated important keyword proportion: we divided keywords with the total number of the words in the title and the abstract. We used this *proportion keywords* variable as an estimate for the importance of proboscis length measurement in the given studies.

We extracted the following information from the articles for *proboscis preparation methodology*: (i) if live or dead specimens were measured; (ii) treatment of live specimens (i.e. immobilization); the methods applied on dead specimens: (iii) preservation; (iv) preparation on dead specimens before fixation, mostly flexibilisation; and (v) fixation.

We extracted the following details for *proboscis length measurement methodology*: (i) the state of proboscis when measured (coiled vs. uncoiled); (ii) landmarks used for measurements; (iii) magnifying devices (e.g. stereo-microscope); (iv) measurement devices (e.g. ruler, digital photograph) and (v) their resolution; (vi) the techniques for reading measurements (e.g. naked eye, software); (vii) and if the repeatability and/or accuracy of measurements were calculated. We also recorded if the authors had referred to other studies for the methods applied.

We extracted further numerical data: (i) the number of investigated species; (ii) the number of all measured individuals; (iii) the year of publication. Furthermore, we assessed the descriptive statistics on proboscis length given in the articles (e.g. mean, standard deviation, range; in some of the articles different statistics were provided for different species and we included all types of these statistics, *see* List A2.1).

In a few publications, the authors used multiple methods for measuring proboscis length, we present them all.

Data analysis

We present descriptive statistics of the extracted variables by providing median, minimum and maximum values, showing box-plots with individual data points and bar-plots. We analysed the following relationships between the variables characterising the measurements:

To investigate how the importance of proboscis length and the scrupulousness in presenting methodology are related, we correlated *proportion keywords* in the title and abstract to (i) the number of missing data (hereafter NA) in the description of the methodology in preparing specimens, (ii) the number of NA-s in measurement descriptions, (iii) resolution estimates for the devices, and (iv) the number of measured individuals. We calculated Kendall’s rank correlation coefficients.

We tested if shorter proboscides were measured more likely in dead, rather than live specimens, because we hypothesised that smaller species are more difficult to measure alive, since fragility increases with decreasing size. We built a mixed effect model, where the response variable was proboscis length and the explanatory variable was measurement condition (dead or alive), and the random factor was the study (Zuur et al., 2009).

We analysed all data in the R 3.4.4 statistical environment (R Core Team, 2018). We used the “lmerTest” 3.1-0 package (Kuznetsova et al., 2017) for the mixed effect model.

Results

We reviewed 135 studies on proboscis length measurements in Lepidoptera, published from 1924 to 2020 (*see* List A2.1, Figure A2.1). Proboscis length was provided only as supplementary descriptive data in 6 cases. The aim of the rest of the studies were to investigate body size relationships in 12, mouthpart morphology and functionality in 33, foraging behaviour strategies in 39, proboscis length and flower depth relationships in 41, pollination effectiveness in 57 and pollinator communities in 19 cases. Many studies (59) had several aims (*see* raw data: Table A2.1).

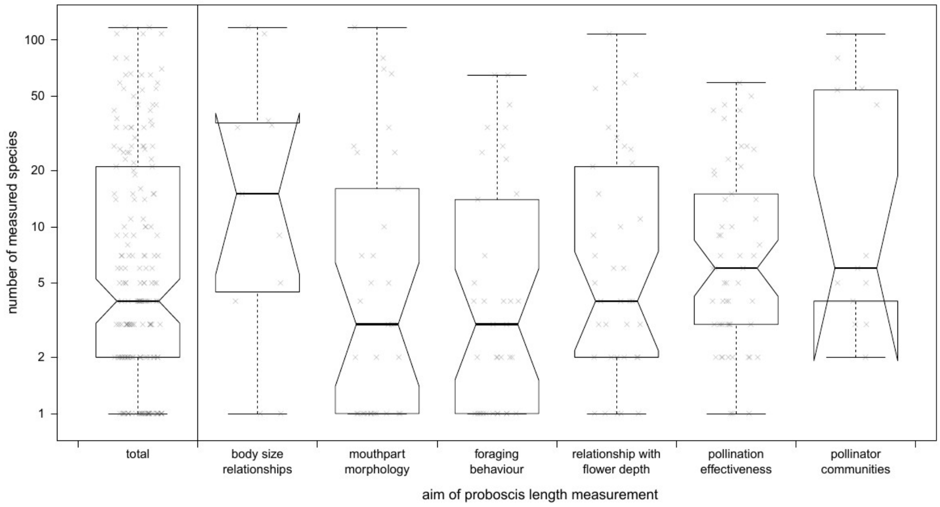

Figure 2.1 Number of the measured species according to the aim of the study. Box-plots show medians, lower and upper quartiles, notches show 95% confidence intervals for medians, whiskers include the range of distribution without outliers. Grey × symbols represent publications, and are jittered on the horizontal axis for better visibility. Vertical axes are log10-scaled.

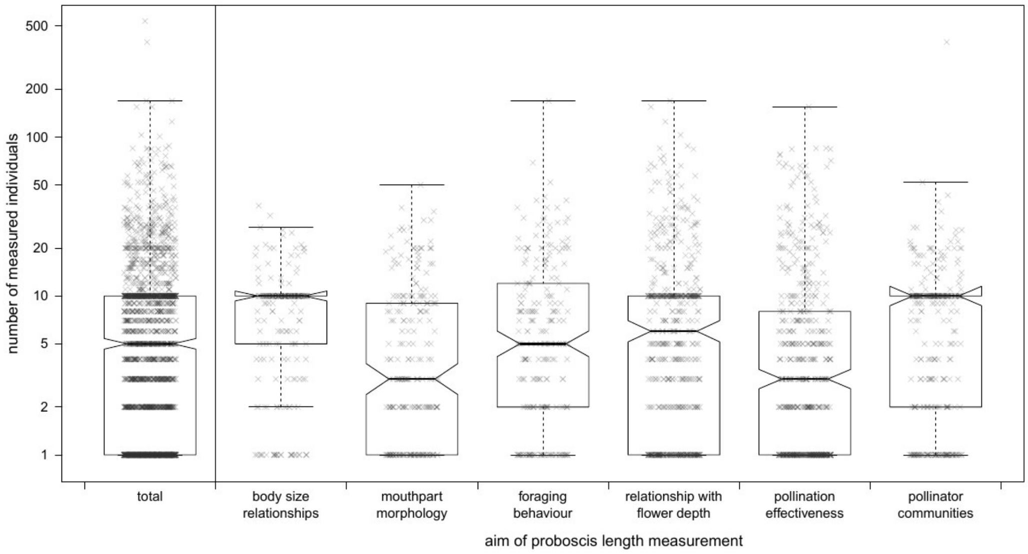

Figure 2.2 Number of the measured individuals according to the aim of the study. Box-plots show medians, lower and upper quartiles, notches show 95% confidence intervals for

medians, whiskers include the range of distribution without outliers. Grey × symbols represent publications, and are jittered on the horizontal axis for better visibility. Vertical axes are log10-scaled.

Authors investigated 1–117 (median: 5; Figure 2.1) lepidopteran species per study. Proboscis length was measured on 4 (median; range: 1–537; Figure 2.2) individuals per species. Altogether, data were published on 13816 specimens of 977 species. Per-species proboscis length means varied between 0.35–280.0 (median: 16) mm, the range of standard deviations was 0.01–32.0 (median: 1.5) mm, and the CV% was between 0.08–122.6% (median: 6.1%). The number of measured species and the number of measured individuals were different for the different aims of the studies (Figure 2.1 & 2.2).

Various methods were used for preparation and for measurements. Many papers fell short to provide a thorough description of the procedures applied, and the reasons why the given methods had been used were often unexplained. For example, 61 (43.3%) studies provided no information on proboscis preparation, 67 (47.5%) on proboscis measurements.

Proboscis preparation

Proboscis lengths were measured in live specimens in 18 (12.8%) studies. Although these specimens probably survived being measured, this was not stated. Sixty-two (44.0%) studies reported using dead specimens, including voucher specimens, and animals captured in their natural habitats or reared and then killed for the measurements. Sixty-one studies (43.3%) did not provide information on whether the specimens were live or dead.

Live individuals were immobilised for measurements by one of the following methods: cooling, anaesthetising with CO_2 or ethyl acetate, stabilizing with styrofoam, fixing on glass slide, fixing on plastic board with clips, or covering with a meshed bag. In some cases, researchers did not use any interventions, or they did not state if live specimens were sedated. Dead specimens were either immediately measured after being killed or they were stored as dried or frozen or kept in ethanol (70% or 95%; *see* Table A2.1). Preparation of the dead specimens before fixation was mostly flexibilisation, e.g. soaking in 20–50% lactic acid, 5–10% KOH, diluted household cleaner, distilled water, or kept in a relaxing chamber (for further details *see* Table A2.1). In some cases, the solutions were heated, in others, specimens were just soaked for a couple of days. In 30 (58.4%) publications the authors did not state using any kind of preparation on dead specimens. The prepared specimens would be mounted on microscope slides, stubs, sample holders, or spreading boards and embedded by different methods (polyvinyl-lactophenol, DPX mountan, Entellan, Canada balsam, Euparal, graphite adhesive tape, transparent tape, etc. *see* Table A2.1). We also found one study where samples for measurements were frozen with liquid nitrogen.

Proboscis measurements

Proboscides were uncoiled in 51 (35.2%) of the measurements. In 3 cases proboscides were not uncoiled, and in further cases this information was not provided. *Magnifying devices* were stereo microscopes, light microscopes, scanning electron microscopes or 3D X-ray technology either or not combined with digital photography. Digital cameras by themselves were also used. *Measurement devices* were analogue and digital callipers, rulers, millimetre scales, ocular micrometers, drawings (drawing tubes and digitalising tablets) and photographs (Figure 2.3). The *techniques for reading measurements* were the naked eye, digital interfaces, or software (Figure 2.3; *see* details in Table A2.1). The applied image analysis software were Amira; AxioVision; Image Tool for Windows; ImageJ; Imaris; Microsoft PowerPoint; Olympus Soft Imaging Solution and Sigma Scan Scientific Measurement System. Only 6 publications referred to other publications for the applied measurement techniques.

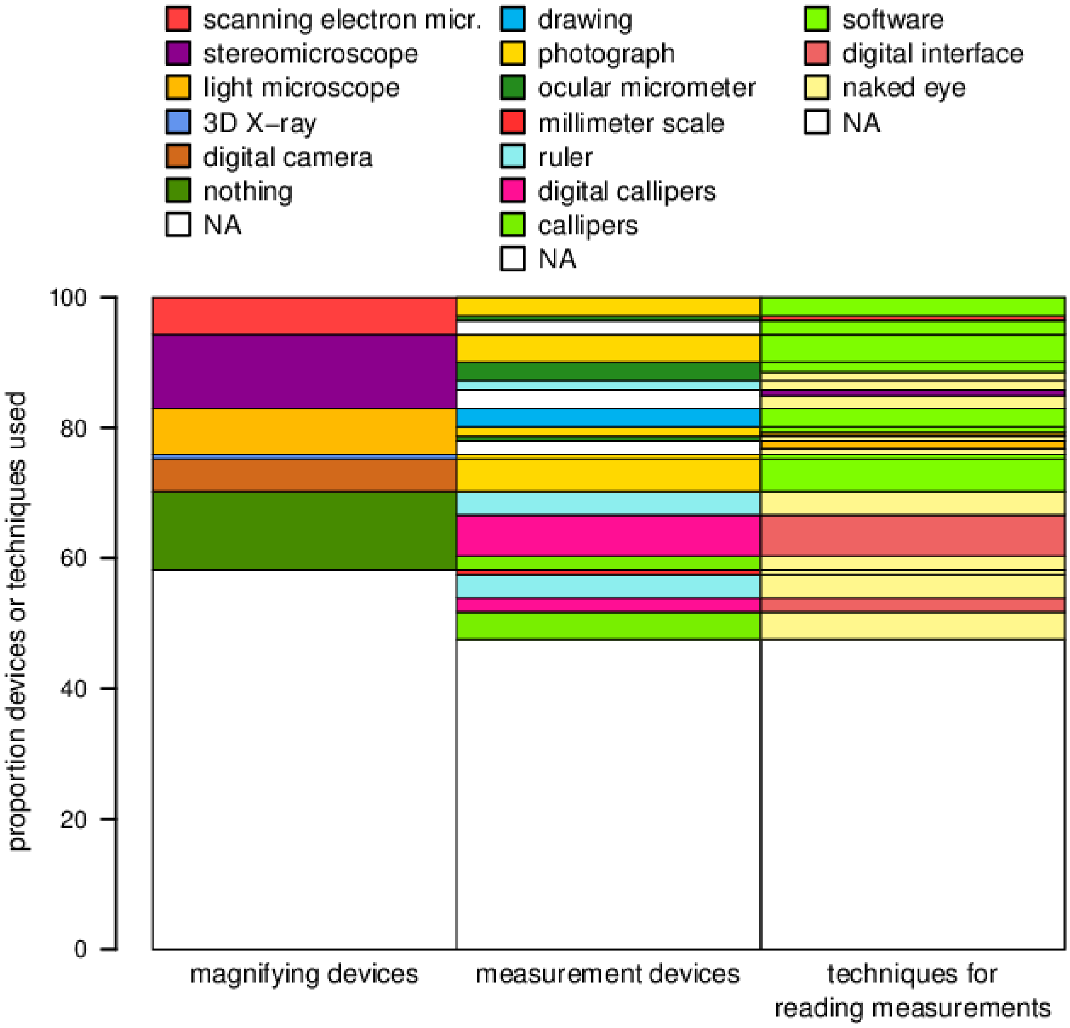

Figure 2.3 Proportion devices or techniques used for measuring proboscis. Columns from left to right are hierarchically organised: e.g., the bars of measurement devices in the range along the y-axis for the magnifying device stereomicroscope represent measurement devices for stereomicroscopy, etc.

Device resolutions ranged between 0.0001 and 1 mm, and most devices measured to the nearest millimetre. The best resolutions were measured from photographs with software. The best measured resolution was 0.5 mm for rulers, and 0.01 for callipers (Figure 2.4). We did not find information on measurement accuracy and precision.

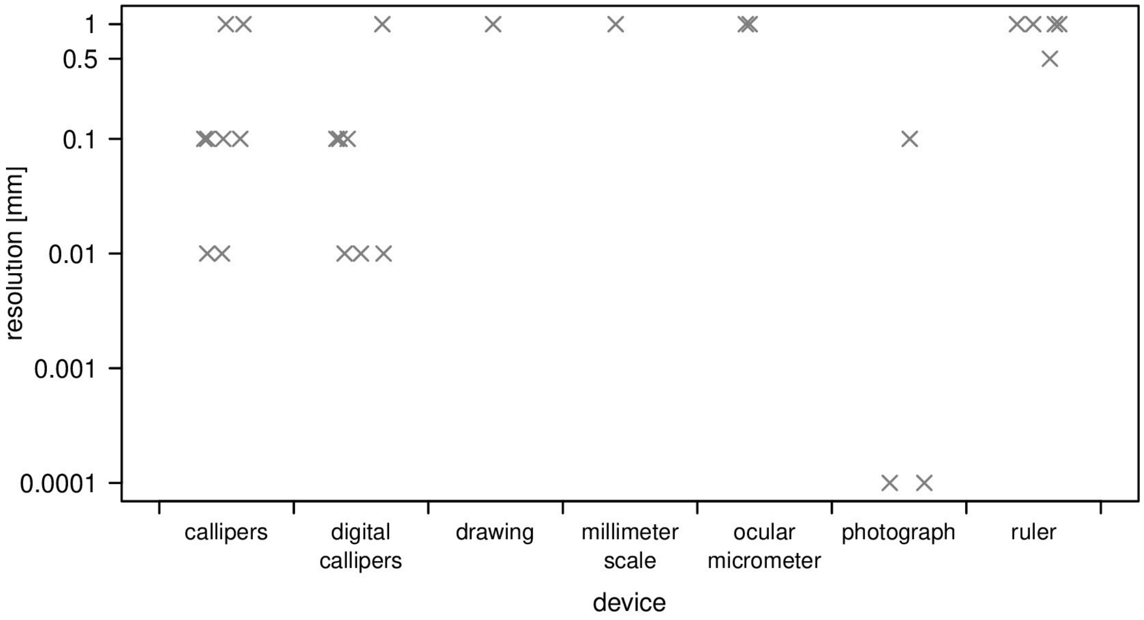

Figure 2.4 Device resolutions used for proboscis measurements. Grey × symbols represent the articles.

Relationships between the variables characterising the measurements

The larger the *proportion keywords* was, including the title and abstract, the lower was the number of NA-s in preparation description (Kendalls's tau = -0.25, $P < 0.001$, $n = 129$; Figure 2.5) and in measurement description (tau = -0.23, $P < 0.001$, n = 129; Figure 2.5). *Proportion keywords* was not related to device resolution (tau = -0.18, $P = 0.100$, n = 51; Figure 2.5) or to the number of measured individuals per species (tau = 0.08, $P = 0.301$, n = 86).

We did not find differences in proboscis length between measurements performed on dead or live specimens ($P = 0.716$, $n_{dead} = 643$, $n_{alive} = 362$; i.e. species with shorter proboscides were not measured more likely as dead than as live specimens).

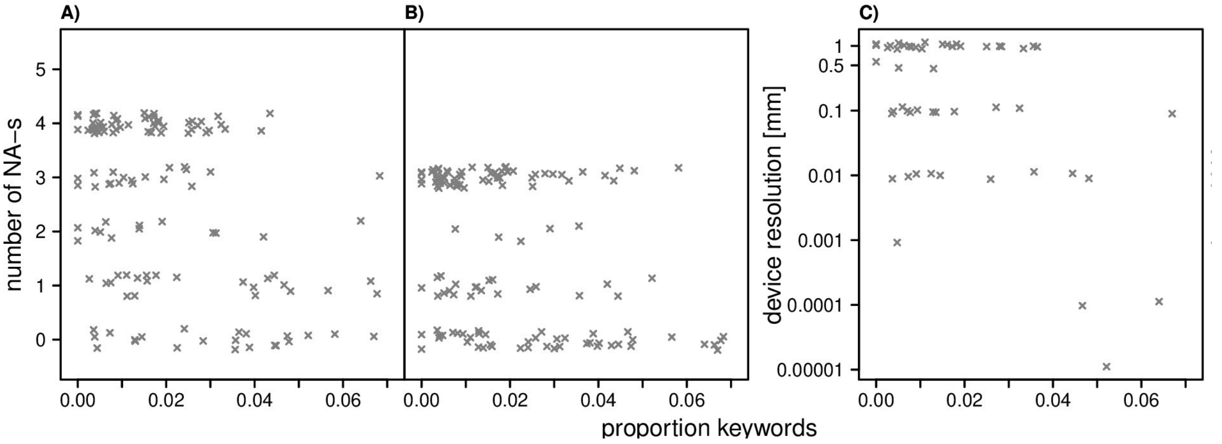

Figure 2.5 Relationship between proportion keywords, in the title and abstract and A) the number of NA-s in preparation description; and B) the number of NA-s in measurement description; and C) the resolution of the provided results of the proboscis length measurements. Grey × symbols represent the articles

Discussion

The number of papers published including lepidopteran proboscis length measurements more than doubled in the last decade, compared to the preceding three decades (Figure A2.1), showing an increased interest. We found various preparation and measurement techniques for quantifying proboscis length. Research aims were different among the reviewed studies, hence the diversity in methodology, e.g. different techniques are needed for studying the sensillas on proboscis by scanning electron microscope or feeding behaviour in the field.

About half of the reviewed studies did not provide information on measurement methodology. This impedes reproducibility and may raise the doubt if these studies were carefully designed with regard to proboscis length measurements and if they took into account the vast range of potential bias (*see* e.g. potential problems in measuring body sizes other than proboscis in insects: (García-Barros, 2015; Knapp, 2012; Van Hook et al., 2012; Von Schiller et al., 2005). Authors provided more methodological information on proboscis length measurement, if information on proboscis length was important from their perspective (*proportion keywords* in the title and abstract, Figure 2.5).

The reviewed studies applied different types of *preparation techniques*. Different techniques may shrink insect body parts in varying degrees (Kearns et al., 1993; Knapp, 2012; Moretti et al., 2017; Van Hook et al., 2012; Von Schiller et al., 2005). In contrast, Fox et al. (2015) suggested that the differences in preparation may not influence proboscis length, since it is mainly built of hard and resistant chitin. Although Fox et al.'s (2015) arguments are reasonable, we did not find studies with suitable data to test this hypothesis. Students measuring live specimens also face further challenges (Blackwell et al., 2006; Van Hook et al., 2012): handling live, fragile specimens and avoiding injuries is difficult. In contrast, anaesthesia, even for relatively short time periods may permanently alter insect behaviour (Chuda-Mickiewicz et al., 2012; Kearns et al., 1993). In a few studies even small species were successfully immobilised and carefully managed by cooling (Bauder et al., 2013; Kunte, 2007; Tiple et al., 2009).

Well-defined *landmarks* are essential for accurate body size measurements (Kouchi et al., 1999; Van Hook et al., 2012), and defining them seems to be relatively easy in the case of lepidopteran proboscis, compared to e.g. the expandable tongue of bees (Harder, 1982; Kearns et al., 1993; Morse, 1977). Only 16 (11.3%) of the reviewed studies specified the landmarks to measure proboscis length. Length was defined as the distance from the anterior edge of the eye to the proboscis tip in most cases (e.g. (Chupp et al., 2015; Corbet, 2000; Kunte, 2007). These landmarks are reasonable, because the proboscis base is not always visible from a lateral view, since it can be covered with the hairy labial palpus. Furthermore, when measuring proboscis from digital photographs, coiled and uncoiled states of the same proboscis should provide different values due to pixel organization, thus for relative estimates, only one of these methods can be used throughout a study.

A measurement is always a comparison between the measured object and a standard scaled device, and are investigated by persons. Photographs, drawing tubes, analogue or digital callipers, rulers or millimetre scales were applied to measure proboscis length. Contrary to Van Hook et al., (2012), who suggested that measuring butterflies' forewings with different devices yield similar results, we suggest that the different methods and devices are likely differ in resolution, accuracy and precision. Non-standardized devices may differ in bias, e.g. plastic rulers could be biased compared to each other, thus incurring random error (Kemper and Schwerdtfeger, 2009; Van Hook et al., 2012). Although measuring from photographs provided the highest resolution, it does not affect accuracy or precision. If the scaling device was a general-purpose ruler or another non-standardized scale, measurement accuracy can be doubtful, although usable for relative estimates if only a single device had been used. A further problem could be optical distortion, especially with low-quality optics (Larson and Chandler, 2010). Measurement duration may also be different across methods, e.g. if the speed of measurements increases bias (Daly, 1985; Kemper et al., 2009; Van Hook et al., 2012). We found that the reviewed studies often gave the resolution of the measurements, while precision, accuracy and repeatabilities were rarely reported. Furthermore, 24.1% of the authors used callipers, rulers and millimetre scales, while these devices can measure only straight objects. Unless mounted on a slide, proboscis is not straight even if uncoiled, since it has a tendency to remain curved, resulting in an underestimate (*see* e.g. Photo 3 of (Ryckewaert et al., 2011)).

We found a large variance in sample sizes among and within studies. However, we did not find a relationship between *proportion keywords* and the number of measured individuals per species. Many authors measured a relatively small number of individuals, similarly to cases measuring other morphological traits in various taxa (Cardini et al., 2015). However, we found a few good examples where sample sizes were carefully chosen (e.g. (Haverkamp et al., 2016; Kawahara et al., 2012; Krenn, 1998). The large variance in proboscis length within species (e.g. found by (Szigeti et al., 2020) and the variance due to preparation and measurement techniques make choosing an appropriate sample size crucial if the aim of the study is to characterise population distribution. Sample size may be deliberately chosen low, in order to avoid the negative impact on natural populations by removing many individuals (Costello et al., 2016; Farnsworth et al., 1993; Fischer and

Larson, 2019). Researchers also have to trade-off sampling different variables, and it is a further constraint to achieve large sample sizes.

Although some studies reported means and standard deviations (*see* Table A2.1), descriptive data on proboscis length were not provided in many cases, similarly to the findings of Stang et al. (2009) and Amorim et al. (2014) for measurements other than proboscis. In several cases the type of the statistics, i.e. if a value was the mean or the median or a single value was not provided (Meerabai, 2012). In a few cases, authors gave different types of descriptive statistics within a single table (Atachi et al., 1989; Singer and Cocucci, 1997). In contrast, some publications provided well detailed descriptive statistics: beside the mean and SD, some gave the range and the number of measured individuals (Grant and Grant, 1983; Kramer et al., 2015). Entire datasets were published only in a few cases (Johnson and Raguso, 2015; Kislev et al., 1972).

Here, we reviewed how lepidopteran proboscis length had been measured. We did not find detailed protocols for proboscis length measurement, but there are some publications with well described measurement methodology (*see* e.g. (Bauder et al., 2014; Krenn et al., 2001). There are a few guidelines to measure bee tongues (Harder, 1982; Kearns et al., 1993), and these may also help students of Lepidoptera. Hereafter, we provide recommendations and a guideline (Table 2.1) based upon this review and our own field experience.

Table 2.1 Guidelines for measurements of lepidopteran proboscis lengths

Recommendations			**Recommended literature**
General	Use the same preparation and measurement methodology throughout the study		(García-Barros, 2015; Kearns et al., 1993; Knapp, 2012; Moretti et al., 2017; Muñoz-Muñoz et al., 2010; Stec et al., 2016; Van Hook et al., 2012; Von Schiller et al., 2005; Walther et al., 2014; Wong et al., 2019)
	Provide detailed descriptions on the applied methods and report detailed descriptive statistics; whole datasets worth to be uploaded to a public data repository		
	Report device resolution; reporting repeatabilities, especially for measurement techniques new or requiring personal practice is recommended; inter-rater repeatabilities are also important if different persons measure different specimens		
Handling live specimens	Immobilization	Fragile species: cooling; anaesthetising with CO2, if altering behaviour is not an issue	General: (Cabrini et al., 2016; Chuda-Mickiewicz et al., 2012; Kearns et al., 1993; Poinapen et al., 2017), Lepidoptera proboscis: (Bauder et al., 2014, 2013; Lee et al., 2017; Xiong et al., 2019)
		Robust species: probably do not need anaesthetisation	
	Stabilization	With styrofoam or with plastic tiles and clips	
	For ecological and behavioural studies and/or		(Costello et al., 2016; Farnsworth et

	for ethical reasons, if possible, try to investigate the effect of handling on survival and follow the behaviour of some individuals for all the investigated species		al., 1993; Fischer et al., 2019; Joint Committee for the Conservation of British Invertebrates, 2002)
Preparation of dead specimens	Killing	Hand-capture: ethyl-acetate	General: (Kearns et al., 1993; Millar et al., 2000; Schauff, 2001), Lepidoptera proboscis: (Peter et al., 2009)
		Traps: with ethanol or water with a few drops of washing up liquid	General: (Kearns et al., 1993; Millar et al., 2000; Schauff, 2001)
	Storing	70% or 95% ethanol	General: (Bauder et al., 2014; Molleman et al., 2005), Lepidoptera proboscis: (Millar et al., 2000; Schauff, 2001)
		Freezing	General: (Millar et al., 2000; Schauff, 2001), Lepidoptera proboscis: (Kramer et al., 2015; Sakagami and Sugiura, 2018)
		Drying	General: (Millar et al., 2000; Schauff, 2001), Lepidoptera proboscis: (Krenn et al., 2001)
	Flexibilisation for measurement	20–50% lactic acid; 5–10% KOH; distilled water; 7% EDTA	General: (Depieri et al., 2010; Kearns et al., 1993; Schauff, 2001), Lepidoptera proboscis: (Kislev et al., 1972; Krenn, 1998; Ramkumar et al., 2010)
	Mounting or embedding the prepared specimens or proboscides	Microscope slides; SEM stubs with tape (depends on the applied devices); Canada balsam, Euparal; etc	Lepidoptera proboscis: (Grant et al., 2012; Kornev et al., 2017; Ramkumar et al., 2010; Zenker et al., 2011)
Landmarks	From the anterior edge of the eye to the proboscis tip		(Chupp et al., 2015; Corbet, 2000; Kunte, 2007)
Measurements	Shoot photo macrographs including a well-calibrated scale		(Bauder et al., 2014; Szigeti et al., 2020)
	Use a macro-lens with the smallest possible geometric distortion		
	Use a tripod in the field or any photo stand in the lab		
	Proboscis and scale should be in the same distance from the lens and parallel with the lens's plane (bubble levels insertable to camera hot shoes can be handy for levelling)		
	Try to standardize the extent of proboscis extension		
	Shoot 2–3 photographs, take measurements on each then calculate averages		(Arnqvist et al., 1998; Blackwell et al., 2006; Muñoz-Muñoz et al., 2010)
	Measure proboscis length from photo macrographs with dedicated software		(Eliceiri et al., 2012; Pennekamp and Schtickzelle, 2013; Rueden et al., 2017)
Sample size	Try to measure about 30 specimens per species to estimate population means and SDs (note: sample size depends on the aim of the		(Cardini et al., 2007; Griffiths et al., 2016; Stec et al., 2016; Van Hook et al., 2012; Wong et al., 2019)

	study, the population variability in the target variables, the effect size of interest, the confidence level needed)	
Ethical consideration	Take into account ethic and nature conservation issues when deciding on measurement methods, including sample sizes	(Costello et al., 2016; Farnsworth et al., 1993; Fischer et al., 2019; Joint Committee for the Conservation of British Invertebrates, 2002)

Recommendations

Primarily, we highlight the importance to provide detailed descriptions on the methods applied. We recommend providing the following information on measurement techniques: if measurements were performed on dead or live specimens; how they were handled, e.g. mounted for measurements; if alive, sedated or not; if dead, how the specimens were stored and proboscides relaxed; if measurements were taken on coiled or uncoiled proboscides; landmarks for measurements; the device used for magnifying the proboscis; measurement technique; how values were read, the software applied for measurements, including version number; and any other equipment used during the measurement procedure. Provide the following descriptive statistics for the measured values: the number of the measured species and individuals, including the number of males and females if determined; mean, SD, minimum and maximum. Access to entire datasets via public repositories is a good practice, since it makes research transparent and more credible (Reichman et al., 2011), and provide data for meta-analyses (Amato and Petit, 2017; Mortelliti et al., 2010; Szigeti et al., 2016b) or for trait-based studies (Moretti et al., 2017; Wong et al., 2019).

If survival is important for the study (e.g. investigating behaviour and/or endangered species), measurement could be achieved either (i) by sedation (e.g. (Bauder et al., 2013; Moré et al., 2012)), or (ii) by mounting specimens on plastic plates while measuring (e.g. (Lehnert et al., 2014; Martins et al., 2007)). However both methods have disadvantages (i) the risk of altering behaviour and (ii) the potential difficulties in small, fragile species. In case of working with freshly collected dead specimens, measurements should be carried out as soon as possible to avoid potential shrinkage due to desiccation. Note that using the same preparation methods within a study still allows taking relative measurements, thus within-study comparisons (Kearns et al., 1993; Van Hook et al., 2012). To safeguard these specimens in collections is beneficial, since they can be used for further studies (Nilsson and Rabakonandrianina, 1988).

We suggest to avoid measuring anything in science by millimeter-paper or a general-purpose ruler (Kemper et al., 2009; Muñoz-Muñoz et al., 2010). We recommend avoiding straight scales, such as callipers or rulers for measuring proboscis with the naked eyes. Rather, shoot photo macrographs including a high resolution printed scale on each photograph, then measure proboscis length with a dedicated software. Accurate scales can be drawn with graphical software. Photography can be used both in the lab or outdoors (e.g.

(Bauder et al., 2013). High-resolution photographs have the advantage of zooming into the picture and adjusting contrast or colour to improve landmark identification. Photographs can be archived and later revisited (Kemper et al., 2009). Pay attention to: (i) using a macro-lens with the smallest possible geometric distortion, (ii) that proboscis and scale should be in the same distance from the lens and parallel with the lens's plane (bubble levels insertable to camera hot shoes can be handy for levelling), (iii) using well calibrated scales and (iv) trying to standardize the measure of proboscis extension as much as possible.

Different *preparation and measurement techniques* may potentially yield different results. *Resolution* (i.e. the smallest readable unit), *precision* (i.e. the random error), *accuracy* (i.e. the systematic error) of the *measuring device* and the influence of the measuring person should be taken into account when planning the study, and these data should be provided. The amount of bias can be accumulated during the procedure of preparation and measurement. This may cause larger error than the investigated differences, i.e. the biological variation (Arnqvist et al., 1998; Van Hook et al., 2012; Yezerinac et al., 1992), thus biasing the conclusion of a study. The size of measurement error is inversely related to the quality of the data, and measurement standardization is the most effective way to minimize these errors (Ulijaszek et al., 1999; Van Hook et al., 2012). We encourage researchers to develop standard preparation and measurement protocols. Repeatability tests are useful, especially for newly developed techniques as well as for checking the reliability within and among the persons conducting measurements. We also emphasise the importance of measurement calibration and the observers' training to further enhance data reliability (Blackwell et al., 2006; Gordon and Bradtmiller, 1992; Kouchi et al., 1999; Van Hook et al., 2012). We agree with Blackwell et al. (2006) to replicate measurements at least twice or thrice and use the mean of the replications to decrease random error, when necessary (Arnqvist et al., 1998). Multiple shots on each specimen may also be useful to check measurement repeatabilities (Daly, 1985; Kemper et al., 2009; Muñoz-Muñoz et al., 2010).

We recommend to chose an appropriate *sample size*. Van Hook et al. (2012) suggested that a sample of 30 specimens is enough per population for measuring wing length in butterflies. Similar sample sizes were recommended for accurate estimates of mean body sizes in other taxa (Cardini et al., 2007; Griffiths et al., 2016; Stec et al., 2016; Wong et al., 2019). Note that although no rule of thumb exist on the minimum sample size upon which the shape of a distribution can be estimated, 30 seems to be a safe minimum for this purpose. We are aware that in many, especially field studies, this sample size can simply be not achieved. In such cases, results should be interpreted with caution. We found considerable within-species variability in proboscis length in some of the studies and others suggest that intraspecific variation in arthropods' traits may have a significant impact on the studied systems (Griffiths et al., 2016; Moretti et al., 2017; Szigeti et al., 2020; Wong et al., 2019). These imply that very low sample sizes are likely to bias distribution estimates severely, although the required sample size could be rather different among species, aims, the required confidence level, and may be different for different analyses (Batterham et al., 2005). Since accurate results require an estimate on the appropriate sample size, we suggest conducting preliminary studies on the target species or on data from related taxa, when feasible. Optimizing sample size is not an easy task, and sample size often depends on the time spent in the field or the number of traps available, hence these could be considered when planning the sampling (Cardini et al., 2015). Lastly, we suggest to take into account ethical and nature conservation issues when deciding on measurement methods or sample sizes (Costello et al., 2016; Fischer et al., 2019).

Conclusion

The array of methods and devices have been increasing in insect morphometrics. New technologies, such as automated measurements with dedicated software from photographs (Bánszegi et al., 2014), 3D photographing (Olsen and Westneat, 2015), microCT (Metscher, 2009), probably will influence the development of morphological measurements. Several studies have already used and thoroughly presented new techniques for measuring proboscis length (Bauder et al., 2013; Grant et al., 2012; Lehnert et al., 2016). In contrast, many publications did not disclose the necessary details on measurement procedures, regardless to using or not modern techniques. Deficiencies in the methods and the results were also found in other types of ecological publications (Moretti et al., 2017; Szigeti et al., 2016b). Insufficient description of methodology is an important problem, since it makes the given study doubtful, and its reproducibility impossible (Moretti et al., 2017). Furthermore, such publications are mostly inappropriate to be included in meta-analyses (Amato et al., 2017; Moretti et al., 2017; Szigeti et al., 2016b). Hence, we emphasise that well-planned methodology and detailed descriptions of the applied methods are essential for accurate conclusions. We think that further methodological development to measure proboscis length is important and general protocols could enhance data quality, thus improving cross-study comparisons. Thoroughly planned studies comparing sampling methodologies and comparing their appropriateness and accuracy at different circumstances are still mandatory.

We could not find sufficiently detailed protocols to measure proboscis in live butterflies by reviewing proboscis length measurement methodologies for Lepidoptera. We needed a method that enabled us to measure dozens of proboscides a day prior to tracking the measured butterflies while they were freely foraging in their natural habitat. Therefore, we developed our own measurement method. This is probably similar to (Bauder et al., 2014, 2013), albeit their descriptions are not detailed enough to assess similarity. We provided a short description of this method in Szigeti et al., (2020; Chapter Three), in which we investigated flower visits on the most frequently visited nectar sources by taking proboscis length and nectar plant corolla length into account. This took place at Hegyesd, 2015 since we shifted our studies from Leány kúti-rét due to population decline.

Chapter Three: Are all butterflies equal? Population-wise proboscis length variation predicts flower choice in a butterfly

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Introduction

Intraspecific morphological and behavioural variation are essential in evolution: interactions of a population phenotype in changing environmental circumstances shape population traits through differential survival and reproduction (Bolnick et al., 2003; De León et al., 2012; Grant et al., 2014; Miller-Struttmann et al., 2015; Pauw et al., 2009; Pratt, 2005). Intraspecific morphological variation fundamentally influences individual resource exploitation (Grant et al., 2014; Pauw et al., 2009). Although the number of studies investigating individual level resource use has rapidly increased in the last few decades (Araújo et al., 2011; Dall et al., 2012; Layman et al., 2015), these studies are still biased towards vertebrates; relatively little is known about individual level variation in resource use by insects and even less on how morphology influences individual foraging behaviour.

Animals select from available resources for optimal intake, and food resource acquisition determines an animal's survival and reproductive success (Stephens et al., 2007). Food resource availability impacts foraging behaviour, as well as population size and, in consequence, the composition of communities (Stephens et al., 2007). Intraspecific variation in mouthpart morphologies has been proved to be the basis of microevolutionary processes through resource use (gastropods: (Watanabe and Young, 2006); birds: (De León et al., 2012; Grant et al., 2014). Although flower-visiting insect pollinators are suitable model organisms to investigate relationships between resource use and morphological traits, their intraspecific variability in morphological traits and their effect on microevolutionary processes have been scarcely investigated (Miller-Struttmann et al., 2015; Pauw et al., 2009). Plant-pollinator networks are appropriate examples of complex bipartite interactions, with highly variable functional traits, including multiple connections formed by coevolutionary processes (Darwin, 1862; Nilsson, 1988; Pauw et al., 2009; Schiestl and Johnson, 2013). Flower-visiting insects have to select from the available floral resources to cover their dietary needs (Goulson, 1999). Variation in the shape and size of flowers and pollinator mouthparts plays an important role in foraging efficiency and in the pollinators' resource use. For example, species with longer tongues are able to feed from deeper flowers (Harder, 1985; Haverkamp et al., 2016; Inouye, 1978; Klumpers et al., 2019; Pauw et al., 2009; Rodríguez-Gironés et al., 2007), while they are less successful at imbibing concentrated nectars available in shallower flowers (Borrell et al., 2006; Harder, 1986; Josens and Farina, 2001; Kim et al., 2011). Although resource use based on flower and mouthpart size is relatively well studied across species, it has scarcely been investigated within populations, except in flies in which individual nectar consumption was related to tongue length (Pauw et al., 2009) and in bumblebees in which individual flower choice was also related to tongue length (Inouye, 1980; Johnson, 1986); but see also (Dohzono et al., 2011).

Butterflies are ideal study systems to address plant-pollinator morphological compatibilities, since they usually have long proboscides, that is, specialized mouthparts evolved as an adaptation to imbibe floral nectar as a primary food resource at the adult stage in most species (Bauder et al., 2011; Erhardt et al., 2009; Krenn, 2000). Butterflies consume nectar by active suction that does not allow them to imbibe highly concentrated nectar because of its high viscosity (Borrell et al., 2006; Josens et al., 2001; Kim et al., 2011). Adult butterflies select from the available nectar plant species (Erhardt et al., 2009; Thomas et al., 2016), and diet choice may vary between species, populations, generations, sexes, age groups and individuals (Erhardt et al., 2009; Szigeti et al., 2019; Thomas et al., 2016; Tiple et al., 2009). Furthermore, nectar consumption, as well as larval food intake, has been proved to affect life span and fecundity in several species (Cahenzli et al., 2013; O'Brien et al., 2004).

We studied flower visits of a small population of Clouded Apollo butterflies, *Parnassius mnemosyne* (Lepidoptera: Papilionidae). The Clouded Apollo is an appropriate model species, because adult feeding is particularly important and foraging behaviour of individuals is easy to observe and monitor by mark-resighting in small, closed populations, where lifetime individual resighting probability can be high (Konvička et al., 1999; Szigeti et al., 2018). Clouded Apollo butterflies select from floral resources and their diets vary between regions and years and within flight periods, following temporal changes in floral resource availability at the population and individual level (Konvička et al., 2006; Kudrna et al., 1991; Szigeti et al., 2019, 2018).

Time shifts in the individuals' presence within a flight period and temporal changes in floral resources together partially explain individual resource use in Clouded Apollo butterflies, albeit a considerably large part of the individual differences in resource use remains unexplained (Szigeti et al., 2019). We hypothesized that variation in flower depth of the visited plant species and/or in proboscis length within a population can explain some of the intraspecific differences in resource use. Based on our field experience, we assumed that one of their locally important nectar plants and one of the best-yielding nectar sources in their diet, the sticky catchfly, *Silene viscaria* (Caryophyllaceae; (Szigeti, 2018; Szigeti et al., 2018), has a flower (corolla) depth of ca. 12 mm (Jennersten and Nilsson, 1993) similar to the 12 mm Clouded Apollo proboscis length (Lara Ruiz, 2011; Paulus and Krenn, 1996). Hence, we hypothesized that *S. viscaria* flowers would be available only for Clouded Apollo butterflies with long proboscides, while those with short proboscides would not be able to use this resource.

We investigated whether (i) variation in proboscis length influenced nectar plant choice of Clouded Apollo individuals within a single population. We hypothesized that flower depths constrain butterflies in flower visits via their proboscis lengths. (ii) We also studied whether feeding on *S. viscaria* was constrained by individual proboscis length. We hypothesized that Clouded Apollo individuals observed visiting *S. viscaria* had longer proboscides than those not observed on this nectar source. Specifically, we measured Clouded Apollo proboscis length within a single population and the corolla length of the six most visited forbs. (i) We compared proboscis length to the flower depth of these six nectar plant species and (ii) investigated whether visits to individual flowering plant species were related to proboscis length, taking into account floral abundance as a confounder.

Methods

Study site and period

We carried out field work at Hegyesd, a 0.5 ha meadow in the Visegrádi-hegység, Hungary, Central Europe (47°45′22.7″N, 19°02′53.4″E, at 295 m above sea level), from late April to the end of May 2015. We sampled butterflies and forbs between 09:00 and 18:00 hours during all days of the Clouded Apollo butterflies' flight period, as weather permitted.

Sampling in the field

We surveyed the whole meadow several times a day throughout the flight period. We walked through the meadow at a slow pace, recording all Clouded Apollo butterflies within a 4 m wide zone on both sides. We aimed to capture all unmarked butterflies with a butterfly net. We measured and marked them individually with a colour combination applied on both forewings' tips with edding® paint markers and then released them (Szigeti et al., 2018). We monitored the meadow regularly throughout the day and identified individuals and their nectar plant species if they were observed feeding (Szigeti et al., 2018).

We measured flower depth and flower abundance of the six plant species Clouded Apollo butterflies visited the most often: *Ajuga genevensis* (Lamiaceae), *Buglossoides purpurocaerulea* (Boraginaceae), *Dianthus giganteiformis subsp. pontederae* (Caryophyllaceae), *Geranium sanguineum* (Geraniaceae), *S. viscaria* (Caryophyllaceae) and *Vicia cracca* (Leguminosae). The cumulative proportion of visits on these six species amounted to 97.4% of the total visits observed and more than 2% of visits were on the sixth species (*A. genevensis*). In contrast, only 0.4% of the visits were observed on the seventh most-visited species.

To sample flower abundance, we prepared a map of our study site using Google Earth aerial photos and adjusted it in the field. About every 3 days, we drew all flowering patches of the six plant species on a map and estimated the number of flowering shoots per patches within the study site by either counting the shoots (< 10 shoots per patch), or assigning rough estimates by tens, as 20, 30, … 100 (> 10 shoots per patch; (Szigeti et al., 2015)). We summarized the number of shoots per plant species per sampling event for the entire site. We also counted the flowers per flowering shoot on the sampled forbs. We calculated flower abundance for the entire study site for each of the six most-visited plants by multiplying the number of flowering shoots by the average of the number of flowers per flowering shoot. We used kernel smoothing (bandwidth = 5) to extrapolate abundances for each day from the 3 days of sampling. Floral abundance was estimated to control for the effect of the considerable observed changes in abundance over time on the feeding behaviour of Clouded Apollos. Sampling was carried out by VS.

Proboscis and corolla length measurements

We measured proboscis length from photo macrographs (Bauder et al., 2013). We mounted live, nonsedated butterflies on a small, scaled board with clips. Then we fixed the board on a tripod under a camera, parallel to the plane of the lens (Nikon d7000 + Micro Nikkor 60mm f/2.8G ED AF-S macro lens). We uncoiled the proboscis and extended it over the board with a hooked pin and we took at least two pictures for each individual. We used FIJI/ImageJ (Schindelin et al., 2012) to measure proboscis length from the photographs and used the average of the lengths measured on the pictures of the same individual.

We measured flower depth with callipers *in situ* (resolution: 0.1 mm). This was the distance between the bottom of the corolla tube, where it meets the receptacle, and the orifice of the flower tube. We defined the orifice as the point where the butterfly can insert the proboscis into the flower.

Ethical note

The Clouded Apollo is a robust butterfly species and we did not observe any injuries caused by our protocol (proboscis measurement and individual marking). Our field work was licensed by the Hungarian Nature Conservation Authorities: KTVF: 31430/2014.

Data analysis

We compared proboscis length to flower depths of the six most visited nectar plant species with a Dunnett's test and differences between flower depths with a Tukey test. To analyse the relationship between visit and proboscis length, we used generalized linear mixed models

(GLMM) with binomial distributions (Zuur et al., 2009), taking into account the effect of flower abundance and the repeated observations on the same butterfly individuals. We fitted binomial models for the six most often visited species separately, so we obtained six different models. The response variable was a visit on a focal plant species (value: 1) or on any of the other visited species (value: 0) at a given visit observed. The explanatory variables were proboscis length and flower abundance of the focal plant species. We included butterfly individual identifiers as a random factor. We used log10-transformed kernel-smoothed daily estimates of the number of flowers of the focal plant species. Note that the results of the six different models are not independent; hence, P values were corrected for multiple comparisons according to Benjamini and Hochberg (1995).

We analysed all data in the R 3.4.4 statistical environment (R Core Team, 2018), using the ‘chron’ 2.3–52 package (James and Hornik, 2015) for calculating dates, the ‘KernSmooth’ 2.23–15 package (Wand, 2015) for kernel smoothing, the ‘DescTools’ 0.99.31 package (Signorell, 2020) for the Dunnett’s test, the ‘agricolae’ 1.2–8 package (de Mendiburu, 2020) for the Tukey test, the ‘lme4’ 1.1–21 package (Bates et al., 2015) for GLMM and the ‘ggeffects’ 0.12.0 package (Lüdecke, 2018) for visualizing GLMM.

Results

We observed 1077 flower visits by 113 Clouded Apollo individuals, for which proboscis length data were available. We observed 9.5 ± 7.5 (mean ± SD; range 1–39) visits per individuals. Clouded Apollo butterflies most often visited the flowers of *D. giganteiformis* (69.5%), *B. purpurocaerulea* (8.9%), *S. viscaria* (7.4%), *V. cracca* (6.1%), *G. sanguineum* (3.6%) and *A. genevensis* (2.2%). Flower abundances and visits changed considerably across species during the flight period (Figure A3.1).

We measured proboscis length in 169 individuals. This varied considerably between individuals (minimum–maximum: 9.98–13.52 mm; mean ± SD: 12.13 ± 0.58 mm; Figure 3.1). The difference between the longest and the shortest proboscis was remarkably large compared to the mean (range 3.53 mm, 29% of the mean). We observed 113 individuals visiting flowers (proboscis length range 10.51–13.52 mm; mean ± SD: 12.19 ± 0.54 mm).

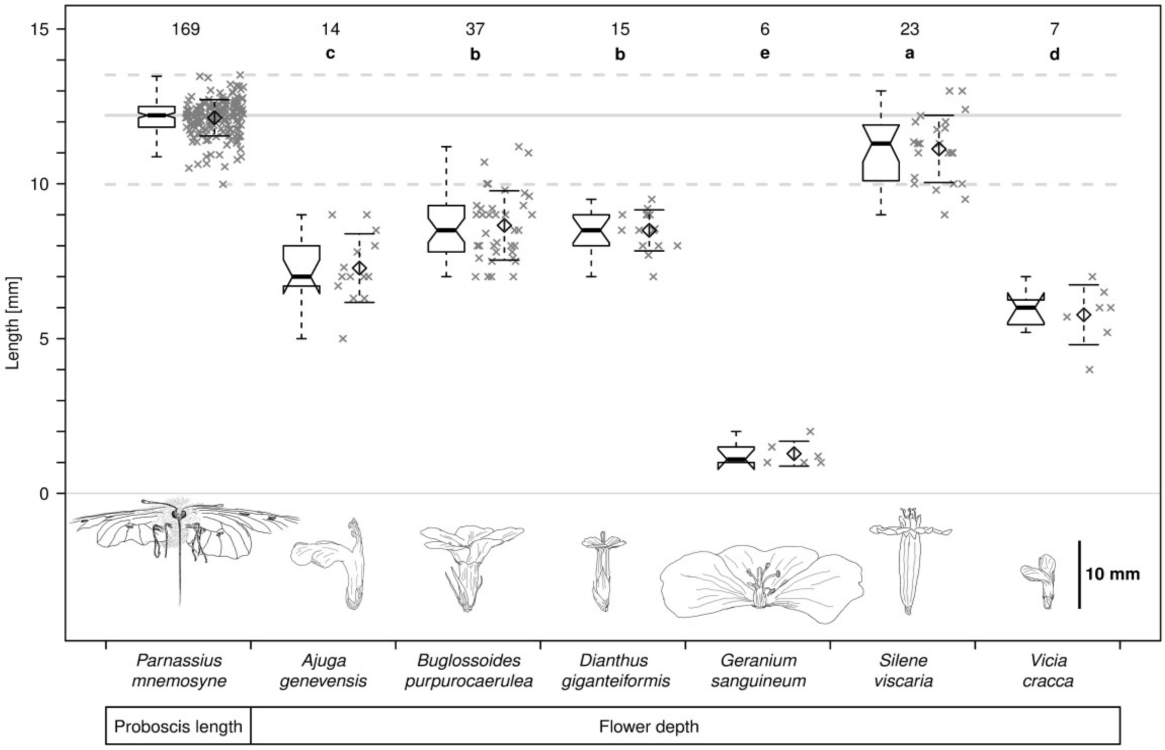

Figure 3.1 Clouded Apollo proboscis length and flower depth of the six most-visited plant species. The box plots show the median and 25th and 75th percentiles; the whiskers indicate the values within 1.5 times the interquartile range. Notches show 95% confidence intervals for the medians. Diamond symbols show the means and their whiskers SDs. Grey crosses represent individual butterflies or flowers and are jittered on the horizontal axis for better visibility. The horizontal dashed grey lines show the minimum and maximum and the solid grey line shows the median proboscis length. Letters above the boxes show significantly different flower depths (Tukey test). Numbers above the letters show the number of measured butterflies or flowers. Drawings illustrating proboscis length and flower depths are scaled.

The proboscis was significantly longer than the flower depth for each of the six most-visited plants (Figure 3.1; Dunnett's test: $P_{adjusted} < 0.001$ for each comparison). Flower depths differed between the six most-visited plants and varied considerably within species (Figure 3.1, Table A3.1). Flowers of *G. sanguineum* were extremely shallow and those of *S. viscaria* extremely deep, within the range of the six species (Figure 3.1). The depth range of *S. viscaria* was similar to the range of proboscis length of Clouded Apollo butterflies (Figure 3.1), and the difference between the deepest and the shallowest *S. viscaria* flowers was large compared to the mean depth (range 4.0 mm, 36% of the mean). Many *S. viscaria* and a few *B. purpurocaerulea* flowers were deeper than the length of the shortest proboscides (Figure 3.1). Flower depths of the other four species were shorter than any proboscis length (Figure 3.1).

The median proboscis length of butterflies observed visiting *S. viscaria* was longer than that of butterflies observed visiting other nectar plants (Figure 3.2). Proboscis length significantly influenced visits to *S. viscaria*: individuals with proboscides 1 mm longer than those of their conspecifics were 2.46× more likely to visit *S. viscaria* (odds ratio; $P < 0.001$; Table 3.1, Figure 3.2). The individual with the longest proboscis (13.52 mm) was estimated to be 14.96× more likely to feed on *S. viscaria* than that with the shortest proboscis (10.51 mm; binomial GLMM; Table 3.1). Proboscis length did not significantly influence visit probability of the other five nectar plant species (Table 3.1, Figure A3.2). The odds ratio of visits significantly increased with increasing flower abundance for *B. purpurocaerulea, D. giganteiformis* and *V. cracca* (Table 3.1, Figure A3.2).

In summary, individuals with longer proboscides visited *S. viscaria* more often than those with short proboscides, but there was no such relationship in the other five frequently visited nectar plant species (Table 3.1, Figure 3.2).

Table 3.1 Clouded Apollo visits related to proboscis length and flower abundance: summary of the generalized linear mixed models.

Dependent variable: a visit observed on the focal or on another species	**Explanatory variables**	**Slope estimate**	**SE**	**P**	**Odds ratio (exp(estimate))**
Ajuga genevensis	proboscis length	-0.08	1.11	0.94	0.92
	log10(flower abundance)	-2.39	2.53	0.52	0.09
Buglossoides purpurocaerulea	proboscis length	-0.87	0.59	0.27	0.42
	log10(flower abundance)	24.03	3.74	<0.001	2.73E+10
Dianthus giganteiformis	proboscis length	0.21	0.24	0.51	1.23
	log10(flower abundance)	2.98	0.42	<0.001	19.69
Geranium sanguineum	proboscis length	-0.81	0.58	0.28	0.44
	log10(flower abundance)	-1.72	0.72	<0.05	0.18
Silene viscaria	proboscis length	0.9	0.24	<0.001	2.46
	log10(flower abundance)	0.41	0.55	0.54	1.51
Vicia cracca	proboscis length	-0.3	0.43	0.54	0.74
	log10(flower abundance)	3.08	1.07	<0.05	21.76

The response variable in binomial models was whether the focal floral species or another species had been visited at a specific observation event. We made separate models for the six species. All models included flower abundance of the focal species on the observation day as a fixed factor and visiting individuals as a random effect.

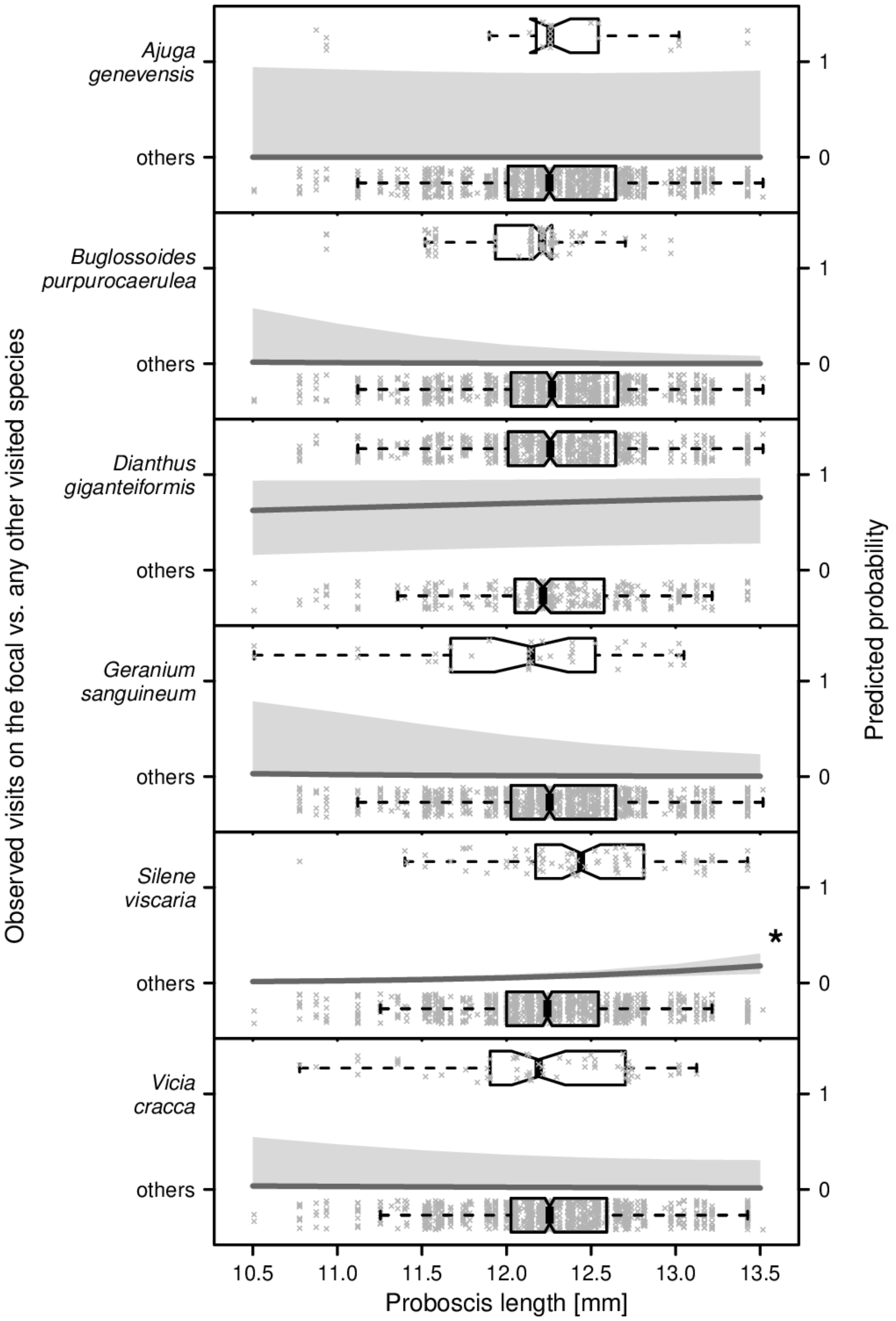

Figure 3.2 Clouded Apollo flower visit and proboscis length relationships: proboscis lengths compared between butterflies observed on focal nectar plants among the six most-visited species versus those observed on any other species; binomial GLMMs were repeated for all the six most-visited plant species as a focal plant. The box plots show the median and 25th and 75th percentiles; the whiskers indicate the values within 1.5 times the interquartile range. Notches show 95% confidence intervals for the medians. Grey crosses represent individual observations and are jittered on the vertical axis for better visibility. Dark grey lines represent regression lines and light grey bands represent 95% confidence intervals for regression lines. The asterisk represents a significant effect (P < 0.05).

Discussion

We investigated the relationships between flower visits and proboscis length of Clouded Apollo butterflies during a single flight period in one population. We found remarkable intrapopulation variation in the length of the butterfly's proboscis, and high intra- and interspecific variation in the flower depth of the six most-visited nectar plant species. Moreover, we showed that the longer the proboscis, the more likely Clouded Apollos were to visit *S. viscaria*, the plant species with the deepest corolla in their diet.

We found large individual differences in Clouded Apollo proboscis lengths, and the median (12.2 mm) was similar to those reported by Paulus et al. (1996) and Lara Ruiz (2011) (12 mm in both studies). Flower depth also varied within and across species, *G. sanguineum* being extremely shallow and *S. viscaria* extremely deep within the range of the six most-visited floral species. Flower depth of *S. viscaria* was similar (median: 11.3 mm) to that reported by Jennersten et al. (ca. 12 mm 1993). Many *S. viscaria* and a few *B. purpurocaerulea* flowers were deeper than the proboscis length of butterflies with the shortest proboscides. Butterflies are considered unable to feed from flowers deeper than their proboscis length and interspecific differences in proboscis length explain differences in flower visits of different butterfly species (Corbet, 2000; Haverkamp et al., 2016; May, 1992)

Some of the variation in proboscis length may result from environmental fluctuations during larval development, because abundance and quality of larval host plants, as well as microclimate, determine adult body size in butterflies (Boggs et al., 2005; García-Barros, 2000). Clouded Apollos with longer proboscides also had longer forewings (mean slope ± SE: 0.31 ± 0.03, $P < 0.001$, R2 = 0.43, N = 169; linear regression; Kis, Kőrösi, Szigeti, & Vajna, 2015; unpublished data). Similar results have been reported for other pollinators (within and between species, (Agosta et al., 2005; Kramer et al., 2015; Kunte, 2007; Peat et al., 2005)). Caterpillars growing in warmer microhabitats and/or places richer in host plants may develop into larger butterflies with longer proboscides. Besides larval nutrition, inherited traits may also influence adult body size in butterflies (Chown and Gaston, 2010; Honek, 1993), thus genetic variation probably also contributes to proboscis length variation. Similarly, differential development may involve flower depth variability due to environmental factors, such as soil humidity (Galen, 2000) along time and space, and flower depth variability may also be influenced by genetic factors (Gómez et al., 2009; Klinkhamer and van der Veen-van Wijk, 1999). Interspecific flower depth variation has been found to be an important environmental factor influencing individual diet choice in pollinators (Harder, 1985; Haverkamp et al., 2016; Klumpers et al., 2019; Rodríguez-Gironés et al., 2007).

Whatever factors determine the morphological variation among foraging individuals, this variation may cause differences in the individuals' food intake as well as in their fitness (Grant et al., 2014; Holbrook and Schmitt, 1992; Pauw et al., 2009; Tammaru, 1998). Changes in any of these factors may alter trait distribution in a population (foragers: (Grant et al., 2014); flower visitors: (Miller-Struttmann et al., 2015; Pauw et al., 2009); food resources: (Lavorel and Garnier, 2002); nectar plants: (Leibman et al., 2018; Little et al., 2005)).

Visits on *S. viscaria* were influenced by proboscis length: individuals with longer proboscides visited *S. viscaria* with a larger odds ratio than those with shorter proboscides. Therefore, our results show that individuals with shorter proboscides were less likely to exploit *S. viscaria* as a nectar source. The distribution of corolla tube length in *S. viscaria* implies that a Clouded Apollo butterfly with the shortest proboscis could reach only 13.2% of *S. viscaria* flowers. Individuals with at least the lower quantile proboscis length could reach 73.3%, with a median length 86.6%, with an upper quantile length 91.6% and with the longest proboscis 100.0% of the *S. viscaria* flowers. We did not find statistically significant effects of proboscis length on visits to the other five plant species. The visit patterns of *S. viscaria* indicate that variability in proboscis length and flower depth alone may partially explain individual floral choice, although other factors such as flower abundance or nectar quantity and quality are probably crucial as well. Morphological variation may cause differences in the individuals' food intake as well as in their fitness: large variance in proboscis length may be maintained by a fluctuating environment where floral resource availability changes rapidly (Pauw et al., 2009; Szigeti et al., 2018). Ultimately, intraspecific corolla length variation may be as important in the individual diet choice of nectarivorous organisms as its interspecific variation.

We further discuss a handful of potential alternative explanations of this large variation in proboscis length and its possible consequences for nectar source use. (i) We hypothesize that butterflies with short proboscides access poorer quality *S. viscaria* flowers or have no access to it at all, compared to those with long proboscides. Thus, they learn to avoid *S. viscaria* and look for alternative sources. This could also be true for any similar situations when butterflies face nectar sources with corolla lengths fluctuating around their reach. Several plant species have been shown to include more nectar when their corollas were longer and thus were richer resources for insect visitors with longer proboscides (Gómez et al., 2008; Lázaro et al., 2015). This hypothesis does not predict natural selection for either long or short proboscides or both; it simply considers variation in length and learning. However, selection is likely to happen and may induce various scenarios. (ii) Long proboscides might be more advantageous than short ones, because they allow the butterfly to imbibe nectar from both long and short corolla flowers (Inouye, 1980; Rodríguez-Gironés et al., 2007). (iii) Although we did not find any significant relationship between individuals with short proboscides and their visits to short corolla nectar plant species, short proboscides might also be advantageous for various reasons. For example, individuals with shorter proboscides may be better able to consume more concentrated nectars from shallower flowers, since viscosity increases with concentration and more viscous solutions require more force to pump through longer tubes of the same diameter (Borrell et al., 2006; Josens et al., 2001; Kim et al., 2011). Shallower flowers (such as *G. sanguineum*) may contain more concentrated nectars than plants with deeper flowers (Borrell et al., 2006; Harder, 1986; Josens et al., 2001; Kim et al., 2011). Furthermore, handling times are shorter for shorter proboscides, because less time is needed to uncoil them and take up the same amount of nectar (Borrell et al., 2006; Harder, 1986; Kunte, 2007); but see (Klumpers et al., 2019; Peat et al., 2005). (iv) Large variance in proboscis length might be associated with within-population resource partitioning: within-population competition may be reduced if butterflies with short proboscides exploit shallower flowers with sparse nectar that has a high sugar concentration and those with long proboscides visit deeper flowers yielding ample, low-concentration nectar. We have no conclusive data to argue for or against these hypotheses. Testing them requires laboratory experiments and conclusions cannot be drawn from observational data, such as those presented here.

To our knowledge, our results provide the first evidence that individual variation in mouthpart length influences diet in Lepidoptera, and generally in solitary nectarivorous insects studied in natural circumstances. Intraspecific relationships between body sizes and diets in insect pollinators have scarcely been investigated and almost exclusively in social forager bumblebees (Dohzono et al., 2011; Johnson, 1986; Peat et al., 2005; Spaethe and Weidenmüller, 2002; Willmer and Finlayson, 2014) and flies (Pauw et al., 2009), but mouthpart length and diet relationships have been thoroughly studied in other taxa (gastropods: (Watanabe et al., 2006); ants: (Davidson, 1978); lizards: (Schoener, 1968); birds: (De León et al., 2012; Grant et al., 2014; Pratt, 2005)). Note that Clouded Apollo butterflies also visited other plant species with short corollas and we found a large scatter in the relationship between visits and proboscis length; hence, we agree with Dohzono et al. (2011) that the morphological fit between proboscis length and flower depth is not the sole determinant of foraging efficiency. Pollinators face a vast range of very different cues regarding whether to land on a plant and probe its flowers (Blüthgen and Klein, 2011; Junker et al., 2015; Kuppler et al., 2016; Szigeti et al., 2019). For example, the use of different nectar sources by Clouded Apollo butterflies varies across the species' distribution range (Konvička et al., 2001; Kudrna et al., 1991; Lara Ruiz, 2011; Szigeti et al., 2018), and even between nearby habitat patches and between consecutive years (Szigeti et al., 2018, 2015). Individuals may encounter different forb species and size distributions of flowers in different habitat patches. Moreover, flower depth may also vary at the intraspecific level due to weather conditions (Carroll et al., 2001; Galen, 2000), and may change during the flowering period (Inouye and Pyke, 1988; Jo et al., 2014). Our results indicate that intraspecific morphological variation in both plants and their pollinators is an essential factor in the choice of nectar source; hence, their interactions are at least partially based on continuous trait variability, rather than on well-defined discrete traits of different taxa, as the pollination syndrome hypothesis implies (Blüthgen et al., 2011; Ollerton et al., 2009).

Differences across years found in the nectar plant species' abundance and visit rates at Leány-kúti rét, 2009–13 (Szigeti et al., 2018) imply strong environmental impact, probably due to weather fluctuation on these traits. We found that abundance was the main factor influencing the butterflies' nectar source choice in Clouded Apollos, although floral structure and colour were also important in one of the habitats (Vajna et al., 2020b; Chapter One). We also showed that proboscis length predicts visit rate on a long-corolla resource in a single year (Szigeti et al., 2020; Chapter Three). If within-species corolla and/or Apollo proboscis lengths also vary across years, these may further contribute to the variety of annual visit patterns. We addressed this problem in Chapter Four. Furthermore, we developed a new technique based on photo macrographs for measuring corolla length. We assume this technique is more reproducible than measuring corolla length with callipers and has the advantage of being archived (Kemper et al., 2009). We briefly outline this method in Chapter Four.

Chapter Four: Annual variation in Clouded Apollo butterflies' proboscis length and their nectar plants' corolla length – a field study

Unpublished manuscript with major contributions from Flóra Vajna, Viktor Szigeti, Ádám Kőrösi, Katalin Pásztor, Ádám Gór, Dorottya Somlay, János Kis

Introduction

There is a complex network between insect pollinators and the plants they visit, and these connections are shaped by co-evolutionary processes (Pauw et al., 2009). These relationships play essential roles in terrestrial ecosystems (Ollerton, 2021). Pollinators visit plants mostly for feeding on floral resources, mainly nectar, partially pollen. While nectar contains water, various sugars in different quantities, amino acids, fats and antioxidants (Abrol, 2012; Alexandersson et al., 2002; Baker et al., 1983; Filella et al., 2013; Nicolson et al., 2007); pollen includes proteins, carbohydrates, water, oils, mineral salts and vitamins (Halmágyi et al., 1991; Nicolson et al., 2007; Willmer, 2011). For plants, it is beneficial to entrust fertilisation on insects or other animal pollinators compared to abiotic carriers, such as wind or water (Willmer, 2011). Since flower visitors are more likely to find the target than currents, thus entrusting fertilisation on a pollinator may considerably reduce the amount of pollen necessary for sexual reproduction, especially in smaller isolated populations (Willmer, 2011). Furthermore, self-fertilisation can be avoided compared to wind or water pollinated plants (Willmer, 2011).

Flower visiting insects' choice among plants is based on their inherited (Schoonhoven et al., 2005) and learnt preferences (Dixit et al., 2020; Patiny, 2014; Schiestl et al., 2013; Broadhead & Raguso 2021; Goyret et al 2008; Goyret & Raguso 2006). Moreover, it is based on body size (Arbulo et al., 2011; Inoue et al., 2006), i.e. a larger flower visitor needs more food, such as nectar (Willmer, 2011), which is produced usually by larger flowers (Gómez et al., 2008; Lázaro et al., 2015). The visitors' feeding efficiency is determined by e.g. the quantity of the available nectar (May, 1988; Stang et al., 2009), the abundance of the interacting species (Stang et al., 2009), the ratio of proboscis length and corolla length (Agosta et al., 2005; Alexandersson et al., 2002; Arbulo et al., 2011; May, 1988; Szigeti et al., 2020; Chapter Three) and the ratio of the corolla width and the diameter of the visitors' head (Jervis et al., 1993).

Most adult butterflies are flower visitors and feed on liquid nutrition with their elongated proboscis, primarily feed on floral nectar (Krenn, 2019; Lee et al., 2014; May, 1992; Monaenkova et al., 2012; Willmer, 2011). They are considered less effective pollinators than e.g. bees since their long proboscis enables them to imbibe nectar without fertilising the flower (Erhardt et al., 2009). Cheating is beneficial for the butterflies because they get more food with less effort. Nevertheless, they proved to be important pollinators for several plants e.g. milkweed *Asclepias syrica*, tobacco *Nicotiana* spp., yucca *Yucca* spp., the senita cactus *Pachycereus schottii*, leafflower trees *Glochidion* and *Breynia* spp., and forbs belonging to the Caryophyllaceae family (Abrol, 2012; Jennersten, 1988; Mertens et al., 2021; Ollerton, 2021).

Proboscis length

Nectar is usually, hidden in the bottom of the corolla (Nicolson et al., 2007). In order to suck up this energy-dense sugar solution, the proboscis should be at least as long as the length of the corolla of the visited flower (Corbet, 2000; May, 1992; Pauw et al., 2009). In contrast, Liang et al., (2021) observed in bumblebees that if the flower tube opening is wider than their intertegular span, then the bees are able to tug into the flower tube not only their heads but also their bodies. Body part sizes, such as proboscis length, are influenced by both inheritance and environmental effects during the larval stage, e.g. the impact of larval nutrition on larval development (Boggs et al., 2005; García-Barros, 2000). These impacts result in individual size differences, including proboscis length (Kislev et al., 1972). Large individual variances in mouthpart morphology such as proboscis length may severely affect individual diet range (Dohzono et al., 2011; Johnson, 1986; Pauw et al., 2009; Peat et al., 2005; Spaethe et al., 2002; Szigeti et al., 2020: Chapter Three; Willmer et al., 2014) and is likely related to behaviour, life history and ultimately, fitness (Clutton-Brock et al., 2010).

Corolla length

Weather conditions (e.g. precipitation and heat distribution during growth) impact several plant traits such as phenology, nectar quality and quantity, as well as plant size (Carroll et al., 2001; Pfeifer et al., 2006). Within-species corolla length variability was found in *Erysimum mediohispanicum* (Gómez et al., 2009), *Lonicera implexa* (Lázaro et al., 2015), *Ajuga genevensis*, *Buglossoides purpurocaerulea*, *Dianthus giganteiformis subsp. pontederae*, *Geranium sanguineum*, *Silene viscaria* and *Vicia cracca* (Szigeti et al., 2020; Chapter Three). Individual corolla length variability may be influenced partially by fluctuating weather conditions (Carroll et al., 2001; Galen, 2000). Corolla length may also be a plant strategy (Krenn et al., 2021) because with longer corolla, it is possible to exclude short-tongued, generalist flower visitors if they are not effective pollinators (Rodríguez-Gironés et al., 2007, 2006), although with short corolla more potential visitors, both short and long-tongued, may be lured (Rodríguez-Gironés et al., 2006). Furthermore, flowers with short corolla are cheaper to maintain because they contain less nectar (Carvalheiro et al., 2014; Galetto et al., 2004; Gómez et al., 2008; Lázaro et al., 2015).

Annual variation in foraging behaviour

Annual variation was found in diet choice together with foraging behaviour in a wide range of vertebrate taxa such as fish (Robert et al., 2008), birds (Garthe et al., 2011; Hamer et al., 2007; Hedd et al., 2002; Kokubun et al., 2010; Rey and Schiavini, 2005), and mammals (Arnould et al., 2011; Walton and Pomeroy, 2003). Atlantic mackerel larvae *Scomber scombrus* of different size classes selected their prey differently over the years (Robert et al., 2008). The breeding success of northern gannets *Morus bassanus* was found especially stable due to the flexibility of the adults' prey choice (i.e. size, species), the duration of foraging trips and the consistency in bearings of foraging trips and behaviour at sea (Hamer et al., 2007). Moreover, their inter-annual foraging tactic was strongly related to prey availability (Garthe et al., 2011). Food supply and growth of Cassin's auklets *Neocalanus cristatus* were different in 3 consecutive years, and the latter was presumably caused by the variability of prey availability (Hedd et al., 2002). The foraging behaviour of thick-billed murres *Uria lomvia* varied over the years with environmental changes associated with prey distribution (Kokubun et al., 2010). Inter-annual variation was found in the prey item proportion of the southern rockhopper penguins *Eudyptes chrysocome chrysocome* (Rey et al., 2005). In Australian fur seals *Arctocephalus pusillus doriferus* and in grey seals *Halichoerus grypus* (Walton et al., 2003) population-wide, inter-annual diet variations were found (Arnould et al., 2011). Annual diet variation was associated with variable food availability in all of these species, except the Australian fur seals, where the annual variation in diet choice was explained by age and the consistent decline of body condition (Arnould et al., 2011).

Szigeti et al., (2018) found annual variability in the flower visitation in Clouded Apollo butterflies related to variability in floral abundances. Considerable intraspecific variability was found both in proboscis and corolla lengths within a single year (Szigeti et al., 2020; Chapter Three). Variability in proboscis and corolla length may affect trophical relationships between pollinators and their nectar plants. If foragers' and their diet's traits change annually, it will likely influence their trophic relationships that may result in the evolution of these traits (Grant et al., 2014; Moreau et al., 2000).

Goals

We aimed to study if annual variation in diet was related to the variation of a forager's mouthpart morphology (proboscis length) and the morphology of their food resources (corolla length). We studied a nectar-feeding insect pollinator, the Clouded Apollo butterfly *Parnassius mnemosyne*. Clouded Apollo adults spend much time feeding while they visit a few plant species frequently, many occasionally. Occasional visits maybe sampling the nectar supply for an informed decision on switching or not among the available nectar plant species (Szigeti et al., 2019). Foraging behaviour is easy to observe and monitor by mark-resighting in small, closed populations (Szigeti et al., 2018). Adult diet is very different across habitats according to the published lists of their visited nectar plant species (Konvička et al., 2006, 2001; Kudrna et al., 1991; Lara Ruiz, 2011; Pecsenye, 2017; Szigeti et al., 2020, 2018; van Helsdingen et al., 1996; Vojnits et al., 2000). Clouded Apollos prefer habitats with open areas with their nectar species available, at forest edges with their larval host plants, *Corydalis spp.*

We assessed if annual variation in nectar plant visitation was related to proboscis length and the corolla lengths of the four most visited flowering plants with corollas comparable in length with proboscis length throughout the study. (1) We expected proboscis length variability across years because body size is determined by many environmental factors, such as temperature and aridity (García-Barros, 2000), and these factors vary among the years. (2) We also expected annual variation in the corolla lengths of the Clouded Apollos' most visited nectar plant species, because droughts, for example, may result in decreasing flower size (Carroll et al., 2001); and these can vary among years. (3) Based on the results of Szigeti et al. (2020; Chapter Three), we investigated the effect of proboscis and corolla length ratio on flower visits over 5 consecutive years. We addressed the questions if (i) length differences between corolla and proboscis affect foraging behaviour, whether (ii) a focal plant species' Clouded Apollo visitors had longer proboscides than their conspecifics not observed visiting that forb and if (iii) visiting probabilities on a focal nectar species are higher for butterflies with longer proboscis. (iv) Analysing data from 5 consecutive years, we could assess how consistently floral choice could depend on corolla and proboscis length ratios.

We present the methods and results of this chapter together with some of the methods and results of Szigeti et al., (2020; Chapter Three) in order to compare them.

Methods

Study site and period

We carried out field work in Central Hungary, Visegrádi-hegység, Hegyesd (47°45'22.62"N, 19°02'49.54"E, 295 m above sea level, 0.5 hectares), 2015–19 from late April to early June during the entire Clouded Apollo flight periods. This colline meadow is rich in insect-pollinated flowering plant species, and it is surrounded by Turkey Oak *Quercus cerris* forest. It is situated on a south-western slope of a hill, located nearby Tahitótfalu; there are signs for former horticultural activities (e.g. presence of quince *Cydonia oblonga* and poet's daffodil *Narcissus poeticus,* not native in the region).

Butterfly sampling and observations on flower visits

We sampled Clouded Apollos with mark-resighting. We scanned the entire habitat several times per day at a slow pace, and we recorded all individuals. We captured, measured and marked individually all unmarked specimens, then released them. They received a number (black permanent marker) on the ventral side of their hind wings, as well as three colour dots (edding® paint marker) on the apex of their front wings' ventral sides, where the wing is transparent, so the colour code is visible from both ventral and dorsal sides. The marks wear out rarely during the butterflies' lifetime, thus, the specimens can be identified without further capture with binoculars. We did not observe any modification in the butterflies' behaviour due to marking. When we observed an individual feeding, we recorded its identity and the visited nectar plant species.

Nectar plant species

We sampled corolla length in nectar plant species most frequently visited by Clouded Apollos through the 5 years. We excluded plant species with especially short corollas from these measurements, since we assumed that all Clouded Apollos could undoubtedly have access to their nectaries (*see* Király (2009) for scaled flower drawings), thus proboscis length can not constrain accessing the nectar. The most frequently visited nectar plant species with long corollas were *Ajuga genevensis, Buglossoides purpurocaerulea, Dianthus giganteiformis subsp. pontederae, Silene viscaria* (Table A4.4). Note that among these, *Silene viscaria* had the longest corolla, comparable to the Clouded Apollos proboscis length (Szigeti et al., 2020); Chapter Three). We sampled the shallow-flower forb *Geranium sanguineum* only in 2015.

Measurement protocols

We measured proboscis length from photo macrographs (Bauder et al., 2013). We mounted live, non-sedated butterflies on a small, scaled board with clips. Then we fixed the board on a tripod under a camera, parallel to the lens plane (Nikon d7000 + Micro Nikkor 60mm f/2.8G ED AF-S or a Sigma 105 mm f/2.8 EX DG OS macro lens). We uncoiled the proboscis and extended it over a scaled board with a hooked pin, and we took at least two pictures for each individual. We used FIJI/ImageJ (Schindelin et al., 2012) to measure proboscis length from the photographs and used the average of the lengths measured on the pictures of the same individual. Proboscis length was defined as the distance from the anterior edge of the compound eye to the tip of the proboscis (Chupp et al., 2015; Corbet, 2000; Kunte, 2007). We addressed measurement repeatabilites by plotting a Bland-Altman plot (Figure A4.3) and computing a paired t-test and an intraclass correlation coefficient (ICC; Section A4.1, Table A4.5) (Bland and Altman, 1986; Giavarina, 2015; Koo and Li, 2016).

Flowers in all years were selected randomly in time and in space throughout the flight period of the Clouded Apollos. In 2015, VS measured flower depth with callipers *in situ* (resolution: 0.1 mm). We also included the results of FV's callipers measurements in 2015, not included in the analyses in Szigeti et al., (2020; Chapter Three). This was the distance between the bottom of the corolla tube, where it meets the receptacle, and the orifice of the flower tube. We defined the orifice as the point where the butterfly can insert the proboscis into the flower. From 2016, we shot photo macrographs on the flowers *in situ*. A spacer was attached to the camera (Nikon d5100 + Micro Nikkor 60mm f/2.8G ED AF-S lens), with an L-shaped frame parallel to the lens' plane, approximately at the minimum focal distance of the lens. The frame was designed to appear at the edges of each picture, and it bore a printed scale. We adjusted the long axis of the corolla to the plane defined by the frame to shoot standardised pictures without injuring the flowers. We took two pictures from each flower, and we used FIJI/ImageJ (Schindelin et al., 2012) to measure corolla length. Corolla length was defined the same way as measuring with callipers. Measuring from photographs is expected to be more reliable and repeatable than measuring with callipers (see: Vajna et al., 2020a; Chapter Two), and the measurements can be repeated on the archived pictures (Kemper et al., 2009). We addressed measurement repeatabilites by plotting a Bland-Altman plot (Figure A4.3 – measurements with photo macrograph & A4.6 – callipers vs. photo macrographs) and computing a paired t-test and an intraclass correlation coefficient (ICC; Section A4.1, Table A4.5).

Photographs on proboscis length were taken by JK and on corolla length a few by JK, the most by FV. All measurements from the photographs were conducted by FV.

Data analysis

We analysed annual variance in proboscis length with a linear model and compared years with Tukey tests. We compared differences in annual variabilities (standard deviations) with Bartlett's test. We analysed annual variances in the corolla lengths of the Clouded Apollos' most visited nectar plant species with ANOVA and Tukey tests. We calculated the variation coefficient (CV%) for both proboscis and corolla lengths. Flower visitation on these plants was analysed with binomial Generalised Linear Mixed Models (GLMMs); the target variable was whether the observation was on the focal plant species or not; proboscis length was the explanatory variable, the butterfly identifier was the random factor, years were analysed separately. In contrast to Szigeti et al. 2020, Chapter Three, these models did not include flower abundance, since continuous abundance data were available only for 2015. The categorical abundance data collected with the "scanning" method and available for 2015–19 prevented model fit, therefore we evaluated the potential effect of floral abundance graphically. We applied box plots for visualising the data. We carried out all the analyses in the R 3.6.3 statistical environment (R Core Team, 2020). We used the "ggeffect 0.14.2" package (Lüdecke, 2018) for creating the proboscis length ~ flower visitation plots, "lme4 1.1-23" (Bates et al., 2015) for applying GLMMs, the "irr 0.84.1" package for calculating repeatabilities (ICC) (Gamer et al., 2019), and the "BlandAltmanLeh 0.3.1" package for creating plots on measurement agreement (Lehnert, 2015).

Results

Measurement reliability

Both proboscis and corolla lengths measurements taken from photo macrographs were repeatable: the lower 95% confidence limit for ICC for proboscis length was ranged between 0.93 and 0.99; for corolla length, it was between 0.98 and 1.00 (Figure A4.3 & Figure A4.4, Table A4.5). Corolla length measurements with callipers vs. photo macrographs were in agreement (Figure A4.6, Table A4.5). Corolla length measurements of two persons (VS and FV) with callipers were not in agreement (Figure A4.5, Table A4.5).

Clouded Apollos' proboscis length

We measured 168, 191, 186, 272 and 203 Clouded Apollo butterflies' proboscis length between 2015 and 2019, respectively. Proboscis lengths were similar in 2015, 2016 and 2017 whereas in 2018 and 2019 they were significantly shorter than in the previous years, albeit longer in 2019 than in 2018 (Tukey test; Figure 4.1 and in Table 4.1). Standard deviations also differed across years (p = 0.0200; Bartlett's test). Proboscis lengths were not different between the sexes (p = 0.3464; Tukey-test; Figure 4.1). The CV% of the proboscis length were found quite stable, ranging 4.40–5.18 through the five years (Table A4.1).

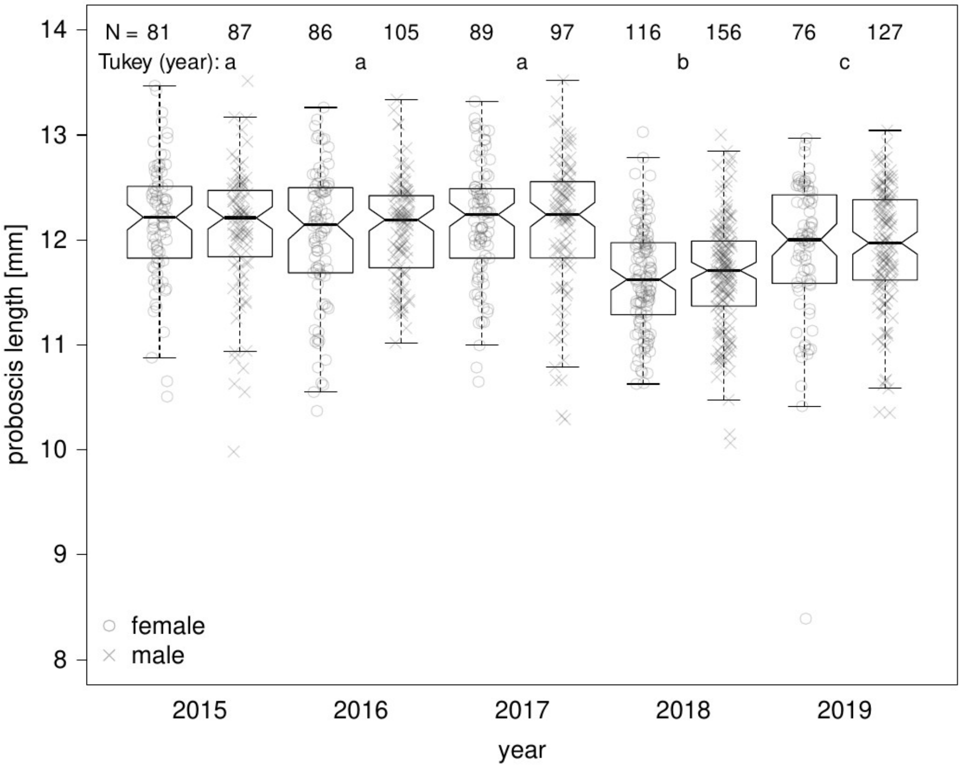

Figure 4.1 Clouded Apollo proboscis length according to years and sexes. The box plots show the median and 25th and 75th percentiles; the whiskers indicate the values within 1.5× the interquartile range extending from below the lower and above the upper quartile. Notches show 95% confidence intervals for the medians. Circles (○) represent individual female,

crosses (×) male butterflies and are jittered on the horizontal axis for better visibility. Letters above the boxes show significantly different annual proboscis lengths (Tukey test). Numbers above the letters show the number of measured butterflies per sex per year.

Table 4.1 Annual variation in Clouded Apollo proboscis length.

	Estimate	**SE**	**P**
intercept	12.13	0.04	0.00
2016	-0.06	0.06	0.31
2017	0.03	0.06	0.56
2018	-0.47	0.06	0.00
2019	-0.2	0.06	0.00

Corolla length of Clouded Apollos' most visited nectar plant species

Over the years, *A. genevensis, B. purpurocaerulea* and *D. giganteiformis* differed in their corolla lengths, whereas *S. viscaria* did not (Tukey tests, Figure 4.2, Table A4.1). Annual changes were different across species. Mean corolla lengths of *A. genevensis* were similar in 2015 and 2018, much longer in 2016, 2017 and 2019. *B. purpurocaerulea* mean corolla lengths were the longest in 2015, similar in 2016 and 2019, longer in 2017 and the shortest in 2018. *D. giganteiformis* corollas were the shortest in 2015, were similar in 2016, 2017 and 2019, but were the longest in 2018. Relative to proboscis length variation, CV%-s are higher for corolla length measurements; the widest range was found in *B. purpurocaerulea* (8.57–15.47), the other CV% values for all the species' corolla length were between these values (Table A4.1).

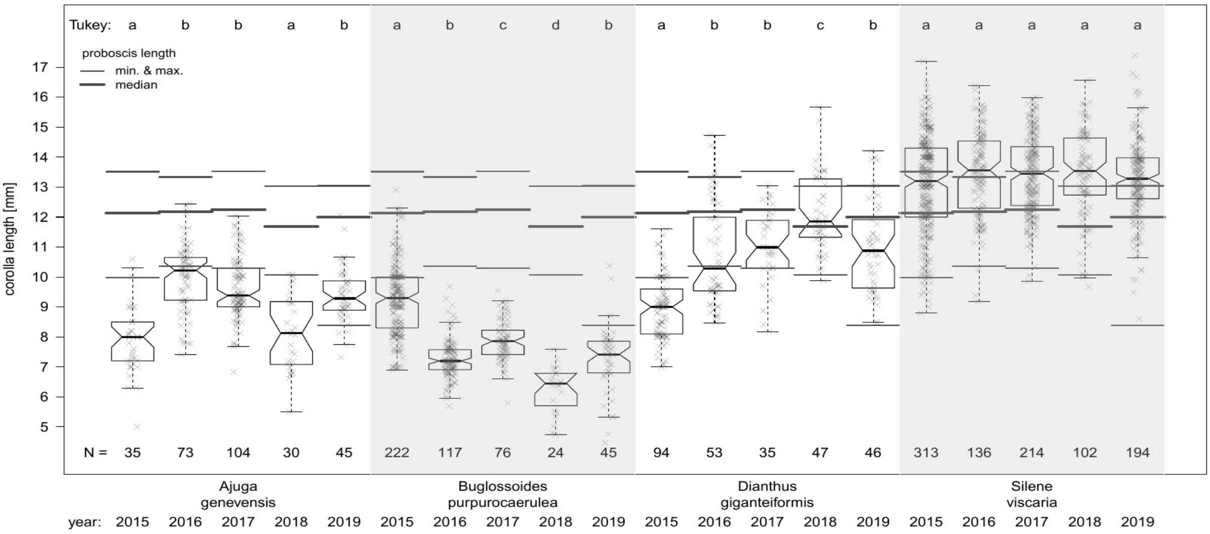

Figure 4.2 Corolla length of the most-visited plant species. The box plots show the median and 25th and 75th percentiles; the whiskers indicate the values within 1.5× the interquartile range extending from below the lower and above the upper quartile. Notches show 95% confidence intervals for the medians. Black crosses represent individual flowers and are jittered on the horizontal axis for better visibility. Horizontal thin lines show the minimum and

maximum, the thick lines median proboscis length. Letters above the boxes show significantly different corolla lengths within a given species (Tukey test). Numbers below the boxes show the number of flowers measured.

Flower visitation of the four most visited plants between 2015–19

We observed 4445 flower visitation by 672 Clouded Apollo individuals throughout the 5 years. We observed 6.7 ± 6.9 (mean ± SD; range: 1–58) visits per individuals. During this time, Clouded Apollos most often visited *D. giganteiformis* (59.5%), *B. purpurocaerulea* (14.8%), *S. viscaria* (6.5%), and *A. genevensis* (5.2%) (Table A4.2). Flower abundances and visits changed considerably across species during the flight periods (Figure A4.1).

Median proboscis length of butterflies observed visiting *S. viscaria* in 2015 was longer than that of butterflies observed visiting other nectar plants. Proboscis length significantly influenced visits to *S. viscaria*: individuals with proboscides 1 mm longer than those of their conspecifics were 2.51× more likely to visit *S. viscaria* (odds ratio; $P < 0.001$; Table 4.2, Figure 4.3). The individual with the longest proboscis (13.52 mm) was estimated to be 15.88× more likely to feed on *S. viscaria* than that with the shortest proboscis (10.51 mm; binomial GLMM; Table 4.2). Median proboscis length of butterflies observed visiting *B. purpurocaerulea* in 2016 was longer than that of butterflies observed visiting other nectar plants. Proboscis length significantly influenced visits to *B. purpurocaerulea* in 2016: individuals with proboscides 1 mm longer than those of their conspecifics were 4.21× more likely to visit *B. purpurocaerulea* in 2016 (odds ratio; $P = 0.02$; Table 4.2, Figure 4.3). The individual with the longest proboscis (13.34 mm) was estimated to be 71.69× more likely to feed on *B. purpurocaerulea* in 2016 than the individual with the shortest proboscis (10.37 mm; binomial GLMM; Table 4.2). Proboscis length did not significantly influence visit probability in any other case.

Table 4.2 Clouded Apollo visits related to proboscis length: summary of the generalized linear mixed models.

Response variable: a visit observed on the focal or on another species	Year	Slope estimate	Standard error	Odds ratio (exp(estimate))	Adjusted P
Ajuga genevensis	2015	-0.56	0.83	0.57	0.69
	2016	1.34	0.61	3.82	0.18
	2017	0.17	0.21	1.19	0.69
	2018	NA	NA	NA	NA
	2019	0.94	2.24	2.55	0.73
Buglossoides purpurocaerulea	2015	-0.33	0.82	0.72	0.73
	2016	1.44	0.48	4.21	**0.00**
	2017	0.12	0.29	1.13	0.73
	2018	NA	NA	NA	NA
	2019	1.31	1.17	3.72	0.69
Dianthus giganteiformis	2015	0.10	0.22	1.11	0.73
	2016	-0.35	0.25	0.71	0.69
	2017	-0.17	0.23	0.84	0.69
	2018	-0.21	-0.21	0.81	0.69
	2019	-0.24	0.28	0.79	0.69
Silene viscaria	2015	0.92	0.24	2.51	**<0.001**
	2016	0.34	0.32	1.40	0.69
	2017	0.21	0.87	1.23	0.81
	2018	0.90	1.17	2.46	0.69
	2019	0.46	0.46	1.58	0.69

The response variable in binomial models was whether the focal floral species or another species had been visited at a specific observation event. We made separate models for the four species. All models included proboscis length as a fixed factor and visiting individuals as a random effect. In the case of $P<0.05$, the number is written in bold; if $P<0.1$, it is written with italics. Note that in 2018 only a single feeding was observed in *A. genevensis*, and none in *B. purpurocaerulea*.

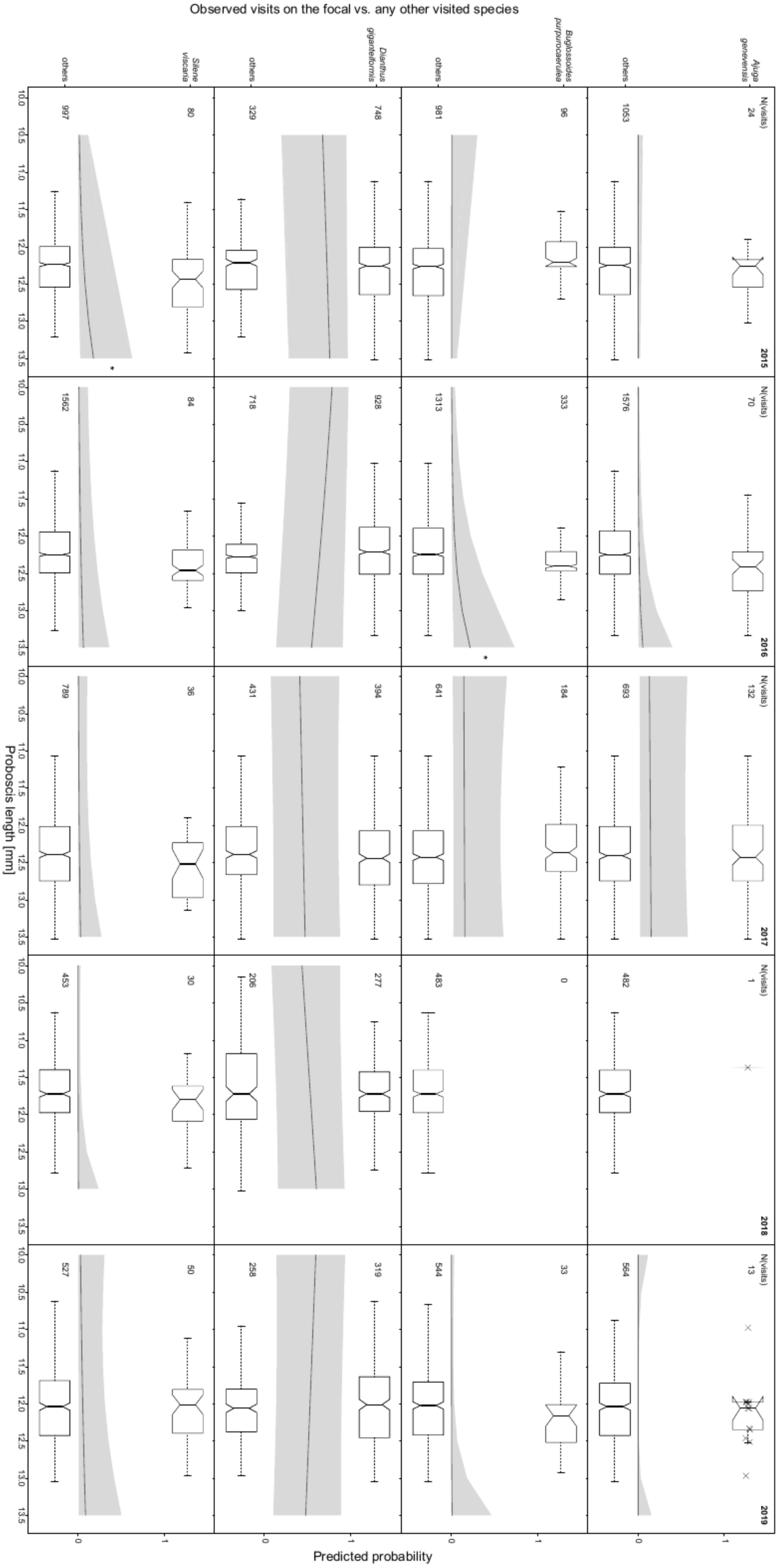
Observed visits on the focal vs. any other visited species
Ajuga genevensis
Buglossoides purpurocaerulea
Dianthus giganteiformis
Silene viscana
others
N(visits)
2015
24
1053
96
981
748
329
80
997
2016
70
1576
333
1313
928
718
84
1562
2017
132
693
184
641
394
431
36
789
2018
1
482
0
483
277
206
30
453
2019
13
564
33
544
319
258
50
527
*
10.0
10.5
11.0
11.5
12.0
12.5
13.0
13.5
Proboscis length [mm]
0
1
Predicted probability

Figure 4.3 Clouded Apollo flower visitation and proboscis length relationships: proboscis lengths compared between butterflies observed on focal nectar plants among the four most-visited species versus those observed on any other species; binomial GLMMs were repeated for all the four most-visited plant species as a focal plant. The box plots show the median and 25th and 75th percentiles; the whiskers indicate the values within 1.5× the interquartile range extending from below the lower and above the upper quartile. Notches show 95% confidence intervals for the medians. Black crosses represent individual observations if less than 20 observations were available and are jittered on the vertical axis for better visibility. Dark grey curves represent regression curves, and light grey bands represent 95% confidence intervals for the regression curves. The asterisk represents a significant effect ($P < 0.05$).

Discussion

Over five consecutive years (2015–19), we investigated the relatedness of Clouded Apollo individuals' proboscis lengths on flower visitations, with corolla lengths of the most visited forbs taken into account. In contrast to our previous study on the same population in 2015 (Szigeti et al., 2020; Chapter Three), where we concluded that in *Silene viscaria*, the species with the longest corolla among those Clouded Apollos visited, corolla length constrained access to nectar, we did not find proboscis and corolla length-dependent visitations *in general*. Proboscis length and visitation were seemingly associated only twice during the five years, in different years and for different plant species.

Proboscis and corolla lengths

We observed considerable individual differences in proboscis length each year (Figure 4.1). Proboscis lengths were not different between the two sexes in any of the years. Similarly to our results Chen et al. (2019) and Peng et al. (2019) did not find differences in proboscis length between the two sexes; contrarily, females had longer proboscis than males (e.g. (Fox et al., 2015; Ramkumar et al., 2010)) or males had longer proboscis than females (e.g. (Martins et al., 2007)); the results can vary across species (Kramer et al., 2018), but most studies did not compare proboscis length between the two sexes (Danaher et al., 2020; Kislev et al., 1972; Martins et al., 2013; Mell, 1940; Nave et al., 2016; Pivnick and McNeil, 1985; Singer et al., 1997). Mean proboscis length varied across years but was similar in 2015, 2016 and 2017 (Figure 4.1); proboscis length CV%-s were stable through the five consecutive years (Table A4.1). Liang et al. (2021) also found tongue length variation in several bumblebee species. Furthermore, in the bumblebees *Bombus balteatus* and *B. sylvicola,* tongue lengths evolved shorter over 40 years (Miller-Struttmann et al., 2015). Another morphological character, forewing shape changed from narrower to broader in the Montane Apollo butterfly *Parnassius apollo* over 30 years (Štefánik and Fedor, 2020). Annual fluctuation was observed in the bill shape (i.e. beak length and depth) of Darwin's finches *Geospiza fortis, scandens*, and *magnirostris* through four decades (Grant et al., 2014).

Corolla lengths were similarly variable within species in each year. While corolla lengths differed in three of the most-visited species, *A. genevensis*, *B. purpurocaerulea* and the overwhelmingly most visited *D. giganteiformis, S. viscaria* corollas were not significantly different across the five years. Interestingly, the relative changes in corolla lengths across the five years were somewhat different in the four nectar species (Figure 4.2); the CV%-s of the corolla length were fluctuating across years, especially in *B. purpurocaerulea* (Table A4.1). Similarly, inter-annual and individual level corolla length variability was found in *Lonicera implexa* (Lázaro et al., 2015); and individual differences were observed in the corolla length and the width of flower tube openings of several plant species visited by bumblebees (Liang et al., 2021). Note that the number of flowers we measured was higher in *S. viscaria* than in the three other species. However, sample sizes were ≥ 30 except in one case, *D. giganteiformis*, 2018, n = 24. Measuring 30 specimens was recommended for accurate morphological studies in several taxa (Cardini et al., 2007; Griffiths et al., 2016; Stec et al., 2016; Van Hook et al., 2012; Wong et al., 2019), thus we think our results are reliable. Some of the corollas of all the four flowers frequently visited by Clouded Apollos were longer compared to the proboscides of a few or many Clouded Apollos in at least some of the years (Figure 4.2, e.g. in case of *D. giganteiformis* of 2018, 21 flowers out of 47 were longer than the upper quartile of the proboscis length of the Clouded Apollos in the same year). In contrast, other frequently visited species (Table A4.2) had much shorter corollas, i.e. Clouded Apollos could easily reach the nectary in all flowers (e.g. *Thymus spp.* (4.9 mm (mean; range 3.0–7.0 mm; CV% 20.8) Méndez-Tovar et al., 2015) or *Ligustrum vulgare* (2.8 ± 0.3 mm (mean ± SE) Barrow and Pickard, 1985). Note that we did not sample corolla length form forbs with obviously short corollas compared to Clouded Apollo proboscis length.

Flower visitations of the four most visited plants between 2015–19

Contrary to our hypothesis, proboscis length did not influence flower visitation, except in *S. viscaria* in 2015 and *B. purpurocaerulea* in 2016. The latter can be explained by the phenology of the species, rather than constraints imposed by long corollas, since in 2016 all *B. purpurocaerulea* corollas were shorter than proboscides (Figure 4.2). Flowers were more abundant at the beginning of the butterflies' flight period (Figure A4.1) and Apollos with longer proboscis lived earlier during this period than those with short tongues (Figure A4.7). In contrast, many *S. viscaria* flowers were longer than most proboscides (Figure 4.2) and flowering phenology was similar to the Apollo's phenology (Figure A4.1). These imply that Clouded Apollos were only constrained by corolla length in visiting *S. viscaria*, while the pattern that long-tongued individuals visited *B. purpurocaerulea* more often than the short-tongued is explained by phenological shifts between flowers and butterflies. Median proboscis length was lower in all years than median *S. viscaria* corolla length, while median proboscis length was larger in all years than median *B. purpurocaerulea* corolla length (Figure 4.2). Clouded Apollo individuals with median proboscis length can access almost every *A. genevensis* and *D. giganteiformis,* all *B. purpurocaerulea,* and approximately half of the *S. viscaria* flowers (Figure 4.2). Although we expected Apollos with only the longer proboscides would visit *S. viscaria* (Szigeti et al., 2020; Chapter Three), proboscis length did not predict its visitation between 2016–19 (Figure 4.3, Table 4.2). These results imply that proboscis and corolla length ratios do not directly impact access to flowers, at least in pollinators not highly specialised to long-corolla plants (in contrast, see e.g. the highly specialised Wallace's sphinx moth *Xanthopan predicta* (Minet et al., 2021)). These indicate that factors other than just proboscis and corolla length ratios may influence foraging patterns in insect pollinators.

Among bumblebee species, longer proboscis was associated with a narrower body (Inoue et al., 2006), implying that insect flower visitors may insert their heads into the flower and reach deeper than proboscis length *per se* would predict, favouring the long-proboscised even more in accessing long corollas. Our results, as well as those of Inoue et al. (2006), contradict the assumption that proboscis length should be longer or at least equal to corolla length for nectar exploitation (Corbet, 2000; May, 1992; Pauw et al., 2009). However, in Clouded Apollos, head width (the longest distance between the perimeters of the eyes, frontal view, as the widest part of the body in butterflies (data from (Somlay, 2021)), increases with increasing proboscis length (Spearman's rho = 0.43, $p < 0.001$, N = 520; details in Table A4.3). It suggests that, contrary to bumblebees, Clouded Apollo individuals with shorter proboscis and narrower head may tug their heads deeper into the corolla than long-proboscised conspecifics, and may able to reach the nectar. The ratio of the diameter of the flowers' corollas and flower visitors' heads could be a limiting factor for pollinators in flower choice (Jervis et al., 1993). However, Liang et al. (2021) found that long-tubed flower species had significantly wider openings than the bumblebees' intertegular span, which indicates that these flower visitors are able to put into these flowers not only their heads but also a part of their bodies. This might be true to smaller Clouded Apollos as well, albeit we did not measure flower entry width, and we are unable to test this hypothesis. Furthermore, different body parts may develop differently across years, and size may vary accordingly, probably due to climatic impacts on larval development (Boggs et al., 2005; García-Barros, 2000). This may also contribute to annual variation in accessing specific resources. Similar processes in plant development (Hatfield and Prueger, 2015; Herrero and Zamora, 2014) may contribute to further variation. Taken together, the proboscis – head width relationship indicates that measuring solely proboscis length may underestimate the reach of an individual, and for both pollinators and flowers, multiple morphological characters should be analysed simultaneously.

The handling- and feeding times of Clouded Apollo butterflies are similar across the most visited species, thus the profitability of these species seems to be similar, and species with similar profitabilities may be interchangeable sources for foragers (Gór, 2017). Although Gór (2017) did not take into account the probability of whether a flower contains nectar or not, it can be rather different in different species (Szigeti, 2018). In our study sites, *D. giganteiformis* and *S. viscaria* provided the highest quantity of "standing crop" nectar (i.e. the amount of nectar of a flower at a given time (Nicolson et al., 2007)) (Vajna, 2016). Nectar production and content depend on several factors (Dohzono et al., 2011; Erhardt et al., 2009; Kay et al., 1984). The composition and amount of nectar influence the flower choice of butterflies (Erhardt, 1991; Erhardt et al., 2009). We propose the hypothesis that similarly to the annual variation of proboscis and corolla length, as well as floral abundance (Szigeti et al., 2018; Vajna et al., 2021b), nectar rewards may fluctuate across years (Kasagi and Kudo, 2003), thus altering annual profitability and the effort to invest into exploiting long-corolla flowers. The composition and quantity of the produced nectar is affected by various environmental factors (Baker et al., 1983; Farkas et al., 2012; Nicolson et al., 2007), possibly resulting in very high inter-specific variability (Witt et al., 1999); so the fluctuation of nectar rewards may manifest even within a day (Galetto et al., 2004). According to Szigeti (2018), among the frequently visited flower species except for *D. giganteiformis* and *S. viscaria*, two species in the longer corolla range among the most visited species in this population, a vast number of flowers contained no detectable nectar. Clouded Apollo individuals visited some of the available nectar species in a large proportion, many others occasionally, and they also avoided several species (Szigeti et al., 2019). In this scenario, butterflies would invest more in long-corolla flowers more challenging to exploit, (e.g. due to longer handling time than forbs with shorter corollas easy to access) in years when nectar yields of the former are extremely high, nectar compositions are especially favourable and/or relative abundances are high.

Similarly, flower abundance might influence the effort individuals with proboscides shorter than corollas would invest in accessing flowers if nectar-rich species are very abundant. *D. giganteiformis* and *S. viscaria* were abundant during 2015–19, while *B. purpurocaerulea* was abundant in 2015–17 but not in 2018–19 and *A. genevenis* was not abundant during any of the flight periods (Table A4.4, Figure A4.1). The overwhelmingly most visited species during 2015–19 was *D. giganteiformis,* although *B. purpurocaerulea* was still visited frequently between 2015 and 2017 (Table A4.4, Figure A4.1). *D. giganteiformis* and *S. viscaria* were visited in a similar proportion compared to their abundances over the 5 years (Figure A4.2). The flowering period of *D. giganteiformis, S. viscaria* and *A. genevensis* overlapped with the flight period of the Clouded Apollos in all years, while the flowering peaks of *B. purpurocaerulea* were before the peak number of the observed Clouded Apollo individuals (Figure A4.1). These imply that by far the most abundant and relatively accessible and nectar-rich *D. giganteiformis* was overall the most profitable forb in all years. This may have reduced the need to exploit other species, resulting in arbitrary visit patterns according to relative proboscis length.

Annual variation

We found significant annual variation in proboscis (Figure 4.1, Table A4.1) and corolla lengths (Figure 4.2). CV%-s indicate larger variation in corolla length than proboscis length (Table A4.1), implying stronger directional selection on proboscis length. The corolla lengths of two out of four plant species were different in 2015 then in the following years (Figure 4.2). This pattern can be (i) an artefact caused by the different measurement methods, i.e. callipers in 2015 and photo macrographs in 2016–19 or (ii) a natural process, e.g. differential development of the flowers driven by environmental factors such as soil water accessibility. If it was a measurement artefact, we expect all corollas to have been shorter or longer in 2015 than in the other years. However, we found that corolla lengths were more or less similar in 2016, 2017 and 2019 and different in both 2015 and 2018 in three species, while they did not vary in *S. viscaria*. We witnessed exceptionally hot and dry weather during the 2018 Clouded Apollo flight period. Drought stress may reduce flower size (Carroll et al., 2001), and *B. purpurocaerulea* corollas were shorter this year than in other years. However, *D. giganteiformis* corollas were longer in this year compared to others, and corolla length changed just the opposite direction as in *B. purpurocaerulea* in 2015 (Figure 4.2). These indicate that different forbs react to similar conditions rather differently, and these are in line with the environmental impact hypothesis. These variations may also partly explain why insect pollinators visiting a range of flowering plants may show fluctuating visit patterns throughout different years.

In Darwin's finches *Geospiza spp*. beak morphology and the success (i.e. fitness) of a certain beak shape was found fluctuating across years, due to weather-driven diet variability (Grant et al., 2014). Evolution of body sizes may happen on a short time scale, i.e. less than a million year. Short term phenotypic fluctuation may therefore be the result of local variation in niche optima due to restricted environmental variation within a stable adaptive zone (Uyeda et al., 2011). We were not able to estimate the Clouded Apollos' reproductive success, nor proboscis length heritability, but in a few years period, we observed proboscis length fluctuation, as well as the fluctuation of the nectar plant supply, albeit no casual relationship of the two can be established. Heritability of butterflies morphological traits was observed in a few studies (Bauerfeind and Fischer, 2008; Ellers and Boggs, 2002; Seko et al., 2006). Corolla length heritability was found by Campbell (1996) and Gómez et al. (2009).

Fluctuation in weather conditions may also impact insects: strong associations between weather and population fluctuations were found in many butterfly species over 2 decades (Roy et al., 2001) and rainfall affected insect abundance in a 5-year-long study

(Denlinger, 1980). Environmental factors may affect several aspects of communities over the years and decades, such as the available nectar sources, phenologies of plants (Song et al., 2020), flower visitors (Dell et al., 2005), as well as plant-pollinator networks (Marshall et al., 2020). Changes in environmental conditions over the years may also contribute to differences in ecological dynamics, such as community composition, indicating the importance of multi-year studies (Werner et al., 2020). Furthermore, human-impacted environmental stress, such as habitat degradation, may affect morphology over years (Štefánik et al., 2020). Including intraspecific trait variation in entomological studies may improve our understanding the underlying natural processes (Gentile et al., 2021). These results imply that single-year studies may rather be snapshots than reveal actual population trends, and highlight the importance of long-term studies (García-Barón et al., 2021; Rodríguez-Muñoz et al., 2019).

General discussion

Our major goal was to identify plant and floral traits, as well as proboscis length related to nectar plant choice in Clouded Apollo butterflies. We began with looking for plant traits as potential drivers of nectar plant species choice (Chapter One), then focused on the role of within-population variation of proboscis length relative to corolla lengths in floral visits (Chapters Three and Four).

The main driver impacting flower visitation was the plant species' abundance, while flower colour and structure had only minor effects (Figure 1.3–1.4). The relative abundances of insect-pollinated plant species differed in two nearby habitats. Clouded Apollos are sequential specialists in their nectar plant species choice: they visited some of the available nectar species in a great proportion, many others occasionally, and they were able to switch to a few nectar sources once they become abundant (Szigeti et al., 2019). Plant type (i.e. herbaceous or woody), flower colour, corolla type (i.e. tubular or non-tubular) influenced choice in many butterfly species belonging the Papilionidae family (Mertens et al., 2021).

The Clouded Apollos' proboscis length varied significantly among individuals within the same generation and across years (Figure 3.1 & 4.1, Table A4.1), as did corolla lengths of the most visited nectar plant species that also varied across species (Figure 4.2, Table A4.1). In some years we observed a slight decline (always less than 1 mm) in proboscis length with the progress of the flight period. This means that individuals entering the population early had longer proboscides compared to those entering late in the flight period (Figure A4.7). Soteras et al. (2020) also observed changes in mean proboscis length of hawk-moths among years, possibly explained by short-term changes in environmental variables. Differences between the proboscis length of two Clouded Apollo individuals measured at first capture the same day can be larger than the average decline along the flight period (Figure A4.7). However, this slight decline might be relevant, since, individuals with proboscides 1 mm longer than those of their conspecifics were 2.51× more likely to visit *S. viscaria* in 2015 and individuals with proboscides 1 mm longer than those of their conspecifics were 4.21× more likely to visit *B. purpurocaerulea* in 2016. The idea to investigate proboscis and corolla lengths originates in field observations hinting that annual variation in the visit rates of the longest corolla forb Clouded Apollos visited in our study sites, could be the result of varying proboscis–corolla ratio. This annual variation was observed in the visit rates of the Sticky Catchfly *Silene viscaria* despite the forbs' more or less constant abundance across different flight periods (Szigeti, 2018). Our first results seemed to corroborate this hypothesis (Szigeti et al., 2020; Chapter Three, Figure 3.2). It would be the first evidence that individual variation in proboscis length affects butterflies' nectar plant choice in natural circumstances. However, these results were based on a single year and investigating further years, albeit with somewhat different methodology, yielded a fluctuating pattern that did not corroborate our previous conclusion. In contrast to our conclusion of Szigeti et al., (2020; Chapter Three), proboscis length did not significantly influence behaviour in general: through the five years of the study, only in two cases did proboscis length relate to flower visitation in two different species, and in two different years (Figure 4.3). In addition, one of these was probably related to a time shift in flowering and flight phenology, rather than proboscis-corolla length ratios (Figure A4.1). Annually fluctuating relationships may be explained by (i) environmental factors affecting plants (Song et al., 2020), flower visitors (Dell et al., 2005) and plant-pollinator networks (Marshall et al., 2020) as well as by (ii) effects not studied here, e.g. the relationship of multidimensional trait matching of Clouded Apollos including proboscis length and head width with corolla length and diameter of its orifice, or nectar yield, etc. These results emphasise the importance of long-term studies since single-year studies may rather be snapshots than reveal general population characteristics (García-Barón et al., 2021; Rodríguez-Muñoz et al., 2019).

This research should be expanded in time and space, including annual monitoring of foraging and floral abundance in multiple habitats across a long-term study. Sampling nectar yields, applying spectrophotometry to assess floral colour instead of human vision-based categories and developing further measurement techniques to better estimate the butterflies' access to their most important nectar plants could significantly improve our understanding of foraging patterns. Since the availability of resources is not constant (Figure A4.1), Clouded Apollo individuals were found switching among the available nectar plant species throughout their lifetime, following the temporal changes of the availability of nectar plants (Szigeti et al., 2019), flower consistency should be include in the evaluation of plant-pollinator relationships.

As Jervis et al. (1993) and Liang et al. (2021) pointed out, the ratio of the diameter of the pollinators' heads and the diameter of the flowers' orifice can be a limiting trait of flower choice and feeding. Studying the location of the nectary related to the corolla orifice may also yield more accurate estimates for the minimum proboscis length required for access (Krenn et al., 2021). We reported methods to measure proboscis length in live butterflies (Szigeti et al., 2020; Chapter Three) and corolla length *in situ* (Chapter Four). We think both methods are sufficiently accurate and relatively easy to apply to a large sample. The advantages of these photographic methods are that they provide high resolution and higher repeatability, are quick in the field and non-invasive to the specimens. Photographs also can be archived and later revisited (Kemper et al., 2009), and the butterflies' behaviour can be observed after taking pictures. Disadvantages are (i) mounting live butterflies under the camera to take pictures requires time and practice, and (ii) measuring on photographs takes considerable time later in the lab. Our proboscis measurement protocol may be similar to Bauder et al. (2013) and Bauder et al. (2014), albeit similarity can not be assessed due to their vague protocol description. Overall, a vast range of studies applied proboscis measurement preceding our studies, but information on protocols were not accurate enough to base our research on already published methods (Vajna et al., 2020a; Chapter Two) and this malpractice seems to be expanding over a wide range in biology from ecology (Mortelliti et al., 2010; Szigeti et al., 2016b) through biomedical research (Glasziou et al., 2014) to cancer research (Errington et al., 2021). We are aware that our descriptions of proboscis and corolla length measurements presented here could be further detailed. We plan the publication of methodology, further expanding to several butterfly species for proboscis length. We have already worked on measuring butterfly head width (Somlay, 2021) while further dimensions of floral structures are yet to be investigated.

Besides the relevance of these results in the studies of plant-pollinator systems and foraging ecology, we think these are also important contributions to Clouded Apollo biology and provide relevant information for planning conservation strategies for this species. The Clouded Apollo is protected by the Bern Convention, the European (van Swaay et al., 2010), and the Hungarian Red List (KöM, 2001). Both its southern and northern European distribution ranges had been shifted polewards in the second half of the 20th century, likely due to climate warming (Parmesan et al., 1999). Insect decline was reported in numerous studies (e.g. (Cardoso et al., 2020; Doré et al., 2021; Gérard et al., 2020; Halsch et al., 2021; Warren et al., 2021; Zattara and Aizen, 2021). We experienced the Clouded Apollos vulnerability: at Leány-kúti rét, their number was radically decreased during 2009–13. One suitable explanation for this phenomenon could be the forest overgrowth, meaning the loss of the essential open patches from their habitat, where the nectar plants live. It is crucial to protect butterflies by protecting the habitat of their nectar plants. Population size has started to decrease sharply also at Hegyesd since 2020 (J. Kis, pers. comm.). Although climatic impacts may underlie this decline, habitats climatically still suitable can be managed informing on important nectar resources.

New scientific results

1. Nectar plant species choice in the Clouded Apollo butterflies was influenced mainly by the plant species' abundance and by the colour and structure of the flowers. Drivers influencing visitation may be different among habitats.
2. By reviewing lepidopteran proboscis length methodologies, we found that a significant portion of the articles had not disclosed descriptions of the methodology sufficiently detailed for reproducibility, making it difficult for newbies to the field to apply their methodologies. We provided detailed recommendations for planning, conducting and publishing lepidopteran proboscis length measurements.
3. We developed non-invasive, repeatable photographic techniques for measuring proboscis length in live Clouded Apollos and corolla length *in situ* of long-corolla flowers.
4. We found considerable individual and annual variation in Clouded Apollo proboscis length (Figure 4.1, Table A4.1), as well as in the corolla lengths of its most visited nectar resources (Figure 4.2, Table A4.1).
5. We found that individual variation in proboscis length may be related to nectar plant choice in natural circumstances in a species not specialised to a single nectar plant. However, studying multiple years proved this finding controversial, probably due at least partially to the high annual variation in corolla and proboscis lengths. Our results are inconsistent with the assumption that in order to access nectar, proboscis length of an individual should be as long or longer than corolla length. We propose investigating multiple morphological traits at once, both on the plant and the pollinator side.

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Appendices

A1: Appendix for Chapter One

From the supplementary material of Vajna Flóra, Szigeti Viktor, Harnos Andrea és Kis János: A kis apollólepke (*Parnassius mnemosyne* (LINNAEUS, 1758)) nektárnövényfajok közti választása, Állattani Közlemények, Volume: 106, Issue: 1–2, Pages: 11–37. Available at: http://www.mbt-biologia.hu/gen/pro/mod/let/let_fajl_kiiras.php?i_faj_azo=2012&b_megnyitas=igaz

Table A1.1 Annual Clouded Apollo flower visit ratios (%) at Leány-kúti rét and Hegyesd. NA-s denote plant species not observed (not available) in specific years.

	Visitation ratio (%)						
	Leány-kúti rét					Hegyesd	
Plant species	2009	2010	2011	2012	2013	2014	2015
Ajuga genevensis	1.1	2.0	1.4	0.4	3.8	3.4	1.7
Ajuga reptans	NA	NA	NA	NA	NA	0.3	0.0
Anacamptis morio	0.2	0.5	0.0	0.0	0.0	0.1	0.0
Anthyllis vulneraria	0.0	0.0	0.0	0.0	0.0	NA	NA
Arabis sp.	0.0	0.0	0.0	0.0	0.0	0.1	0.0
Berberis vulgaris	0.0	0.0	0.0	0.0	0.0	NA	NA
Buglossoides purpurocaerulea	20.8	0.3	1.1	0.0	8.2	4.8	8.9
Campanula persicifolia	NA	NA	NA	NA	NA	0.0	0.0
Campanula rapunculus	0.0	0.0	0.1	0.0	0.0	0.0	0.0
Capsella bursa-pastoris	NA	NA	NA	NA	NA	0.0	0.0
Cerastium sp.	NA	NA	NA	NA	NA	0.0	0.0
Clematis integrifolia	0.0	0.0	0.0	0.0	0.0	NA	NA
Colutea arborescens	NA	NA	NA	NA	NA	0.0	0.0
Cornus mas	NA	NA	NA	NA	NA	0.0	0.0
Cornus sanguinea	NA	NA	NA	NA	NA	0.1	0.0
Cota tinctoria	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Crataegus monogyna	0.0	0.0	0.0	0.4	0.0	0.0	0.0
Cydonia oblonga	NA	NA	NA	NA	NA	0.0	0.0
Cynoglossum officinale	NA	NA	NA	NA	NA	0.0	0.0
Dianthus collinus	0.0	0.0	0.0	0.0	0.0	NA	NA
Dianthus giganteiformis subsp. pontederae	21.3	48.3	15.2	60.4	42.8	73.6	70.3
Dictamnus albus	0.0	0.0	0.0	0.0	0.0	NA	NA
Digitalis grandiflora	NA	NA	NA	NA	NA	0.0	0.0
Eremogone procera	0.0	0.2	0.6	0.4	0.6	NA	NA
Euonymus verrucosus	0.0	0.0	0.0	0.0	0.0	NA	NA
Euphorbia cyparissias	0.2	0.2	0.2	0.0	0.0	0.2	0.0
Euphorbia epithymoides	NA	NA	NA	NA	NA	0.0	0.0
Ficaria verna	0.0	0.0	0.0	0.0	0.0	NA	NA
Filipendula vulgaris	0.0	0.2	0.3	0.7	0.0	NA	NA
Fragaria viridis	6.2	9.2	16.2	15.9	1.3	0.5	0.3
Fraxinus ornus	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Galium glaucum	NA	NA	NA	NA	NA	0.1	0.0
Galium sp.	0.0	0.0	0.0	0.0	0.0	NA	NA
Genista tinctoria	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Geranium robertianum	0.0	0.0	0.0	0.0	0.0	NA	NA
Geranium sanguineum	NA	NA	NA	NA	NA	2.6	3.3
Geum urbanum	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Helianthemum ovatum	0.0	0.0	0.0	0.0	0.0	NA	NA
Helianthemum sp.	NA	NA	NA	NA	NA	0.0	0.0
Hieracium bauhini	0.0	0.0	0.0	0.0	0.0	0.3	0.0
Hypochaeris oligocephala	NA	NA	NA	NA	NA	0.0	0.0
Inula hirta	0.0	0.2	0.5	0.0	1.3	0.2	0.2
Iris graminea	NA	NA	NA	NA	NA	0.0	0.0
Iris variegata	NA	NA	NA	NA	NA	0.0	0.0
Lamium purpureum	0.0	0.6	0.0	0.0	0.0	0.1	0.0
Lathyrus latifolius	0.0	0.0	0.0	0.0	0.0	NA	NA
Lathyrus nissolia	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Lathyrus tuberosus	NA	NA	NA	NA	NA	0.0	0.0
Leopoldia comosa	NA	NA	NA	NA	NA	0.0	0.0
Lepidium campestre	0.0	0.0	0.0	2.2	2.5	0.0	0.0
Leucanthemum vulgare	0.0	0.0	0.1	0.0	0.0	0.1	0.1
Ligustrum vulgare	0.0	0.0	0.0	0.0	0.0	NA	NA
Lithospermum arvense	NA	NA	NA	NA	NA	0.0	0.0
Lotus corniculatus	0.0	0.0	0.1	0.0	0.0	NA	NA
Melampyrum cristatum	0.0	0.0	0.0	0.0	0.0	NA	NA
Myosotis stricta	0.0	0.3	0.1	0.0	0.0	0.1	0.0
Orchis mascula	NA	NA	NA	NA	NA	0.0	0.0
Ornithogalum orthophyllum subsp. kochi	0.0	0.0	0.0	0.0	0.0	NA	NA
Orobanche sp.	NA	NA	NA	NA	NA	0.0	0.0
Plantago sp.	0.0	0.0	0.0	0.0	0.0	NA	NA
Polygala comosa	4.4	9.1	3.9	0.4	2.5	0.0	0.0
Polygonatum odoratum	NA	NA	NA	NA	NA	0.0	0.0
Potentilla sp.	0.0	0.0	0.4	0.0	0.0	0.1	0.0
Primula veris	NA	NA	NA	NA	NA	0.0	0.0
Prunus spinosa	NA	NA	NA	NA	NA	0.0	0.0
Pseudolysimachion spicatum	0.0	0.0	0.0	0.0	0.0	0.1	0.0
Ranunculus acris	NA	NA	NA	NA	NA	0.3	0.0
Ranunculus illyricus	0.0	0.0	0.0	0.0	0.6	NA	NA
Ranunculus polyanthemos	0.5	4.4	3.2	1.9	1.3	NA	NA
Rhinanthus minor	0.0	0.0	0.0	0.0	1.3	NA	NA
Robinia pseudoacacia	NA	NA	NA	NA	NA	0.0	0.0
Rosa canina	0.0	0.0	0.0	0.0	0.0	0.1	0.0
Rosa gallica	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Salvia nemorosa	0.0	0.0	0.4	3.7	0.0	NA	NA
Salvia pratensis	NA	NA	NA	NA	NA	0.1	0.0
Sambucus nigra	0.0	0.0	0.0	0.0	0.0	NA	NA
Sanguisorba minor	0.0	0.0	0.0	0.0	0.0	NA	NA
Saxifraga bulbifera	0.2	0.2	1.0	0.0	0.0	0.1	0.0
Scorzonera laciniata	0.0	0.0	0.1	0.0	0.0	NA	NA
Silene nutans	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Silene viscaria	36.5	9.8	44.5	8.9	18.2	7.4	8.0
Silene vulgaris	NA	NA	NA	NA	NA	0.0	0.0
Stachys recta	NA	NA	NA	NA	NA	0.0	0.0
Stellaria graminea	0.0	0.0	0.0	0.0	0.0	NA	NA
Stellaria holostea	0.0	0.2	0.0	0.0	0.0	0.0	0.0
Symphytum tuberosum	NA	NA	NA	NA	NA	0.0	0.0
Syringa vulgaris	NA	NA	NA	NA	NA	0.2	0.1
Tanacetum corymbosum	0.0	0.0	0.0	0.0	0.0	NA	NA
Taraxacum officinale	0.0	0.0	0.0	0.0	0.0	NA	NA
Thymus odoratissimus	0.2	3.3	1.0	0.0	10.7	0.7	0.3
Tragopogon orientalis	NA	NA	NA	NA	NA	0.0	0.0
Trifolium alpestre	0.0	0.2	1.0	0.0	0.0	2.1	0.2
Trifolium campestre	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Trifolium montanum	1.3	4.2	3.2	4.1	5.0	0.5	0.0
Trifolium pratense	4.5	3.1	2.5	0.0	0.0	NA	NA
Trifolium repens	0.0	0.0	0.3	0.0	0.0	NA	NA
Valerianella locusta	NA	NA	NA	NA	NA	0.0	0.0
Verbascum phoeniceum	0.0	0.2	0.0	0.0	0.0	0.1	0.0
Veronica austriaca	0.0	0.0	0.0	0.0	0.0	NA	NA
Veronica chamaedrys	0.3	0.3	0.7	0.0	0.0	0.0	0.0
Veronica teucrium	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Vicia angustifolia	0.0	0.0	0.0	0.0	0.0	0.4	0.3
Vicia cracca	2.4	3.3	1.9	0.0	0.0	1.9	5.6
Vicia sp.	NA	NA	NA	NA	NA	0.0	0.0
Vincetoxicum hirundinaria	0.0	0.0	0.0	0.7	0.0	0.0	0.0
Viola arvensis	0.0	0.0	0.0	0.0	0.0	0.3	0.0

Table A1.2 Distribution of the number of plant species according to Clouded Apollo visit ratios (categorised) in the studied years at Leány-kúti rét and Hegyesd. Column sub-headers show visit categories (from left to right: non-visited, visited <1%, >1% except the 4 most visited, the 4 most visited species in a specific year; all visited species and all insect-pollinated species with grey background.

			not visited	<1%	1%< (except the 4 most visited)	annually 4 most visited	all visited	all insect pollinated
number of nectar plant species	Leány-kúti rét	2009	56	6	5	4	15	71
		2010	48	13	6	4	23	71
		2011	45	16	6	4	26	71
		2012	58	6	3	4	13	71
		2013	57	2	8	4	14	71
		2009-13	36	18	10	7	35	71
	Hegyesd	2014	49	19	3	4	26	75
		2015	57	12	2	4	18	75
		2014-15	44	24	2	5	31	75

Table A1.3 Visitation ratio [%] and median flower abundance of the 5 most visited species in each of the observed years.

species name	location	year	visitation ratio [%]	median flower abundance
Silene viscaria	Leány-kúti rét	2009	3.53	5
Dianthus giganteiformis subsp. pontederae			21.27	5
Ajuga genevensis			20.78	4
Fragaria viridis			6.17	5
Trifolium pratense			4.55	2
Dianthus giganteiformis subsp. pontederae		2010	48.28	5
Silene viscaria			9.84	4
Fragaria viridis			9.22	5
Polygala comosa			9.06	5
Ranunculus polyanthemos			4.38	4
Silene viscaria		2011	44.52	2
Fragaria viridis			16.24	4
Dianthus giganteiformis subsp. pontederae			15.24	3
Polygala comosa			3.88	3
Ranunculus polyanthemos			3.19	5
Dianthus giganteiformis subsp. pontederae		2012	60.37	3
Fragaria viridis			15.93	3
Silene viscaria			8.89	2
Trifolium montanum			4.07	2
Salvia nemorosa			3.70	1
Dianthus giganteiformis subsp. pontederae		2013	42.77	3
Silene viscaria			18.24	2
Thymus odoratissimus			10.69	4
Ajuga genevensis			8.18	1
Trifolium montanum			5.03	5
Anacamptis morio			3.77	0
Dianthus giganteiformis subsp. pontederae	Hegyesd	2014	73.59	5
Silene viscaria			7.44	3
Buglossoides purpurocaerulea			4.83	2.5
Ajuga genevensis			3.35	0.5
Geranium sanguineum			2.61	3
Dianthus giganteiformis subsp. pontederae		2015	70.26	4
Buglossoides purpurocaerulea			8.94	4
Silene viscaria			8.03	3
Vicia cracca			5.57	2
Geranium sanguineum			3.28	3

A2: Appendix for Chapter Two

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Table A2.1 https://static-content.springer.com/esm/art%3A10.1007%2Fs00435-020-00507-z/MediaObjects/435_2020_507_MOESM1_ESM.xls

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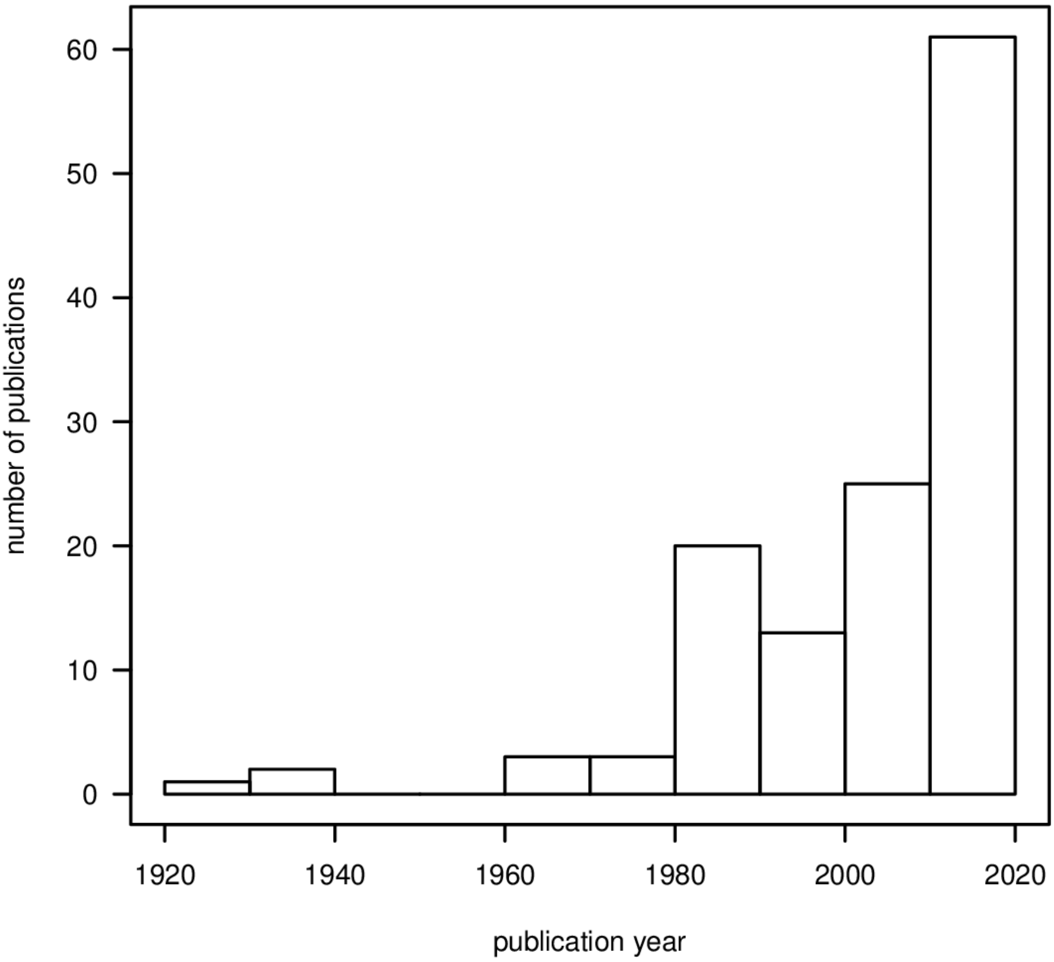

Figure A2.1 Changes in the number of publications investigating lepidopteran proboscis length in the last 100 years.

A3: Appendix for Chapter Three

From the supplementary material of Viktor Szigeti, Flóra Vajna, Ádám Kőrösi, János Kis: Are all butterflies equal? Population-wise proboscis length variation predicts flower choice in a butterfly. Animal Behaviour, Volume 163, May 2020, Pages 135-143. Available at: https://www.sciencedirect.com/science/article/pii/S0003347220300683#gs1.

Table A3.1 Flower depth comparisons among the six species visited the most by Clouded Apollos.

Species-pairs compared	**P**
Buglossoides purpurocaerulea-Ajuga genevensis	0.0005
Dianthus giganteiformis-Ajuga genevensis	0.0211
Geranium sanguineum-Ajuga genevensis	<0.0001
Silene viscaria-Ajuga genevensis	<0.0001
Vicia cracca-Ajuga genevensis	0.0225
Dianthus giganteiformis-Buglossoides purpurocaerulea	0.9955
Geranium sanguineum-Buglossoides purpurocaerulea	<0.0001
Silene viscaria-Buglossoides purpurocaerulea	<0.0001
Vicia cracca-Buglossoides purpurocaerulea	<0.0001
Geranium sanguineum-Dianthus giganteiformis	<0.0001
Silene viscaria-Dianthus giganteiformis	<0.0001
Vicia cracca-Dianthus giganteiformis	<0.0001
Silene viscaria-Geranium sanguineum	<0.0001
Vicia cracca-Geranium sanguineum	<0.0001
Vicia cracca-Silene viscaria	<0.0001

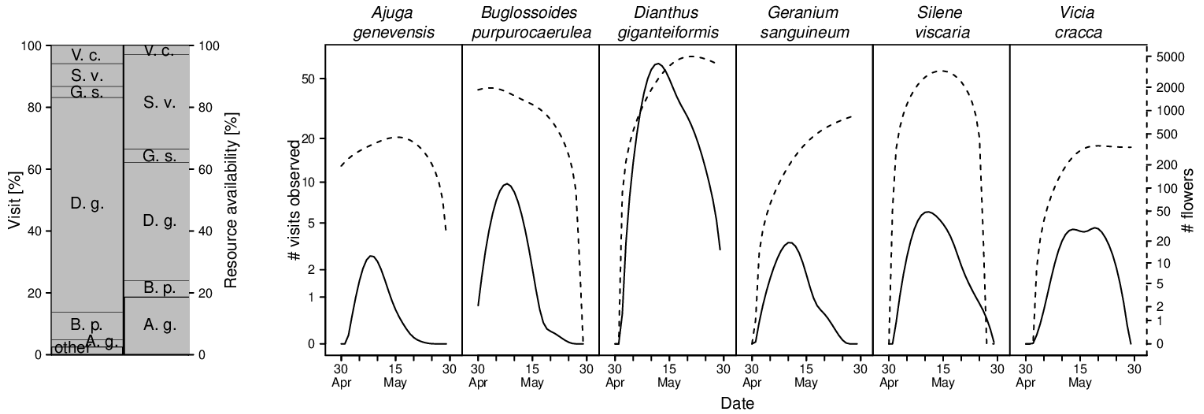

Figure A3.1: (a) Clouded Apollo annual visit rates and resource availability of the six most-visited nectar plants (abbreviations correspond to species names in (b)) and (b) temporal changes during the Clouded Apollo flight period in flowering and visits for the six most-visited nectar plants. Dashed curves show changes in flower abundances and solid curves changes in the number of visits observed. All curves are kernel smoothed. Note that both vertical axes in (b) are $\log_{10}$ scaled.

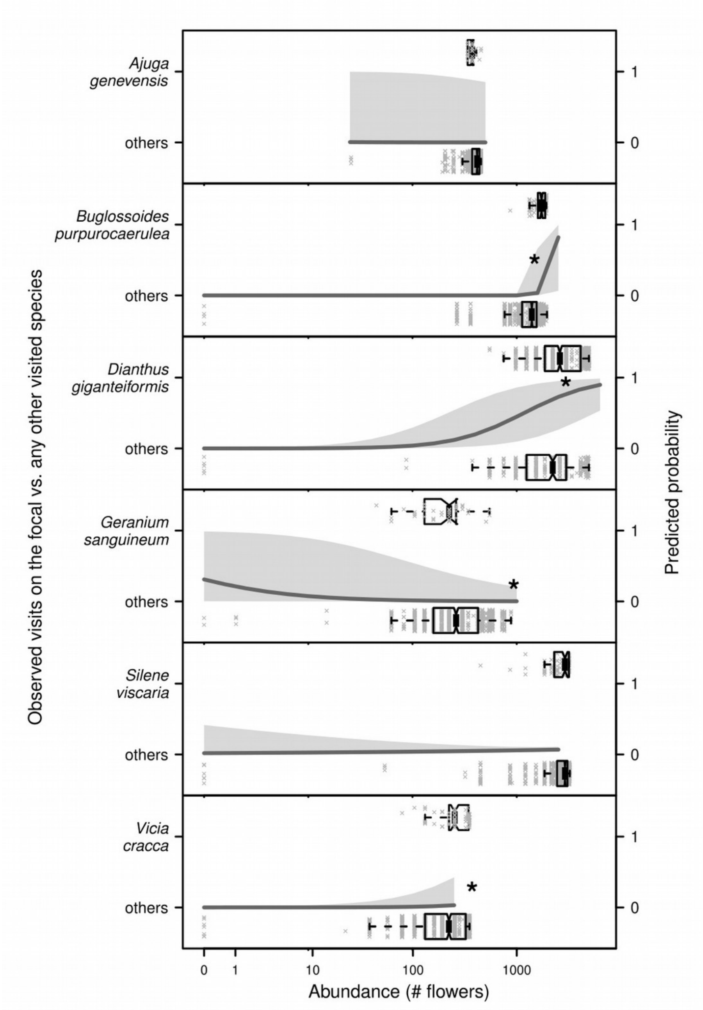

Figure A3.2: Clouded Apollo flower visit and flower abundance relationships. The box plots show the median and 25th and 75th percentiles; the whiskers indicate the values within 1.5 times the interquartile range. Notches show 95% confidence intervals for the medians. Grey crosses represent individual observations and are jittered on the vertical axis for better visibility. Dark grey lines represent regression lines and light grey bands represent 95% confidence intervals for the regression lines. Asterisks represent a significant effect ($P < 0.05$). The x-axes are $\log_{10}$ scaled.

A4: Appendix for Chapter Four

Annual variation in Clouded Apollo butterflies’ proboscis length and their nectar plants’ corolla length – a field study

Table A4.1 Annual variation in corolla length of the most visited nectar plant species and the proboscis length of the Clouded Apollo butterflies.

	Species	Year	Mean	SD	CV%	Min	LQ	Median	UQ	Max	N
Corolla	*A. genevensis*	2015	7.90	1.16	14.71	5.00	7.20	8.00	8.50	10.60	35
		2016	9.99	1.12	11.22	7.41	9.23	10.22	10.65	12.45	73
		2017	9.62	1.02	10.60	6.83	9.01	9.38	10.28	12.04	104
		2018	8.15	1.25	15.37	5.50	7.11	8.12	9.17	10.09	30
		2019	9.41	0.92	9.77	7.32	8.90	9.29	9.87	12.07	45
	B. purpurocaerulea	2015	9.25	1.16	12.57	6.90	8.30	9.30	10.00	12.90	222
		2016	10.76	1.68	8.95	5.69	9.54	7.20	12.00	9.68	117
		2017	11.03	1.22	8.57	5.80	10.30	7.86	11.89	9.56	76
		2018	12.16	1.28	12.19	4.74	11.33	6.45	13.27	7.59	47
		2019	11.00	1.52	15.47	4.47	9.67	7.42	11.87	10.37	45
	D. giganteiformis	2015	8.97	1.02	11.35	7.00	8.10	9.00	9.60	11.60	94
		2016	7.26	0.65	15.62	8.47	6.91	10.29	7.58	14.73	51
		2017	7.87	0.67	11.10	8.18	7.42	10.99	8.22	13.04	35
		2018	6.27	0.76	10.55	9.87	5.80	11.86	6.78	15.67	24
		2019	7.27	1.13	13.82	8.49	6.80	10.88	7.86	14.21	46
	S. viscaria	2015	13.09	1.62	12.37	8.80	12.00	13.20	14.30	17.20	313
		2016	13.47	1.41	10.49	9.18	12.30	13.56	14.54	16.38	136
		2017	13.40	1.29	9.63	9.86	12.39	13.45	14.34	15.98	214
		2018	13.43	1.44	10.72	9.68	12.74	13.54	14.64	16.57	102
		2019	13.28	1.34	10.08	8.61	12.62	13.28	13.97	17.40	194
Proboscis	*P. mnemosyne*	2015	12.13	0.58	4.83	9.98	11.80	12.21	12.50	13.52	168
		2016	12.07	0.56	4.64	10.37	11.71	12.18	12.46	13.34	191
		2017	12.17	0.60	4.97	10.29	11.83	12.24	12.53	13.52	186
		2018	11.66	0.51	4.40	10.07	11.32	11.69	11.99	13.03	272
		2019	11.93	0.62	5.18	8.39	11.61	12.00	12.39	13.04	203

CV% shows variation coefficients. LQ is the lower quartile (25% percentile), UQ is the upper quartile (75% percentile).

Table A4.2 Annual and years-pooled flower visitation ratio of the most visited nectar plant species in 2015–19. Species are listed, if their summarised visit ≥ 1%. Thirty species had <1% pooled visitation ratio.

flower species	2015	2016	2017	2018	2019	2015–19
Dianthus giganteiformis	70.4	57.1	48.4	65.5	56.8	59.5
Buglossoides purpurocaerulea	9.5	21.3	22.4	0.0	6.0	14.8
Silene viscaria	7.7	5.1	5.3	7.5	9.5	6.5
Ajuga genevensis	1.8	4.2	16.1	0.2	2.4	5.2
Vicia cracca	4.8	2.0	0.7	12.4	2.4	3.4
Geranium sanguineum	3.2	3.0	2.2	2.2	2.7	2.8
Thymus sp.	0.3	2.6	1.7	1.7	5.5	2.1
Inula hirta	0.2	0.8	0.2	2.4	3.3	1.0

Table A4.3 Head width, the widest part of the body, of Clouded Apollos was measured from photo macrographs as the longest distance between the perimeters of the eyes, frontal view (Somlay, 2021). Number of females: 434, number of males: 86.

	Estimate	**SE**	**P-value**
intercept	3.98	0.68	<0.0001
head width	3.07	0.26	<0.0001
males	4.44	2.09	0.0346
head width : males	- 1.83	0.72	0.0109

In this linear model, the response variable is proboscis length, the explanatory variables are head width, sex and their interaction.

Table A4.4 The cumulated number of visits per year and the annual mean abundance of the most visited nectar plant species in 2015–2019.

	species	**2015**	**2016**	**2017**	**2018**	**2019**
Cumulated number of visits per year	*Ajuga genevensis*	19	66	132	1	13
	Buglossoides purpurocaerulea	103	337	183	0	33
	Dianthus giganteiformis	762	904	396	270	312
	Silene viscaria	83	81	43	31	52
	others	115	196	64	110	139
Percent of visits per year	*Ajuga genevensis*	1.76	4.17	16.14	0.24	2.37
	Buglossoides purpurocaerulea	9.52	21.28	22.37	0.00	6.01
	Dianthus giganteiformis	70.43	57.07	48.41	65.53	56.83
	Silene viscaria	7.67	5.11	5.26	7.52	9.47
	others	10.63	12.37	7.82	26.70	25.32
Annual mean abundance	*Ajuga genevensis*	1.75	1.45	1.09	1.14	1.07
	Buglossoides purpurocaerulea	3.44	3.24	2.91	1.57	1.27
	Dianthus giganteiformis	3.54	3.58	2.71	2.10	2.00
	Silene viscaria	2.73	2.85	3.13	2.11	2.92
Annual median abundance	*Ajuga genevensis*	1.5	1	1	1	1
	Buglossoides purpurocaerulea	4	3	3	1	1
	Dianthus giganteiformis	4	4	2	2	2
	Silene viscaria	2	3	3	1	3
Visits relative to abundance	*Ajuga genevensis*	1.00	2.86	14.79	0.21	2.21
	Buglossoides purpurocaerulea	2.76	6.58	7.69	0.00	4.75
	Dianthus giganteiformis	19.90	15.93	17.84	31.21	28.42
	Silene viscaria	2.81	1.80	1.68	3.56	3.25

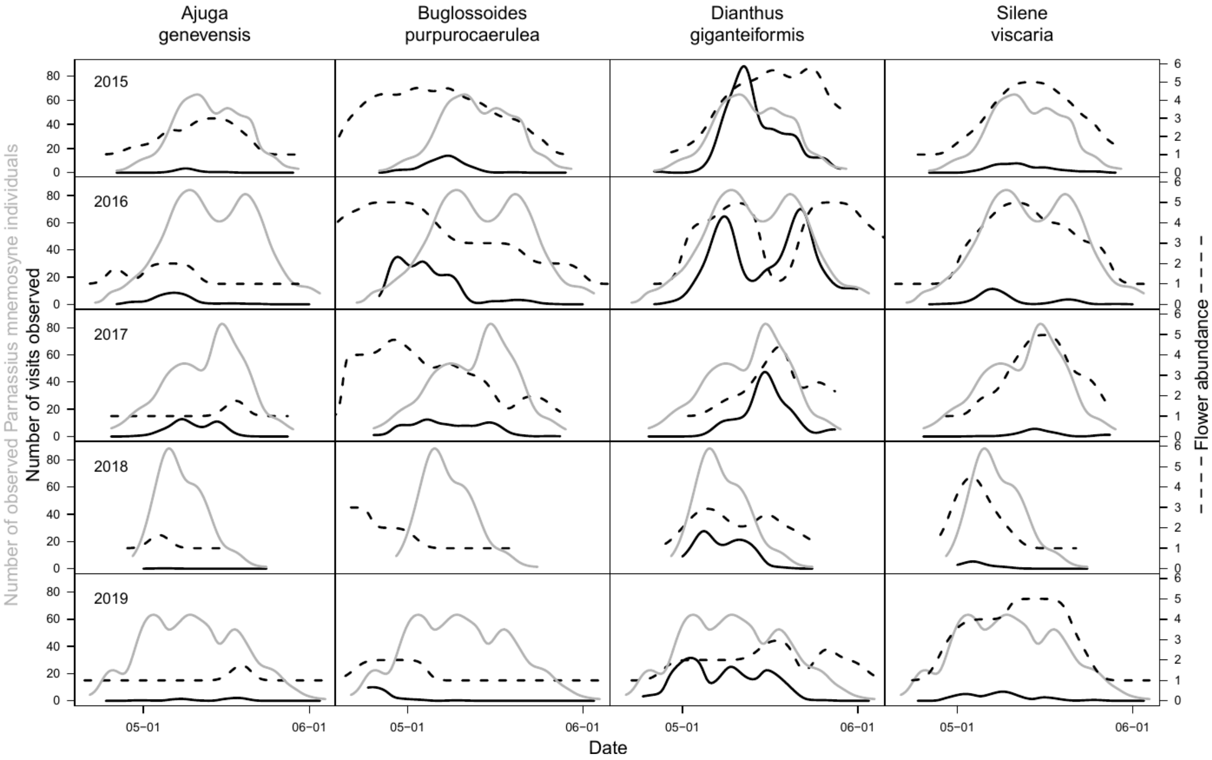

Figure A4.1 Kernel-smoothed distributions of the number of Clouded Apollo individuals (solid grey lines), number of observed visits (solid black lines) and flower abundances (dashed black lines) in 2015–19 in the four most visited nectar plant species.

Flower abundance was sampled as a categorical variable described in Vajna et al., (2020b; Chapter One), and according to Szigeti et al., (2016a). Note that this method yielded much rougher estimates compared to the finer-scale flower abundance sampling method described in Szigeti et al., (2020; Chapter Three). The finer-scale estimate required much more research investment compared to the categorical and is available only for 2015, whereas we have data from the rougher estimate for 2015–19. However, using categorical abundance as a covariate in binomial flower visit models turned the models unstable, hence the solely graphical argumentation.

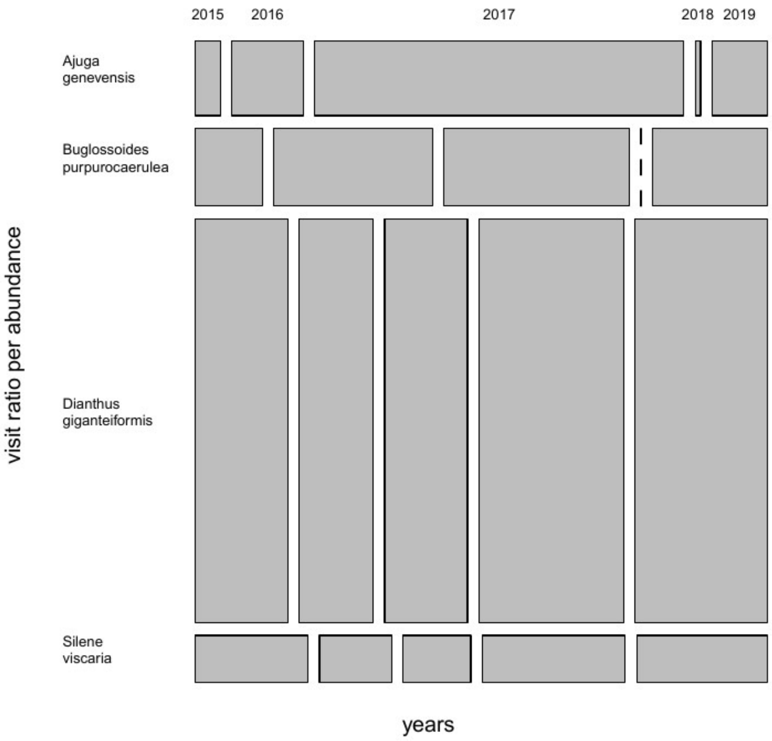

Figure A4.2 Flower visitation ratio of the four most visited nectar plants divided by their abundance categories in 2015–2019.

Section A4.1 Measurement agreement estimates

Intraclass correlation coefficient (ICC) is not advised to be used for calculating repeatability of two different devices or persons, because ICC is sensitive to the bias of different tools (Altman and Bland, 1983). Correlation is also not advised, since it can be misleading (correlation can be high, but values may be different). Instead a graphical tool, the Bland-Altman plot is recommended, combined with paired t-tests (Bland and Altman, 1986; Giavarina, 2015). Bland-Altman plots show the difference as a function of the mean of the measurements; mean ± 1.96 × SD (bold red and blue dashed lines in the Figures A4.3–6), and their 95% confidence intervals (thinner red and blue dashed lines). If the majority of the points are within the range of the mean ± 1.96 × SD, then the two methods' or persons' measurements are in agreement, although accepting a specific value as difference shall depend on the research question (Giavarina, 2015). To estimate repeatabilities of multiple measurements of the same object by the same person and with the same device, the intraclass correlation coefficient (ICC) is appropriate (*see* Table A4.5); if ICC > 0.75, repeatability is excellent, if ICC > 0.40 repeatability is acceptable (Koo et al., 2016). However, Bland-Altman plots and paired t-tests are also recommended in these cases.

In spite of different methodology, the two different analyses for 2015 point to the same conclusion. Annual variances in corolla length (Figure 4.2) presenting all years also suggest that this variation is not the result of the different methodology applied (different species' corollas show opposing direction of change in consecutive years compared to 2015), but natural variation.

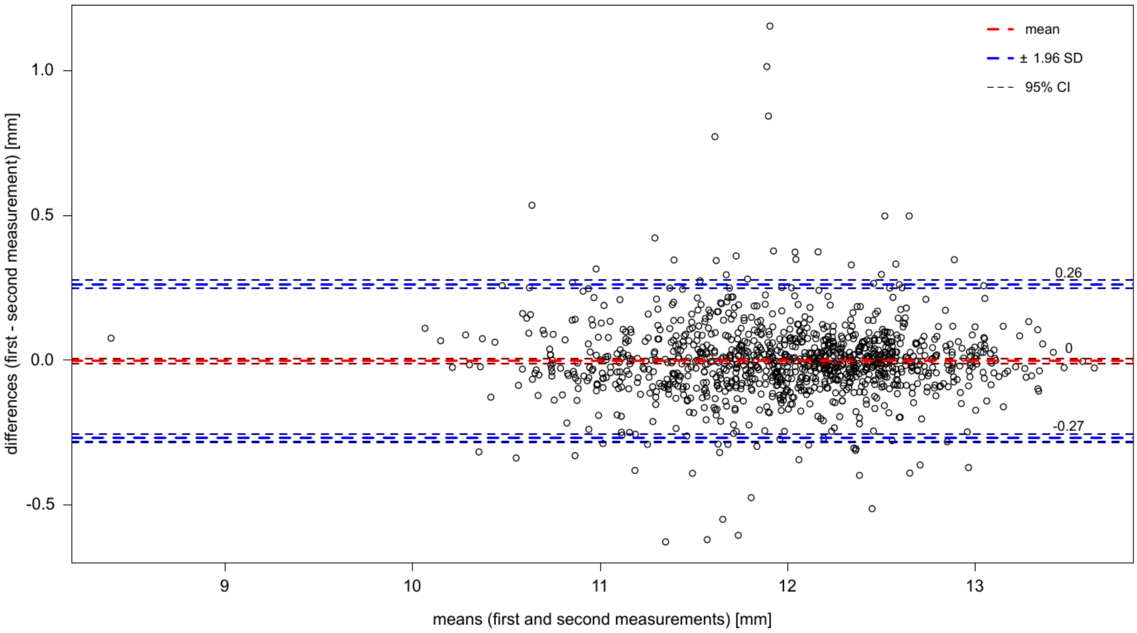

Figure A4.3 Proboscis length measurement agreement on 2 photo macrographs of the same individual; Bland-Altman plot.

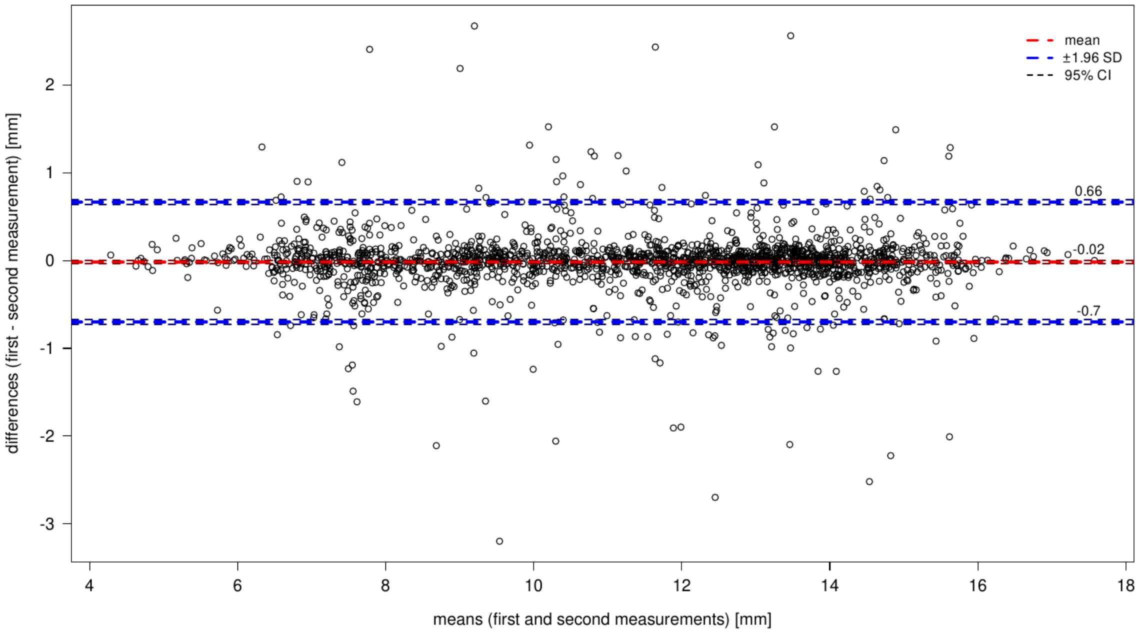

Figure A4.4 Corolla length measurement agreement on 2 photo macrographs of the same flower; Bland-Altman plot.

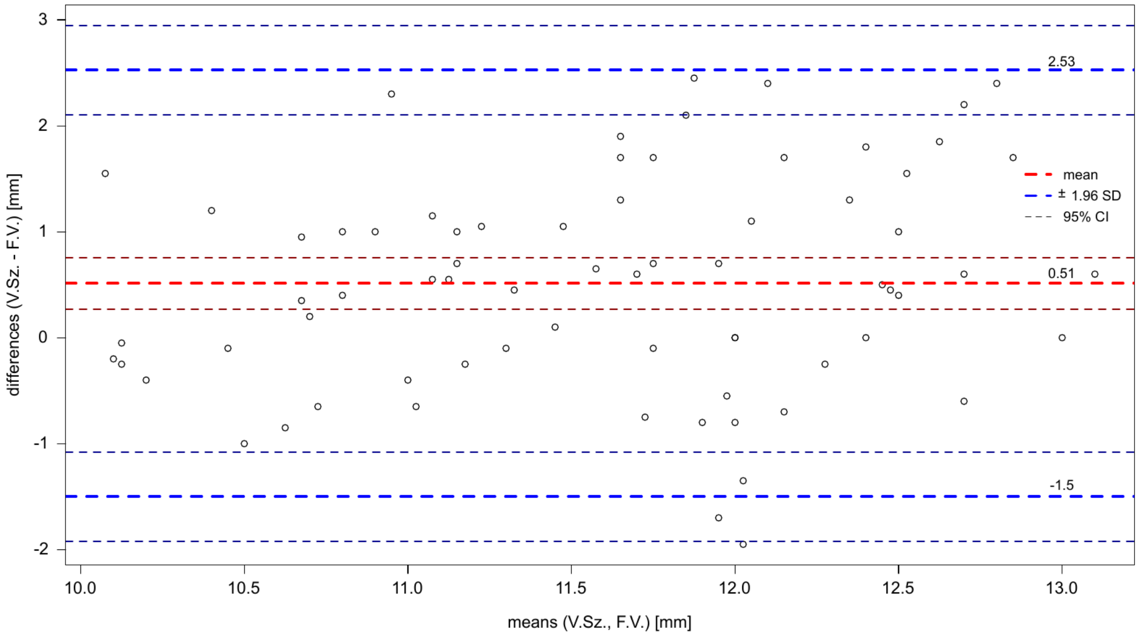

Figure A4.5 Corolla length measurement agreement between two persons; with callipers, of the same flower; Bland-Altman plot.

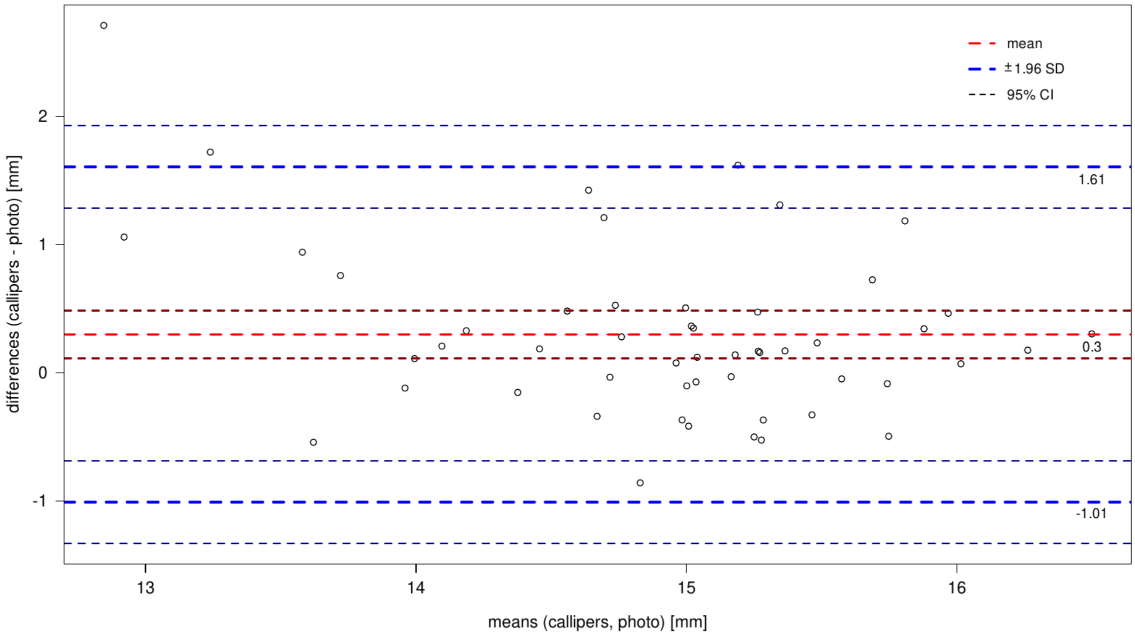

Figure A4.6 Corolla length measurement agreement between callipers and photo macrographs of the same flower; Bland-Altman plot.

Table A4.5 Proboscis and corolla length measurement agreements; paired t-tests and intraclass correlation coefficients (ICC). Although ICC is not recommended for the last two comparisons, we provide them for a didactic purpose.

	Average difference	**t-value**	**DF**	**P**	**± 95% CI**	**h_0**	**ICC**	**Lower 95% CI**	**p**
Proboscis length measurement from photo macrographs (2016–19) – first and second measurement	0.00	-0.77	1106	0.4438	-0.01, 0.00	ICC>0.75	0.976	0.974	<0.0001
Corolla length measurement from photo macrographs (2016–19) – first and second measurement	0.02	-2.28	1856	0.0223	-0.03, -0.00	ICC>0.75	0.992	0.991	<0.0001
Corolla length measurement with callipers – 2 measuring person (2015)	0.51	4.21	70	<0.0001	0.27, 0.76	ICC>0.75	0.322	0.136	1
Corolla length measurement callipers vs. photo macrocraphs (2015)	0.30	-3.23	51	0.0021	-0.48, -0.11	ICC>0.40	0.659	0.509	0.0046

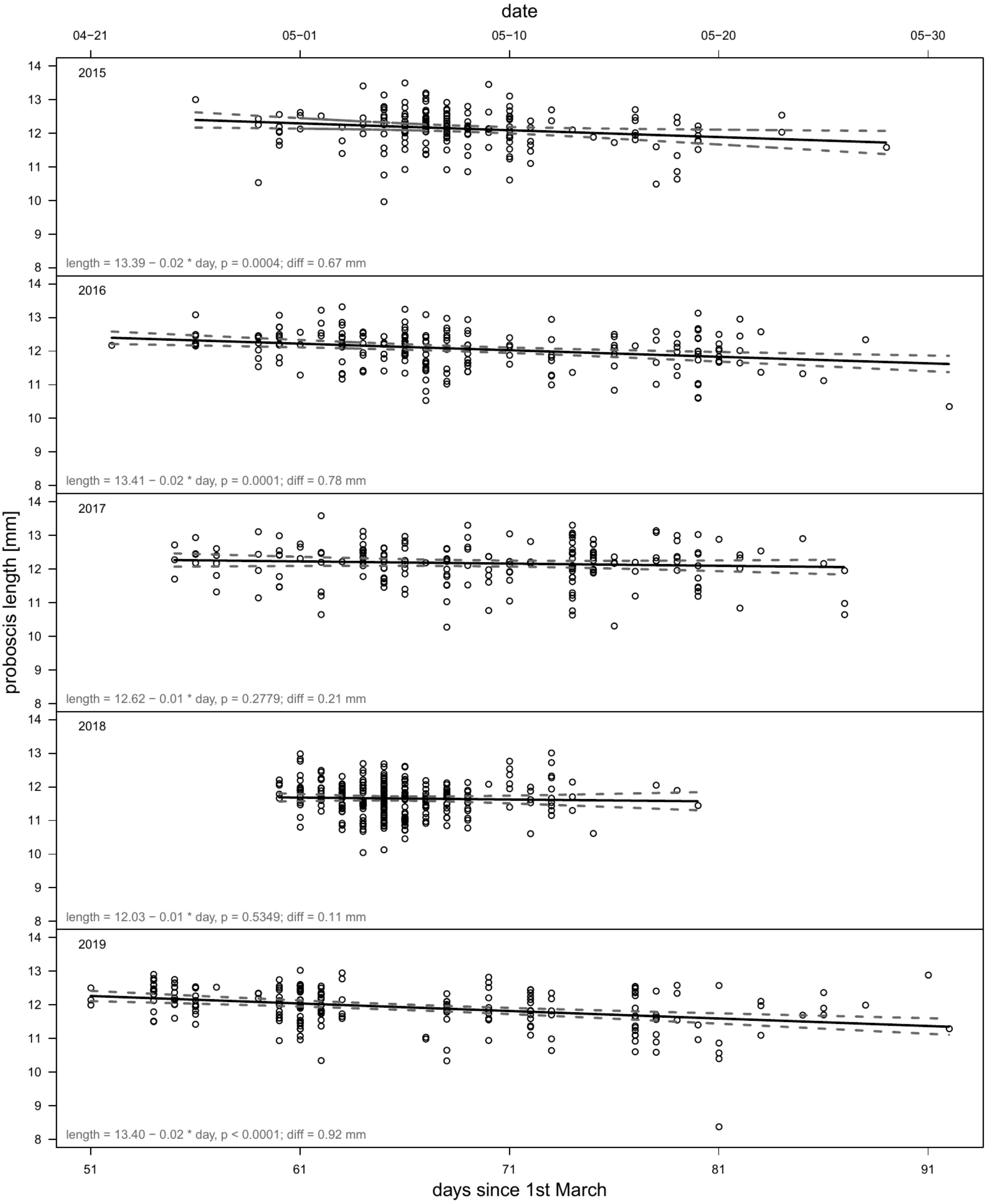

Figure A4.7 Proboscis length changes over the flight period. Circles represent Clouded Apollo individuals, their proboscis was measured at the day of capture. Solid black lines show linear regression; grey dashed lines show 95% confidence intervals.

Glossary

Flower abundance sampling methods

The method applied in Chapter One and Four

We estimated flower abundance with scanning (Szigeti et al., 2016a) at Leány-kúti rét every 3 days (median: 3, range: 2–6 dependent on the weather), at Hegyesd also in every 3 days (median: 3, range: 1–5). Sampling was started on the second day of the flight period the latest, and was finished at the earliest two days before the flight period ended. During the approximately one-hour long sampling, we walked through the entire meadow and listed all the flowering, insect-pollinated plant species; we estimated their abundance. Abundance categories were estimated for all forbs only for the opened, non-withered flowers for the entire meadow: 0: extremely rare, 1: rare, 2: more or less rare, 3: more or less frequent, 4: frequent, 5: extremely frequent. Abundance of a given species' flowers was estimated in relation to all the flowers in the studied habitat. We tried to handle these as approximately equal-distanced categories. Sampling was carried out by JK and VS.

The method applied in Chapter Three

To sample flower abundance, we prepared a map of our study site using Google Earth aerial photos and adjusted it in the field. About every 3 days, we drew all flowering patches of the six plant species on a map and estimated the number of flowering shoots per patches within the study site by either counting the shoots (< 10 shoots per patch), or assigning rough estimates by tens, as 20, 30, … 100 (> 10 shoots per patch; (Szigeti et al., 2015)). We summarized the number of shoots per plant species per sampling event for the entire site. We also counted the flowers per flowering shoot on the sampled forbs. We calculated flower abundance for the entire study site for each of the six most-visited plants by multiplying the number of flowering shoots by the average of the number of flowers per flowering shoot. We used kernel smoothing (bandwidth = 5) to extrapolate abundances for each day from the 3 days of sampling. Floral abundance was estimated to control for the effect of the considerable observed changes in abundance over time on the feeding behaviour of Clouded Apollos. Sampling was carried out by VS.

Hungarian names of the mentioned species

Ajuga genevensis - közönséges ínfű
Buglossoides purpurocaerulea - erdei gyöngyköles
Dianthus giganteiformis subsp. pontederae - magyar szegfű
Fragaria viridis - csattogó szamóca
Geranium sanguineum - piros gólyaorr

Parnassius mnemosyne - kis Apolló-lepke
Polygala comosa - üstökös pacsirtafű
Silene viscaria - enyves szegfű
Thymus odoratissimus - közönséges kakukkfű
Trifolium montanum - hegyi here
Vicia cracca - kaszanyűg bükköny

Standing crop nectar sampling
amount of nectar of a flower at a given time (Nicolson et al., 2007).

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