Summary of the PhD thesis

BEHAVIOUR, MORPHOLOGY AND ECOLOGICAL CONSTRAINS IN BUTTERFLIES

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Budapest, 2022

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General introduction and aims

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.

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

Animals choose among food resources according to their nutritional needs and opportunities. Butterflies are ideal model organisms to study resource use, since adults select among food resources, and are capable to adapt to dynamically changing supplies, although they usually feed regularly on the same plant species sequentially. Our aim was to study which plant species are visited by Clouded Apollo butterflies (*Parnassius mnemosyne* (LINNAEUS, 1758)) from all the available insect-pollinated plants, and to understand which floral traits determine their choice. We monitored the butterflies by mark-resight for 5+2 years in two closely-situated meadows. We estimated flower abundance by scanning. Floral traits were collected from the Biolflor database. Annual visit ratios changed significantly among plant species (Table A1.1). We found great variability in the traits of available flowers and in flower availability between the investigated meadows (Figure 1.1, Table A1.1). Choice was influenced by flower abundance, colour and structure, and the importance of insect pollination in one meadow (Figure 1.3), and only by abundance and colour in the other (Figure 1.4). Floral traits influencing choice may be related to each other. Flower visit patterns imply strong selectivity, and the differences between meadows suggest environmental impact. Clouded Apollos probably visit the most beneficial nectar-sources the most often. The presence of larval host-plants is essential for a butterfly to occupy a habitat. We believe that the presence of nectar plants is also a must, at least for some butterfly species.

Chapter Two: Measuring proboscis length in Lepidoptera - a review

Mouthpart morphologies relate to diet range. Differences among or within species may result in resource partitioning and speciation. In plant-pollinator interactions, mouthpart length has an important role in foraging efficiency, resource partitioning and pollination, hence measuring nectarivorous insect mouthparts' morphological variation is important. Most adult lepidopterans feed on nectars and participate in pollination. Although a vast range of studies applied morphometric measurements on lepidopteran proboscis (tongue) length, general recommendations on methodologies are scarce. We review available proboscis length measurement methodologies for Lepidoptera. Focusing on how proboscides have been measured, how accurate the measurements were, and how were these constrained by sampling effort, we searched for research articles investigating lepidopteran proboscis length and extracted variables on the aims of measurements, preparation and measurement methodology, and descriptive statistics. Different methods were used both for preparation and measurements. Many of the 135 reviewed papers did not provide descriptions of the procedures applied (Figure 2.3). Research aims were different among studies. Forty-four percent of the studies measured dead specimens, 13% measured living specimens, and 43% were unclear. Fifteen percent of the studies used callipers, 9% rulers, 1% millimetre scales, 4% ocular micrometers, 3% drawings and 14% photographs; 55% were non-informative. We emphasise the importance to provide detailed descriptions on the methods applied. Providing guidelines (Table 2.1) for future sampling and measurements, we encourage fellow researchers planning measurements to take into account the effect of specimen preparation techniques on the results, define landmarks, consider resolution, accuracy, precision, choose an appropriate sample size and report details on methodology.

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

Intraspecific morphological variation fundamentally influences individual resource exploitation. In plant-pollinator systems, variation in floral morphologies and pollinator mouthparts may affect pollinators' resource use. This relationship has frequently been studied across species, but hardly ever at the intraspecific level in natural circumstances. We studied flower visits of Clouded Apollo butterflies, *Parnassius mnemosyne*. (i) We investigated whether proboscis (mouthpart) length variability influenced individual nectar plant choice within a single population. We hypothesized that flower depths would constrain butterflies' flower visits via their proboscis lengths. (ii) We studied whether individual proboscis length constrained feeding on the sticky catchfly, *Silene viscaria*, a species with ample nectar and the deepest corolla among the plants visited. We hypothesized that individuals observed visiting *S. viscaria* had longer proboscides than those not observed on this nectar source. We captured Clouded Apollos, then measured proboscis length. We surveyed the population daily, identified marked individuals and recorded feeding on nectar plant species. We compared proboscis length to the flower depth of the six most-visited nectar plants and investigated whether individual visits on flowering plants were related to proboscis length. We found large intrapopulation variation in proboscis length, and high intra- and interspecific variation in flower depth of the six nectar plants (Figure 3.1). Flower depth of *S. viscaria* largely overlapped with proboscis length, while the other five plants had shorter flowers (Figure 3.1). Individuals with longer proboscides visited *S. viscaria* flowers more often than those with short proboscides (Figure 3.2, Table 3.1). These results indicate the importance of morphological variation in the interaction between plants and pollinators. We provide the first evidence that individual variation in mouthpart length affects lepidopteran foraging in natural circumstances. We suggest that interactions between species in plant-pollinator systems are partially based on individually different continuous traits, rather than on well-defined discrete traits of different taxa as implied by the pollination syndrome hypothesis.

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

We showed in Chapter Three that proboscis length predicts visit rate on a long-corolla resource in a single year. If within-species corolla and/or Clouded Apollo butterflies' proboscis lengths also vary across years, these may further contribute to the variety of annual visit patterns. Different environmental factors (e.g. precipitation distribution) might influence the variation in proboscis and corolla lengths and flower abundances, and these factors may fluctuate over years. We investigated the effect of the ratio of proboscis and corolla length on flower visitation over 5 years. Our questions were (i) do Clouded Apollo individuals visiting a given plant species have longer tongues than those conspecifics not observed on that nectar plant species and (ii) how consistent is visitation frequency across flower species over years. Methodological changes were made to the measurement of corolla length (using a callipers in 2015, then macro photographs in 2016–19). The detailed flower abundance estimate (a continuous variable) of 2015 was not available in further years when we used only a categorical estimate referred to as “scanning”. This rough estimate available in 2015–19 could not be included in the visit rate *vs.* proboscic length models. We found considerable individual variation both in proboscis (Figure 4.1) and corolla lengths (Figure 4.2). Annual variation was found in proboscis length and in corolla length for 3 out of 4 important nectar plant species (Figure 4.1 & 4.2, Table 4.1). Proboscis length was associated with flower visitation in two cases: in 2015 for *Silene viscaria* and in 2016 for *Buglossoides purpurocaerulea*, individuals with longer proboscis were more likely to visit the nectar plant species (Figure 4.3, Table 4.2). The latter can be explained by the phenology of the species (Figure A4.1), 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 and flowering phenology was similar to the Apollo’s phenology (Figure A4.1). These imply that Clouded Apollos were constrained by corolla length only when 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.

These results do not support the hypothesis that only those nectar-feeding insects are able to imbibe nectar from flowers that have at least as long a proboscis that the corolla itself. This hypothesis overlooks other structural factors, such as head width and corolla orifice diameter that may also influence the foragers' access to nectar. Annual variability found both in proboscis and corolla lengths supports the necessity of multi-year studies to understand the dynamics of plant-pollinator relationships.

New scientific results

1) Nectar plant species choice in the Clouded Apollo butterflies was influenced mainly by the plant species' abundance and 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. We provide 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.

The author's publications

1. Full text publications in peer-reviewed journals with an impact factor assigned

Szigeti V., Vajna F., Kőrösi Á., Kis J.: Are all butterflies equal? Population-wise proboscis length variation predicts flower choice in a butterfly, Animal Behaviour, 163, 135-143, 2020, DOI: 10.1016/j.anbehav.2020.03.008, ($IF_{20.21}$: 2.844)

Vajna F., Szigeti V., Kis J.: Measuring proboscis length in Lepidoptera - a review, Zoomorphology, 144, 1-15, 2021, DOI: 10.1007/s00435-020-00507-z, (IF_{2020}: 1.18)

2. Full text publications in peer-reviewed journals with no impact factor assigned

Vajna F., Szigeti V., Harnos A. Kis J.: A kis apollólepke (*Parnassius mnemosyne* (LINNAEUS, 1758)) nektárnövényfajok közti választása, Állattani Közlemények, 106, 1-27, 2021., DOI: 10.20331/AllKoz.2021.106.1-2.1

3. Oral presentations at conferences

Vajna F.: Kis Apolló-lepkék (*Parnassius mnemosyne*) pödörnyelvhosszának kapcsolata nektárforrásaik pártacsőhosszával. SZIE-ÁOTK Tudományos Diákköri Konferencia. Budapest, 2015

Vajna F., Szigeti V., Kis J.: Kis Apolló-lepkék (*Parnassius mnemosyne*) pödörnyelvhosszának kapcsolata nektárforrásaik pártacsőhosszával. Magyar Etológus Konferencia. Dobogókő, 2015

Vajna F., Szigeti V., Kis J.: Kis Apolló-lepkék (*Parnassius mnemosyne*) pödörnyelvhosszának kapcsolata nektárforrásaik pártacsőhosszával. Akadémiai Beszámolók. Budapest, 2016

Vajna F., Szigeti V., Kis J.: Hogyan befolyásolja a kis Apolló-lepkék pödörnyelvének hossza, hogy milyen virágokon táplálkoznak?. II. Élettudományi Liftbeszéd Fesztivál, Budapest, 2016

Vajna F., Szigeti V., Kis J.: Testméretfüggő táplálékválasztás kis Apolló-lepkéknél. Szünzooloógiai Szimpózium. Budapest, 2016

Vajna F., Szigeti V., Kis J.: Virágmélység és pödörnyelvhossz kapcsolata a kis-Apolló lepkénél. II. Országos Lepkész Találkozó. Szögliget, 2016

Vajna F., Szigeti V., Kis J.: Kis Apolló-lepkék nektárnövény-látogatása egy hosszú pártacsövű

virágfajon. Magyar Etológus Konferencia. Dobogókő, 2017

Vajna F., Szigeti V., Kis J.: Kis Apolló-lepkék nektárnövény-látogatása egy hosszú pártacsövű virágfajon, A Magyar Rovartani Társaság 860. előadóülése. Budapest, 2018

Vajna F., Szigeti V., Kis J.: Kis Apolló-lepkék nektárnövény-látogatása egy hosszú pártacsövű virágfajon. Akadémiai Beszámolók. Budapest, 2019

Zorkóczy O., Kőrösi Á., Vajna F., Szigeti V., Kis J.: Body size-related survival in a natural population of Clouded Apollo butterflies, *Parnassius mnemosyne*. Reproductive strategies from genes to societies – Frontiers in animal and plant reproduction research. Debrecen, 2019

Gór Á., Lang Zs., Vajna F., Szigeti V., Kis J.: Prolonged mate-guarding and male investment dynamics in Clouded Apollo butterflies, *Parnassius mnemosyne* (Papilionidae: Parnassiinae). Reproductive strategies from genes to societies – Frontiers in animal and plant reproduction research. Debrecen, 2019

Zorkóczy O., Kőrösi Á., Vajna F., Szigeti V., Kis J.: Body size-related survival in a natural population of Clouded Apollo butterflies. Integrating social behaviour and population demography for conservation of wild populations, Debrecen, 2020

Vajna F., Szigeti V., Kis J.: Kis Apolló-lepkék nektárforrás-választása. Akadémiai Beszámolók. Budapest, 2020

Zorkóczy O., Kőrösi Á., Vajna F., Szigeti V., Kis J.: A testméret kapcsolata a túléléssel kis Apolló-lepkénél. Akadémiai Beszámolók. Budapest, 2020

4. Poster presentations at conferences

Vajna F., Szigeti V., Kis J.: Virágmélység és pödörnyelvhossz kapcsolata a kis-Apolló lepkénél (*Parnassius mnemosyne*). I. Országos Lepkész Találkozó. Ispánk, 2015

Vajna F., Szigeti V., Kis J.: Virágmélység és pödörnyelvhossz kapcsolata a kis-Apolló lepkénél *(Parnassius mnemosyne).* 10. Magyar Ökológus Kongresszus*.* Veszprém*,* 2015

Vajna F., Szigeti V., Kis J.: Kis Apolló-lepkék nektárnövény-látogatása egy hosszú pártacsövű virágfajon. XI. Magyar Természetvédelmi Biológiai Konferencia. Eger, 2017

Vajna F., Szigeti V., Kis J.: Proboscis length influences flower visitation on species with long corolla in Clouded Apollo butterflies, Student Conference on Conservation Science. Tihany, 2018