Theses of the doctoral (PhD) dissertation

The reproductive system of Clouded Apollos (*Parnassius mnemosyne*)

Ádám Kristóf Gór

Supervisors: Dr. János Kis

Dr. Zsolt Lang

UNIVERSITY OF VETERINARY MEDICINE

Doctoral School of Veterinary Sciences

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Supervisors

…….
János Kis, PhD
Department of Zoology

…….
Zsolt Lang, PhD
Department of Biostatistics

Made in one copy.

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Ádám Kristóf Gór

1. Premise and objectives of the dissertation

Sexual conflict refers to the evolutionary discordance in the interests between males and females over reproductive resources and strategies. One manifestation of this conflict is mate guarding, a behaviour observed in many species where males actively prevent other males from accessing a female to ensure their paternity. This protective behaviour is driven by the male's motivation to maximise its reproductive success and minimise the risk of females mating with rivals. In some species, mating plugs add another layer to the sexual conflict dynamics. Mating plugs are structures or substances deposited by males in the female reproductive tract to impede the access of rival males, reducing the likelihood of sperm competition. This adaptation serves as a form of post-copulatory mate guarding, ensuring that the male's genetic material has a better chance of successfully fertilising the eggs and releasing the male from consorting the female.

The investment in guarding may depend on several factors. (i) The level of competition among males for mating increases with the increasing ratio of males in a population, thus selecting for males to invest more in mate-guarding. In insects, last male sperm precedence is common, when the last mated male fertilises most of a multiply mated female’s

eggs, thus male benefits from postcopulatory mate-guarding are further enhanced. (ii) The amount of affordable resources and time to spend on guarding may depend on individual variation, e.g. condition among competitors. (iii) The expected reproductive value of the mating partner may also depend on individual variation, e.g. the variance in the conditions among potential partners. The latter may result in males assessing female quality before mating. In insects, the general pattern is that larger female body size is associated with better quality, i.e. higher fecundity, consequently more eggs produced during lifetime. Therefore, males may benefit from investing more in the guarding of larger than smaller females.

Males of many insects, including butterflies, produce mate-guarding devices, such as mating plugs, to prolong guarding and prevent future female matings in the male's absence. In most lepidopteran taxa, internal plugs are common, while in two butterfly families, Nymphalidae and Papilionidae, large, structured external mate-guarding devices, called sphragides (singular: sphragis), evolved independently of each other. The term ‘sphragis’ originates from the Greek word meaning ‘seal’, and it describes the function of this structure. Sphragides are waxy or proteinaceous substances secreted by males, covering the

female copulatory opening and may persist on females throughout their postcopulatory life. These devices do not block oviposition since most lepidopterans are ditrysian, i.e. the copulatory opening is separated from the oopore. From internal plugs to large elaborate sphragides, various, species-specific forms exist in butterflies, differing in size relative to body size, structure, composition, shape, colour, as well as the means how males produce and fix them on the female. In many insects, including butterflies, as well as sphragis-bearing species, cryptic female choice involves the storage of sperm within specialised structures known as spermathecae. The spermatheca serves as a reservoir for received sperm, allowing females to control the timing and utilisation of sperm for fertilisation. There is evidence that the females of sphragis-bearing butterflies, despite being sealed with sphragides, may mate multiple times allowing them to choose between male spermatophores, i.e. capsules or masses containing sperm.

Although the study of sphragides, including arguments on how these prevent butterfly remating, has been started more than a century ago, data on within-species size and morphological variation are scarce. To our best knowledge, quantitative studies on how this variation impacts the guarding devices' persistence on females are absent to date.

Moreover, previous studies focused on typifying species-specific mate-guarding devices in a few specimens of many species. In contrast, we investigated a sphragis-bearing butterfly's alternative mate-guarding devices in detail and behaviours related to those, and aimed to introduce their English and Hungarian terminology. We conducted an extensive observational field study through six years based on mark-recapture in a natural Clouded Apollo (*Parnassius mnemosyne*) population. Inspecting 492 females, we identified three different types of devices, termed as Copulatory opening Appendices (CAP) and all CAP-types were found in several different populations in all the years investigated. Filaments, stopples (together named as small CAPs) and shields (i.e., sphragides) increased in size and structural complexity in the respective order, implying differential male investment and effectiveness in securing paternity. We expected large variation in CAP size and shape as males have varying reserves that can be allocated to mate-guarding. Shield dimensions, colour and all devices' shapes varied considerably. Some devices were lost, and a few were video-recorded when removed by males, showing the role of different parts of the male external genitalia.

Our further aim was to reveal CAP-replacement dynamics within females during their lifetime and to

understand how male investment into small CAPs or shields was (i) related to CAP-persistence on the female, i.e., securing paternity, (ii) associated with female quality, measured as size and (iii) with actual adult sex ratio. We expected that males invest more in larger than smaller females, since larger female insects were found to be more fertile. Moreover, males were expected to invest more in guarding females at higher rates of male-male competition, i.e. male-biased sex ratios. Finally, we predicted that males invest more in mate-guarding early in the flight period than later, since they are younger and have more reserves, and also encounter younger and larger females which are more fertile, thus more rewarding for the males.

To estimate CAP-replacement risks, we used multistate survival models and to reveal the connection between the ratio of shields to all CAPs and adult sex ratio (ASR) we used time series analyses with GLM. Shields were the most frequent mate-guarding devices and were more persistent than small CAPs, often lasting for life, excluding future matings. Most females bearing a shield were deprived of postcopulatory female choice, thus the genetic variance in their offspring may be reduced compared to females bearing small CAPs that may allow mating more often and with multiple males. The ratio of shields to all CAPs gradually decreased towards the end of the

flight period. Males were more prone to produce a shield when mating females with wider thoraces and when the ratio of males (i.e. male-male competition) was higher in the population.

The results are consistent with our expectations, but since our data are observational, no causality can be established.

2. New scientific results

1. We found that besides large sphragides, smaller, alternative mate-guarding devices had regularly been produced by males within a butterfly species. This is the first evidence in the genus *Parnassius*.
2. We found considerable variation in the size and shape of all mate-guarding devices.
3. We provide photographic and video evidence on mate-guarding device removals.
4. We found that multiple and different types of mate-guarding devices may occur consecutively on females.
5. The persistence of mate-guarding devices on the females increased with increasing male material investment in these devices.
6. The size and type of the male produced mate-guarding devices were associated with female size and adult sex ratio (ASR).

3. Publications related to the topic of the study

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

1. Gór, Á., Fónagy, A., Pásztor, K., Szigeti, V., Lang, Z., Kis, J. **Facultative male investment in prolonged mate-guarding in a butterfly.** Behaviour, 160(6), 515-557, 2023. https://doi.org/10.1163/1568539X-bja10219, ($IF_{2022-23}$: 1.672)

2. Gór, Á., Lang, Z., Pásztor, K., Szigeti, V., Vajna, F., Kis, J. **Mate-guarding success depends on male investment in a butterfly.** Ecology and Evolution, 13, e10533, 2023. https://doi.org/10.1002/ece3.10533, ($IF_{2022-23}$: 3.167)

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

1. Gór, Á., Fónagy, A., Pásztor, K., Szigeti, V., Lang, Z., Kis, J. **Változó hím befektetés a nőstény további párosodását akadályozó képletekbe kis apollólepkénél (*Parnassius mnemosyne* (LINNAEUS, 1758)) [Variable male investment in devices**

impeding female remating in the Clouded Apollo butterfly (*Parnassius mnemosyne* (LINNAEUS, 1758))]. Állattani Közlemények 108(1-2), 2023. http://dx.doi.org/10.20331/AllKoz.2023.108.1-2.3

3.3. Oral presentations at international and Hungarian conferences

1. Gór, Á. **Erényövek és párzási dugók cserélődési rátája kis Apolló-lepkéknél (*Parnassius mnemosyne*).** XXXIV. Országos Tudományos Diákköri Konferencia, Budapest, 2019.

2. Gór, Á., Kis, J. **Erényövek és párzási dugók cserélődési rátája kis Apolló-lepkéknél (*Parnassius mnemosyne*).** Akadémiai Beszámolók, Budapest, 2019.

3. Gór, Á., Lang, Z., 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.

4. Gór, Á., Lang, Z., Vajna, F., Szigeti, V., Kis, J. **Erényövek és párzási dugók cserélődési rátája kis Apolló-lepkéknél (*Parnassius mnemosyne*).** Magyar Etológus Konferencia. Mátrafüred, 2019.

5. Pásztor, K., Kőrösi, Á., Gór, Á., Kis, J. **Multilevel variation in body mass and thorax width in a natural butterfly population.** XXII. European Congress of Lepidopterology. Laulasmaa, Estonia, 2022.

6. Pásztor, K., Kőrösi, Á., Gór, Á., Kis, J. **How weather and body size affect survival and detectability in a natural butterfly population.** 8th Student Conference on Conservation Science Europe. Balatonvilágos, 2023.

7. Kis, J., Gór, Á., Lang, Z., Fónagy, A., Pásztor, K., Szigeti, V., Vajna, F. **Guarding success depends on facultative male investment in mate-guarding in Clouded Apollo butterflies.** Magyar Etológus Konferencia. Debrecen, 2023.

3.4. Poster presentations at international and Hungarian conferences

1. Pásztor, K., Kőrösi, Á., Gór, Á., Kis, J. **Change of body mass and thorax width with age in a natural butterfly**

population. 2021 Annual Meeting of the Lepidopterists' Society. Online conference, 2021.

2. Gór, Á., Pásztor, K., Szabó, K., Kis, J. **Does sexual dimorphism in wing colouration relate to sphragis shape in Apollo (*Parnassius*) butterflies?** XXII. European Congress of Lepidopterology. Laulasmaa, Estonia, 2022.

3. Pásztor, K., Kőrösi, Á., Gór, Á., Kis, J. **Change of body mass and thorax width with age in a natural butterfly population.** 7th Student Conference on Conservation Science Europe. Balatonvilágos, 2022.

4. Pásztor, K., Kőrösi, Á., Gór, Á., Kis, J. **How weather and body size affect survival and detectability in a natural butterfly population.** International Conference on the Biology of Butterflies. Prague, Czech Republic, 2023.