Final report

*"Biotic and abiotic effects on changing parental role specialization"*

K 112670

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# 1 Introduction

Our aim in this project was to contribute to the understanding of an evolutionary enigma: the evolution and maintenance of cooperation between unrelated individuals. One of the most common form of cooperation between two unrelated individuals is biparental care for the offspring that is reminiscent of public good games: both parents enjoy the benefits of care (the surviving young), however, they pay the costs individually. Therefore care is prone to conflict about who provides and how much care.

We investigated parental care in a biparental beetle species the *Lethrus apterus* (Fig. 1). This species is an interesting member of the Hungarian fauna. The western edge of the Eurasian distribution of this beetle is in Hungary, and it has a remarkable breeding system. Adults are active only for the short breeding period between mid March and late June. After emerging from the ground in spring, the parents prepare a 50-70 cm deep nest in the ground where the female lays 6-8 eggs in separate brood chambers and the parents prepare a food ball from cut leaves for each individual offspring. These food balls serve as solely food source for the larvae during their development. The sexes are dimorphic (males are larger and have two tusks, i.e. ventral mandibular processes), so it is easy to distinguish them in the field. There is intense competition between males, and tusks are used during the male-male fights (Fig. 1).

*Figure 1: Fighting males of Lethrus apterus. Photo by Z. Barta.*

According to the literature there is a clear division of labour between the sexes: the males collect the leaves while the females prepare the food balls. However, our recent observations suggest that parental roles may have shifted recently as leaf collection is done largely by females in the populations studied in Hungary. This apparent flexibility in parental care makes this species to an ideal model to investigate effects influencing parental role specialization.

## 2 Results

Over the project period we run several field studies that led to publications in international behavioural, zoological or entomological journals, furthermore there are a few further studies where publication is still in progress.

### 2.1 The effects of adult sex ratio and density on parental care (Rosa et al. 2017. *Animal Behaviour*, 132, 181–188)

We carried out a factorial experiment where density and sex ratio of the individuals were manipulated in seminatural enclosures of 1 × 2 m fenced off by plastic flower bed edges. We had three levels of density (4, 8, 12 individuals) and three levels of sex ratio (proportion of males: 0.25, 0.5, 0.75) manipulations, giving 9 manipulation groups in total. We had three replicates for each manipulation group, resulting in 27 enclosures. The behaviour of the individuals were recorded using a webcam fixed on a purpose-built tripod above the enclosure and connected to a netbook computer. Four times eight hour behavioural data were collected for the enclosures over the breeding season, and the over 800 hours of video recordings were analysed and parental provisioning (number of leaf-collecting events) and nest attendance (time spent inside the burrow) were determined. The nests in the enclosures were dug out in the autumn, and the number and sex of offspring were also determined. We found that as either the density of individuals or the proportion of males increased, pairs showed higher nest attendance, furthermore parental provisioning decreased with increasing proportion of males. Male-biased groups also produced fewer offspring under high and low densities. Our results support the notion that as the level of intrasexual competition between males increases males invest more in paternity assurance that leads to a possible conflict of interest between the sexes over paternity and brood size.

### 2.2 Contest behaviour between resident and intruder males (Rosa et al. 2018. *Behavioural Processes*, 149, 65–71)

One of the suggested role of the presence of males at the nest is defence against intruders. To investigate this proposition, we carried out a resident-intruder contest experiment. We put either smaller than average or larger than average males to random nests where resident males were present, and recorded by video cameras the behaviour of the resident and the intruder. We tested 76 nests altogether and analysed the video records. We showed that residents won most of the encounters and small intruders were less willing to engage in a contest and were less successful in their takeover attempts than large ones. However, contrary to many earlier studies, the duration and escalation of the fight did not differ between the two intruder size categories. These findings

suggest that in this species, small and large intruders adjust their decisions about fights to their competitive abilities but once a fight started, they behave similarly during the contest.

### 2.3 Size-dependent investment in tusk length, testis size and sperm length (Rosa et al. 2019. *Journal of Zoology*, 309, 106–113,)

In *Lethrus apterus* there is a big size difference in males in body size (thorax width ranges between 8.40 and 13.80 mm). Extreme small and large males may experience different levels of pre- and postcopulatory sexual selection, and this may result in alternative ways to gain fertilization. In order to investigate precopulatory (tusk length) and postcopulatory (testis size and sperm length) traits we collected, measured and dissected 15 small (thorax width ≤ 11.0 mm) and 15 large (thorax width ≥ 13.0 mm) males caught at the beginning and at the end of the breeding season (60 individuals in total). We measured tusk length by callipers, and the total area of the 12 testicular follicles and the mean length of 60 randomly chosen sperm from two follicles from microscope photographs using the ImageJ software. We demonstrated that there is male polymorphism in *L. apterus* as large males had longer tusks relative to their body size than small ones, and showed that testis size but not sperm length differ between large and small males, and that testis size has different seasonal change in the two size groups. The non allometric relationship between tusk length and body size and the lower decrease in testis size over the breeding season in case of small individuals might indicate the presence of alternative mating tactics in this species.

### 2.4 Seasonal changes in immune response and reproductive investment (Kiss et al. 2020. *Journal of Insect Physiology*, 121, 104000)

We tested the effect of sex, body size, time within the reproductive season and parasite load on the relationship between two physiologically demanding processes: parental investment and immunocompetence. We quantified immune response by encapsulation response and by the bacterial growth inhibition ability of the haemolymph in 33 males and 39 females collected in March and May. The reproductive investment of individuals was determined after dissection where we measured the sum area of the eggs produced by females and the sum area of testicular follicles in males. Parasite load were estimated by counting the number of mites on the individual beetles. We found that the encapsulation response is condition-dependent, whereas antimicrobial capacity was significantly higher in females than in males. In case of antimicrobial activity there was also a seasonal change in the relationship between immunity and reproductive investment under heavy mite load. Reproductive investment was influenced by the interaction between body size and season (in females) and by body size and season (in males). Furthermore, the interaction between antimicrobial activity and season in case of females indicated that reproductive investment

increased with antimicrobial activity early in the reproductive season. We can conclude that investments into these two important life history traits (immunity and reproduction) are governed by complex interactions between physiological and environmental factors.

### 2.5 Seasonal changes of an oxytocin/vasopressin-like neuropeptide, the inotocin (Nagy et al. 2017. *PeerJ*, 5, e4047; Nagy et al. *Hormones and Behavior* under revision)

Several members of the highly conserved oxytocin/vasopressin neuropeptide family are involved in the regulation of reproductive and social behaviours in numerous vertebrate and invertebrate species. We investigated gene expression patterns of inotocin, the insect ortholog of this peptide family, and its receptor to describe their possible role in the control of reproductive behaviour. We detected both the inotocin and its receptor in the draft genome of *Lethrus apterus*, and we intended to investigate expression patterns of inotocin and its receptor by real-time quantitative polymerase chain reaction (RT-qPCR). This method, however, requires stably expressed reference genes for normalisation, however, there was a lack of reference gene studies for insects from natural populations. Therefore, we evaluated 11 candidate housekeeping genes as reference genes and determined which genes can be used to study the hormonal regulation of reproduction and parental care.

Because one of the ancient roles of the oxytocin/vasopressin-like peptide family is related to the regulation of water balance, in an experiment we investigated whether inotocin is related to the control of water balance in *L. apterus*. For this purpose, three experimental groups were created (dried silica gel, saturated silica gel and control), each consisting of five males and five females. Individuals were collected in the field during their normal daily activity and for control group samples were taken immediately, whereas sampling of individuals from experimental groups was performed after four hours of treatment (dry or saturated). Our result suggests that inotocin may not have a role in body water regulation as expression patterns did not change as a response to the treatments.

We analysed the expression levels of (i) inotocin using samples of 50 individuals collected in one population at five occasions over the breeding season, and (ii) its receptor using samples of 84 individuals (collected in two populations, in one at five occasions, and in the other at three occasions). Expression levels of both the hormone and its receptor increased over the reproductive season, i.e. when behaviour shifts from pair formation to parental care. These results suggest that inotocin might be involved in the regulation of parental care in this insect species. As there was no difference between the sexes in expression of the hormone or its receptor, inotocin may play a similar role, potentially related to pair-bonding, in both sexes.

## 2.6 Movement and activity patterns of sexes

(Kiss et al. 2020. *Ecological Entomology* early view)

Different parental roles may require different time and energy budgets that are expected to influence the activity and space use of sexes. We investigate movement patterns by using a grid of 11 × 11 pitfall traps covering a square area of 100 $m^2$ in the natural habitat of *Lethrus apterus*. Traps were open between 08:00 and 19:00 (as weather permitted) on 16 days for a total of 88 h. Traps were checked hourly and any unmarked individual caught was marked with paint marker and the date, the position of trap in the grid, and number and IDs of caught individuals were noted. We found that the sexes differed in their movement pattern: females frequently travelled short distances, whereas males were detected less often, but when detected, they travelled significantly longer distances than females. Thus, these results are consistent with the notion that efficient parental food provisioning requires more localised movement. Furthermore, the long distance movements of some males may indicate active mate searching behaviour.

## 2.7 Microsatellite development

(Rácz et al. 2017. *Acta Zoologica Academiae Scientiarum Hungaricae*, 63, 355–360)

To increase the resolution of our genetic marker set in *Lethrus apterus*, 13 new microsatellite primers were developed and tested further to the already published 15 species specific primers developed earlier by our group. These primers are utilized in the phylogeograpy study (see 2.8 below). Furthermore, we performed a field experiment to investigate the genetic mating system and parentage in *L. apterus*. In this experiment males were either removed from the active nests or not (control nests) and we will compare parentage in the two groups. At the end of the breeding season we dug 24 nests and took DNA samples from potential parents and the larvae for parentage analysis. We genotyped the samples and will use Colony software to estimate relatedness between individuals.

## 2.8 Phylogeography of *Lethrus apterus*

(Sramkó et al. in preparation)

To reconstruct the recent evolutionary history of *Lethrus apterus* we investigated 38 populations that represent the whole distribution area from Hungary to Russia. All populations were analysed for 13 nuclear microsatellite (SSR) loci (5-30 specimens per population), and all but one populations for mitochondrial cytochrome oxidase I (COI) variability (4-10 specimens per population). The 192 specimens examined for COI possessed 43 haplotypes that showed shallow divergence; Bayesian phylogenetic tree reconstruction could only partially resolve relationship

between the haplotypes, and identified a differentiated lineage characteristic of the Pannonian Basin. Phylogeographic clustering of mitochondrial variation identified three clusters: one at the Pontic Steppe, and two less differentiated in Northern Bulgaria and the Pannonian Basin. These two latter regions were identified as potential refugial areas by this analysis. We detected a highly significant isolation-by-distance with SSRs of 531 specimens that showed a longitudinal geographic pattern. Additionally, more genetic variation was found at the western part: 12 genetic groups on the west versus eight on the east. Genetic clusters were separated by main rivers on the eastern part of the area, but not on the west. This implies a west-to-east colonisation, and argues for an evolutionary recent arrival of this species to its current main distribution area, the Pontic Steppe.

### 2.9 Experimental manipulation of the ability and time of food collection (Kiss et al. in preparation)

In the original project proposal we planned an experiment to investigate the effect of decreased abundance of leaves around the nest on parental behaviour and reproductive success. Based on our experience after the submission of the proposal, we modified this experiment to a manipulation that is expected to cause less disturbance to the animals. We had three experimental groups. In the first group we increased the energy expenditure of females during foraging by attaching a small weight on them, in the second group we restricted the animals from collecting leaves by fencing off the nest entrance for part of the day, whereas the third group was a control without manipulation. The leaf collecting behaviour of the parents were recorded by video cameras and we collected data from 48 nests altogether (16 control and 32 manipulated nests). During the autumn the reproductive success was determined by digging out the nests. Video footages were coded and the preliminary analyses suggest that treatment had an effect on both the behaviour and reproductive success.

## 3 Deviations

In the project proposal we planned studies not only in the natural environment of the beetles but also the establishment of a laboratory population. The main aim of the laboratory studies were to investigate underground behaviour that is not possible in the field without severe disturbance of the beetles.

After our first trials we realized that not only light but also temperature and humidity should be controlled accurately, therefore we developed a purpose built climatic chamber with adjustable temperature, photoperiod and humidity conditions where pairs of *Lethrus apterus* could be housed separately. The boxes housing the individuals were planted with turf from the original habitat of the beetles. Individuals were collected after emergence in the field and transported to the laboratory.

Later we further developed the system and built formicarium inspired artificial tunnels carved into 10 × 20 × 60 cm Ytong blocks covered by a plexiglas to enable observation of the behaviour of beetles underground. We were able to keep individuals for several weeks but unfortunately in none of the years beetle started to breed, thus we were not able to augment our field observations with laboratory investigations of breeding behaviour.

# 4 Publications related to the project

During the course of the project we published two papers that uses not beetles but plovers as model system, however, the questions investigated in these publications are very closely related to the core question of this project, i.e. parental cooperation. Two of the senior participants of the project (Z. Barta and A. Kosztolányi) were involved in these studies and the support by the K 112670 grant is acknowledged in these publications.

## 4.1 Parental cooperation in a changing climate

(Vincze et al. 2017. *Global Ecology and Biogeography*, 26, 347–358)

We investigated the effect of an abiotic factor, the temperature on parental cooperation in shorebirds. In 36 populations of 12 shorebird species we collected data on parental cooperation during incubation and showed that not only average temperature but also stochasticity and seasonality of temperature may have profound effect on parental care division. These results are important because show that not only increasing temperatures, but also increasing number of extreme, stochastic events caused by climate change may influence parental cooperation, and thus reproductive success and viability of populations.

## 4.2 Effect of adult sex ratio on parental cooperation

(Eberhart-Phillips et al. 2018. *Nature Communications*, 9, 1651)

We investigated the causes of adult sex ratio (ASR) bias using demographic modelling and the effect of ASR on social behaviour. Using detailed survival, fecundity, and behavioural data from six populations of five plover species, we showed that these closely related species and populations have remarkably different ASRs and that the bias in ASR may have an effect on parental cooperation.

# 5 Summary

In a series of observational and experimental studies we made advances in understanding factors influencing breeding behaviour in a system with role specialization that resembles more the ancient human mating and parental care system than the often investigated avian biparental care. Our

advances in investigating breeding behaviour of a relatively understudied beetle species showed that *Lethrus apterus* can be a good model system. Our results are important not just from the viewpoint of scientific merit, but the project provided also valuable knowledge for the conservation of the species and gave opportunity for students to join research. During the curse of the project three BSc theses (Sebestyén Flóra Zsuzsa 2015, Bővíz Marcell 2016, Rohrer Violetta 2016) and an MSc thesis (Kiss Johanna 2015) were prepared. Furthermore, also a PhD student defended successfully her dissertation on the investigation of mating system in *L. apterus* (Rosa Márta Erzsébet 2020).

# 6 Publications

## 6.1 Publications in the topic of the project


Kiss, J., Németh, Z., Kosztolányi, A., Barta Z. 2020. Differential movement and activity patterns of sexes in a biparental beetle during the reproductive season. *Ecological Entomology* early view, DOI: 10.1111/een.12920, **IF: 1.848, Q1**

Kiss, J., Németh, Z., Kocsis, B., Kosztolányi, A., Barta Z. Experimental manipulation of ability and time of food collection. In preparation, proposed journal: *Behavioral Ecology and Sociobiology*

Kiss, J., Rádai, Z., Rosa, M.E., Kosztolányi, A., Barta Z. 2020. Seasonal changes in immune response and reproductive investment in a biparental beetle. *Journal of Insect Physiology*, 121, 104000, DOI: 10.1016/j.jinsphys.2019.104000, **IF: 2.246, D1**

Nagy, N.A., Németh, Z., Juhász, E., Póliska, S., Rácz, R., Kosztolányi, A., Barta, Z. 2017. Evaluation of potential reference genes for real-time qPCR analysis in a biparental beetle, *Lethrus apterus* (Coleoptera: Geotrupidae). *PeerJ*, 5, e4047, DOI: 10.7717/peerj.4047, **IF: 2.118, Q1**

Nagy, N.A., Németh, Z., Juhász, E., Póliska, S., Rácz, R., Kosztolányi, A., Barta, Z. Inotocin, a potential modulator of reproductive behaviours in a biparental beetle, *Lethrus apterus*. *Hormones and Behavior*, under revision.

Rácz, R., Bereczki, J., Kosztolányi, A., Horváth, A., Sziráki, S., Barta, Z. 2017. Additional polymorphic microsatellite loci for detailed population genetic studies of *Lethrus apterus* (Coleoptera: Geotrupidae). *Acta Zoologica Academiae Scientiarum Hungaricae*, 63, 355–360, DOI: 10.17109/AZH.63.3.355.2017, **IF: 0.846, Q2**

Rosa, M.E., Barta, Z., Fülöp, A., Székely, T., Kosztolányi A. 2017. The effects of adult sex ratio and density on parental care in *Lethrus apterus* (Coleoptera, Geotrupidae). *Animal Behaviour*, 132, 181–188, DOI: 10.1016/j.anbehav.2017.07.023, **IF: 3.067, D1**

Rosa, M.E., Barta, Z., Kosztolányi A. 2018. Willingness to initiate a fight but not contest behaviour depends on intruder size in *Lethrus apterus* (Geotrupidae). *Behavioural Processes*, 149, 65–71, DOI: 10.1016/j.beproc.2018.02.004, **IF: 2.008, Q1**

Rosa, M.E., Kiss, J., Barta, Z., Kosztolányi A. 2019. Size-dependent investment in tusk length, testis size and sperm length in a biparental geotrupid beetle. *Journal of Zoology*, 309, 106–113, DOI: 10.1111/jzo.12704, **IF: 1.724, Q1**

Sramkó, G., Kosztolányi, A., Laczkó, L., Rácz, R., Varga, Z., Barta, Z. Range-wide phylogeography of the flightless steppe beetle, *Lethrus apterus* (Laxmann) (Coleoptera: Geotrupidae) reveals recent arrival to the Pontic Steppe. In preparation, proposed journal: *Scientific Reports*

6.2 Publications related to the project

Eberhart-Phillips, L.J., Küpper, C., Carmona-Isunza, M.C., Vincze, O., Zefania, S., Cruz-López, M., Kosztolányi, A. Miller, T.E.X., Barta, Z., Cuthill, I.C., Burke, T., Székely, T., Hoffman, J.I., Krüger, O. 2018. Demographic causes of adult sex ratio variation and their consequences for parental cooperation. *Nature Communications*, 9, 1651, DOI: 10.1038/s41467-018-03833-5, **IF: 11.878, D1**

Vincze, O., Kosztolányi, A., Barta, Z., Küpper, C., Alrashidi, M., Amat, J.A., Argüelles Ticó, A., Burns, F., Cavitt, J., Conway, W.C., Cruz-López, M., Desucre-Medrano, A.E., dos Remedios, N., Figuerola, J., Galindo-Espinosa, D., García-Peña, G.E., Del Angel, S.G., Gratto-Trevor, C., Jönsson, P., Lloyd, P., Montalvo, T., Parra, J.E., Pruner, R., Que, P., Liu, Y., Saalfeld, S.T., Schulz, R., Serra, L., St Clair, J.J.H., Stenzel, L.E., Weston, M.A., Yasué, M., Zefania, S., Székely, T. 2017. Parental cooperation in a changing climate: fluctuating environments predict shifts in care division. *Global Ecology and Biogeography*, 26, 347–358, DOI: 10.1111/geb.12540, **IF: 5.958, D1**