



SZENT ISTVÁN UNIVERSITY

**INVESTIGATION OF THE MATING SYSTEM IN A
BIPARENTAL BEETLE SPECIES**

Theses of doctoral (PhD) dissertation

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Gödöllő

2020

Doctoral school: Biological Sciences

Discipline: Biology

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1. Introduction and objectives

1.1. The actuality and importance of the topic

The emergence and maintenance of cooperative behaviour between unrelated individuals is one of the most intriguing questions in evolutionary biology. One example of such interaction is biparental care where both parents care for the offspring. Thus, the aim of my doctoral work was to investigate the reproductive behaviour of a supposedly biparental beetle species, *Lethrus apterus* (Laxmann, 1770, Coleoptera: Geotrupidae). In this species, paired beetles prepare deep burrows in which the female lays 6-8 eggs in a sequential manner. The parents gather leaves from the vicinity of the burrow, that will serve as a food supply for the larvae. According to early studies, there is a clear division of labour between the sexes as males are responsible for collecting leaves, while females use these leaf parts to form food balls for the larvae. However, this division of duties has been questioned in a recent study that reported predominant female leaf-collecting behaviour in a Hungarian *L. apterus* population. This indicates that there may have been a change in the division of labour between the sexes in the last century making this species an especially interesting model system. My goal was to better understand the mating system of *L. apterus* by investigating different social effects, intrasexual competition and by conducting research on pheromones.

1.2. The aim of the research

1. The effects of adult sex ratio and density on parental care

Theoretical models suggest that skewed adult sex ratio (ASR) and increased population density can influence parental roles by reducing the mating opportunities of the commoner sex and by changing the intensity of sperm competition. In biparental species, with a high risk

of extrapair paternity and continuous egg laying during the breeding period, males are expected to reduce parental investment and spend more time on mate guarding, or copulate more frequently with their social partner, if male density is high. In my first study, I tested this hypothesis in a field experiment by investigating leaf collection (parental provisioning), nest attendance (copulations and mate guarding take place inside the burrow) and reproductive success of individuals from nine treatment groups differing in ASR (three levels) and individual density (three levels). Under a high level of intrasexual competition among males (i.e. under male-biased ASR or high population density), I predicted an increase in the total time spent inside the burrow as well as a reduction in leaf collection and offspring number or size.

2. Intruding behaviour and male-male competition

Since fighting males can be frequently seen in front of the burrows, I hypothesized that intrasexual competition plays an important role in the reproductive system of *L. apterus*. Hence, in a field experiment, I tested the agonistic behaviour of large and small intruders against randomly selected resident males. My predictions were the followings: (1) Intruders win fewer fights than residents (2) small intruders initiate fewer fights and are more likely to lose when engaging in a fight than large intruders (3) encounters are longer and escalate more quickly when the intruder is large (4) contest duration increases during the breeding season due to the decreased number of vacant burrows.

3. The presence of alternative mating tactics

According to my hypothesis, males of extreme small size are less successful in the competition for mates, and may gain reproductive

success by employing alternative (e.g. sneaking) mating tactics. As a result, small males may experience higher risk of sperm competition and, as a consequence, are expected to invest less in pre- and more in postcopulatory reproductive traits than large individuals. To test this hypothesis, first, I investigated the presence of male polymorphism in *L. apterus*, and then compared tusk length (mandibular process that is expected to be under precopulatory sexual selection) as well as testis size and sperm length (postcopulatory traits) of large and small males to estimate their expenditure on pre- and postcopulatory reproductive traits. I also examined temporal changes in the above reproductive traits to test whether the relative advantage of large and small males differ early and late in the breeding season.

Chemical communication of *L. apterus*

Since sex pheromones are essential chemicals for the intersexual communication of most insect species, I hypothesized that they may play an important role in mate choice and mate recognition in *L. apterus*. My aim was to determine whether and where such sex pheromones are produced in this species. First, I conducted a behavioural test using an Y-olfactometer, where I observed the orientation of males towards the direction of female odour. Then I used electroantennography (EAG) to examine the responses of isolated antennae to odour mixtures obtained from different body parts or from the air above alive individuals and frass.

2. Materials and methods

2.1 1. The effects of adult sex ratio and density on parental care

The field study was conducted near Dorogháza in 2014. To manipulate ASR and individual density, I established enclosures by fencing off plots of size 2×1 m in the field. Individuals were randomly assigned to one of the nine possible experimental treatments created by the full factorial combination of three ASR (proportion of males: 0.25, 0.5, 0.75) and three density levels (4, 8, 12 individuals). I had three replicates of each treatment combination totalling in 27 plots containing 216 individuals. Above-ground activity was recorded using a webcam from 10 am to 6 pm. The plots were observed at four occasions with the exception of four plots that were observed only at three occasions. Video footages were analysed to determine the time spent inside the burrow (nest attendance) and the number of leaf-collecting events. Both variables were corrected for the number of individuals inside the plots. The nests in the experimental plots were dug out at the end of the breeding season and the number of offspring per females and the sex, weight and size of the offspring were recorded.

2.2 Intruding behaviour and male-male competition

The field experiment was carried out in the natural habitat of a *L. apterus* population at Csobánka in 2016. I selected 76 wild-caught males from two size categories: large (pronotum width ≥ 13.0 mm, $n=38$) and small (pronotum width ≤ 12.0 mm, $n=38$) defined by the upper and lower quartiles of the study population (determined by 107 males). Focal males (future intruders) were retained in captivity for a short time and then placed in front of a burrow where the resident male was known to be inside. I did not collect data about the resident's size as any disturbance prior to the behavioural test could have increased the chance of abandonment of the burrow. The behaviour of the

individuals was recorded by an action camera for 15 min. During the analysis of the video footages the following variables were defined: the time until the intruder reached the entrance of the burrow, the time, frequency and latency of intrusion (the intruder is not or only partly visible in the tunnel) and the different above-ground stages (facing, grabbing and turning). In case of lunging (small movements toward the opponent but without physical contact), I registered the number of events and the status (resident or intruder) of the individual that showed the behaviour. The outcome of the contest was determined from the intruder's point of view: leaving (the intruder left the vicinity of the burrow without a fight), losing (the intruder left after losing a fight), staying (the intruder stayed in the burrow without a fight) or winning (the intruder stayed inside after winning a fight). The outcome of the contest was also analysed using a binary variable where leaving and losing together were called 'failure', while winning and staying were considered as 'success'.

2.3 The presence of alternative mating tactics

Field work was carried out in Debrecen in 2017. Tusk length and pronotum width of 231 males were measured with a calliper to determine the presence of male polymorphism in the population. Investment into precopulatory (tusk length) and postcopulatory (testis size and sperm length) traits was analysed using male beetles of two extreme size categories (small: pronotum width ≤ 11.00 mm, large: pronotum width ≥ 13.00 mm) early (n=15 small and 15 large individuals) and late (n=9 small and 14 large individuals) in the breeding season. After measuring tusk length, the beetles were dissected and testis size (the summed area of the 12 testis follicles per individuals) and sperm length (the mean length of 60 sperms per male) were determined with the aid of the ImageJ software. Relative tusk length and relative testis size were also

calculated by dividing the measurements with the pronotum width of the individuals.

2.4 Chemical communication in *L. apterus*

I conducted a behavioural test using an Y-olfactometer, an instrument to test the luring ability of volatile chemicals. As usually it is the females that are expected to produce sex pheromones, I decided to test the orientation of 32 males towards the direction of the odour of three alive females. After each run, I recorded the direction of the stimulus and the way that the focal male had chosen at the intersection of the Y-tube (female odour or fresh air).

In the subsequent studies I used electroantennography (EAG), that is a method for recording and measuring electrical potentials from isolated but electrophysiologically-active antennae, to analyse the antennal response of female and male individuals to different odour mixtures. First, I planned to locate the pheromone glands by extracting various body parts (tip, underside and top of the abdomen, and forelegs) of 20 male and 20 female beetles in hexane, and then measuring the antennal response of 11 individuals (five males and six females) to the stimuli. In the second round, I tested the EAG response to samples that were collected by 24-hour volatile collection from alive individuals of four different group composition (separately kept males and females, a male and a female kept together, and two males kept together). Individuals were placed inside clean glass tubes and the volatile compounds from the air around them were trapped on activated charcoal. In total 36 individuals were used at six volatile collection occasions. The resulting samples were tested on three male and three female antennae. In a similar way, I also performed a 4-hour volatile collection from frass that was collected from the first (March and April, n=9 females and 40 males) and second half (May, n=55 females and 26 males) of the breeding season. The stimuli were tested on three male and four female antennae. In the EAG tests 10 μ l pure hexane

(the solvent used for the pheromone samples) was used as the control odour. The pheromone research was done at the Plant Protection Institute of the Centre for Agricultural Research of the Hungarian Academy of Sciences between 2015 and 2017.

3. Results

3.1 1. The effects of adult sex ratio and density on parental care

The duration of nest attendance increased significantly with the increasing ASR. Parental provisioning (leaf collecting) tended to decrease with the increasing ASR, however, the effect of density was not significant.

In total 72 offspring were counted in the 27 plots. For offspring number, there was a significant interaction between ASR and density: the number of offspring tended to be lower under male-biased ASR, but only when density was low or high. ASR and the density of individuals did not have any effect on offspring sex ratio, weight, thorax width or, in case of male offspring, tusk size.

3.2 Intruding behaviour and male-male competition

All individuals reached the entrance of the burrow, however large intruders approached it significantly quicker than small intruders. Intrusions occurred 60 times out of the 76 trials and large intruders spent significantly more time inside the burrows than small ones. Above-ground contests were observed in 17 cases, all of which were preceded by an intruding event. Intruder size did not predict the occurrence, time or latency of the contests or its stages. Intruders, regardless of their size category, presented more lunging events than the residents. The duration of the above-ground contest was independent of the time of the breeding season.

Residents won most of the contests (13/17, 76,5%). Large intruders were more successful than small ones, meaning that they stayed inside the burrow without a fight or won more times, whereas small males were more likely to leave without a fight. The two size categories did not differ in their chance to stay, lose or win, however, it is worth mentioning that all the four intruders that won a contest belonged to the large size category.

3.3 The presence of alternative mating tactics

The plot of tusk length against pronotum width indicated a non-linear, continuous S-shaped relationship. From the eight fitted continuous models, the two-breakpoint segmented model performed best. Similarly to the population data, males from the large size category had longer tusks even after controlling for body size. These results indicate male polymorphism in relation to tusk length.

In case of absolute testis size, there was a significant interaction between size and the time of the breeding season. Large males had significantly bigger testes than small ones over the breeding season, however, it showed a significant decreased in large, but not in small individuals. Large males also had bigger testes relative to their body size than small ones, but relative testis size decreased significantly over the breeding season in both size categories. Sperm length did not differ between small and large males and was not affected by the time of the breeding season.

3.4 Chemical communication in *L. apterus*

During the Y-olfactometer test, males chose the direction of the stimulus and the control source with an equal chance. EAG response of male antennae was higher to the extract of the ventral side of the female abdomen than to the control odour, although their response to the ventral side of the male abdomen was also relatively high. The antennae of females did not show significant

response to any of the body extracts. In contrary, only female antennae gave higher responses to most of the odour samples of the alive individuals (the only exception was the odour of the males that were kept together), while the male antennal response was not different from the control in any cases. All frass samples elicited a high antennal response from males but not from females.

3.5 Summary of the results and new scientific achievements

- I showed that under high levels of intrasexual competition among males (caused by either a male-biased ASR or high individual density), individuals stay inside their burrows more, possibly to guard their mate. I also found a tendency toward reduced parental investment under male-biased adult sex ratio in *L. apterus*.
- I found that male-biased groups produce fewer offspring under high and low densities, indicating a possible conflict of interest between the sexes over paternity and brood size.
- In case of the intrasexual contests, I showed that residents win most of the contests and that only a few large intruders, with superior competitive abilities, are able to win the trial.
- Small intruders seem to be less willing to engage in contests and are less successful in their takeover attempts than large males. However, contrary to our predictions, the duration and escalation of the fight did not differ between the two intruder size categories and did not change over the breeding season.
- I also demonstrated evidence for male polymorphism as extremely large males had longer tusks relative to their body size than small individuals.

- Large males had bigger testes than small ones throughout the breeding season, however, sperm length did not differ between the two size categories. These results do not prove (or deny) the presence of alternative mating tactics in *L. apterus*, but indicate that tusk length and testis size depend mostly on the size category of the individuals, while sperm may have an optimal length regardless of body size.
- According to the results of my pheromone studies, it seems likely that males are sensitive to the odour of the ventral side of the female abdomen and frass, however, only females presented higher response to the stimuli collected from alive individuals. Given the contradictory results and the small sample sizes, these results are difficult to interpret.

4. Conclusions and proposals

The results of my doctoral work suggest that the extent of intrasexual competition may play an important role in shaping the parental system of *L. apterus*. As egg laying is continuous throughout the time of care, the risk of sperm competition is present over the entire breeding season. When the level of intrasexual competition increases, males may spend more time and energy on mate guarding and on fighting off rivals, at the expense of care giving, ultimately resulting in fewer offspring. According to my results, a male-biased adult sex ratio and high individual density are able to increase the level of intrasexual competition, and the outcome of these contests are mostly determined by status of residency and male size. Such contests can be frequently seen under natural circumstances, and the findings, that small and large males differ in their contest behaviour, and that males are polymorphic with respect to tusk length raise the possibility of the presence of alternative mating tactics. Although, large males had not only larger tusks but bigger

testes and equally long sperm as small individuals and so I could not prove that such alternative mating tactics exist in *L. apterus*. In light of these results, I suspect that it is possible that the changes that happened in the last century in the species' parental system could be due to an increase in the level of intrasexual competition in the Hungarian populations. An underlying cause may be habitat fragmentation or the shortening of the breeding season as a result of climate change.

5. Publications

Publications on the topic of the dissertation:

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). In: *Animal Behaviour*, 132, 181-188 p. (MTMT ID: 3272383).

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). In: *Behavioural Processes*, 149, 65-71 p. (MTMT ID: 3362517).

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. In: *Journal of Zoology*. (MTMT ID: 30801759).

Publications related to the topic of the dissertation:

McNamara K.B., Robinson S.P., **Rosa M.E.**, Sloan N.S., Van Lieshout E., Simmons L.W. (2016): Male-biased sex ratio does not promote increased sperm competitiveness in the seed beetle, *Callosobruchus maculatus*. In: *Scientific Reports*, 6. (MTMT ID: 3083158).

Kiss J., Rádai Z., **Rosa M.E.**, Kosztolányi A., Barta Z. (2020): Seasonal changes in immune response and reproductive investment in a biparental beetle. In: *Journal of Insect Physiology*. (MTMT ID: 31032520).

National and international conference presentations:

Rosa M.E., Barta Z., Fülöp A., Székely T., Kosztolányi A. (2015): The effects of adult sex ratio and density on parental care in *Lethrus apterus*. XVII. Congress of the Hungarian Ethological Society, Dobogókő, Hungary (talk in Hungarian).

Rosa M.E., Barta Z., Kosztolányi A. (2016): Intruding behaviour and contest outcome in relation to intruder size in *Lethrus apterus*. XVIII. Congress of the Hungarian Ethological Society, Debrecen, Hungary (poster in Hungarian).

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). ASAB Winter Meeting, London, UK (poster in English).