K-115970: FINAL REPORT 01.10.2015-15.12.2020

The major goals of this project focusing on a natural population of a passerine bird were to
(i) uncover how observed phenotypic variation in three different behaviours (singing, aggression and risk-taking) can be decomposed into within- and between-individual variations (objs. 1,5),
(ii) understand the proximate and ultimate mechanisms that shape such hierarchical organization (objs. 3,4) and
(iii) provide deeper insights about the evolutionary ecology of behavioural plasticity and consistency (objs. 2-5).

For several years, we routinely assayed various behavioural traits of male collared flycatchers (*Ficedula albicollis*), one of the focal species in this project, under completely natural conditions. Earlier studies have demonstrated at the phenotypic level how these behaviours correlate with each other and with physiological condition and fitness. To extend this line of research, we applied an integrative approach that amalgamated the long-term monitoring data with targeted within-individual sampling along different time scales, and the characterization of genetic relationships between individuals based on molecular markers. We designed manipulative experiments to test predictions originated from our correlative approaches.
The particular objectives are follows:
1) Decomposing phenotypic variance into within- and between-individual variances
2) Identifying proximate mechanisms that mediate behavioural variations within individuals
3) Identifying ultimate mechanisms that mediate behavioural variations within and between individuals
4) Decomposing phenotypic variance into genetic and environmental components
5) Between-year variance of behaviours and its correlations with ecological and social environmental factors

Achievements by objectives
Objective 1
100% achievement. We have already fulfilled this objective in the early parts of the project. Based on the available long-term data, we have performed the appropriate statistical analyses for song traits and other behaviours, showing how these traits vary within individuals at different temporal scales.

We have published several papers on this topic using collared flycatcher as model species: Garamszegi et al. 2015, Jablonszky et al. 2017, Zsebők et al. 2017, Hegyi et al. 2019: *Ibis*, in addition to methodological papers: Allegue et al. 2016, Zsebők et al. 2018: *J Ornithol*, 2018: *Behav Ecol Sociobiol*, 2019, 2020.

Objective 2
100% achievement.
We have published a paper that suggests that measuring nutritional reserve trajectories in addition to actual condition may reveal functionally important processes underlying signal-condition as well as behavioural correlations (Hegyi et al. 2019: *Behav Ecol Sociobiol*). We have also published a paper that investigates the relationship between health state, oxidative damage and behaviour in the collared flycatcher (Szabó et al. 2020). Another paper investigating the predictions of the objective shows that behaviour in a novel environment varies independently of physiological state as reflected by body condition (Jablonszky et al. 2020).

In a pilot study, we applied a fluoxetine treatment to manipulate the physiological state of the flycatchers. Fluoxetine is a selective serotonin re-uptake inhibitor, and is known to affect aggressive and risk-taking behaviour in poultry. During the chick-feeding period we subsequently monitored the activity and risk-taking behaviour of 20 randomly selected males (half of them received fluoxetine, the rest were treated with physiological water as control) when feeding young. We found no statistically identifiable difference in these traits between the experimental and control birds. Given these technical difficulties some predictions for this objective were tested in other model organisms.
We manipulated individual experience with territorial conflicts during the courtship period by forcing 13 individuals to lose in a series of repeated social challenges by intruder males. Loser males were those, who were not allowed to retain their original territories after exposing them to a decoy male, as we had closed the entrance of their nest box with a flycatcher dummy. This manipulation forced them to seek another territory, which potentially had a lower quality, thus causes a negative experience. The behaviour of these individuals (novelty avoidance, aggression, risk-taking) was monitored on the newly occupied territory, and was compared to the behaviour of males that had been allowed to re-occupy their original territory, thus their winner position was kept reinforced. These comparisons revealed no difference between the two groups of males having different winner/looser history in territorial conflicts. However, individuals that lost their original territory altered their songs at their newly occupied territory, which raised within-individual variance in song traits along the change in the quality of the environment (Zsebők et al. 2017).

Papers for this topic using collared flycatcher as a model species: Krenhardt et al. 2016, Szöllősi et al. 2016, Zsebők et al. 2017, Hegyi et al. 2019a, Jablonszky et al. 2020, Szabó et al. 2020, Krenhardt et al. submitted.
Papers for this topic on alternative organisms: Sandel et al. 2016, Horváth et al. 2017, 2019, Winternitz et al. 2017, Urszán et al. 2018.

Objective 3
100 % achievement.
We have compiled a database and performed the appropriate statistical analyses to determine the within-individual repeatability of reaction norms for song traits, as a new methodology (double hierarchical modelling) has become available to study different components of reaction norms (plasticity and predictability). Given that double modelling is a data hungry exercise as it requires a large number of within-individual repeats, we have published papers using this statistical approach in other model organisms to test the biological predictions for other behavioural traits: Horváth et al. 2019, Urszán et al. 2018. We still have one sub-project for this objective to be terminated: we compiled database, performed statistical analyses and started drafting a manuscript for a study that uses double hierarchical modeling to decompose the different components of reaction norms (plasticity and predictability) for song traits. We expect to submit the manuscript soon (Jablonszky et al. in prep).
Concerning the link between behaviour and fitness, we have uncovered the relationship between territorial aggression and reproductive success and survival. (Szász et al. 2019).We have published another manuscript that investigates the predictions of the objective in a different model organism, in which life-time reproductive success can be reliably estimated for all individuals in the population under controlled laboratory conditions (Canal et al. 2021 in press). In the collared flycatcher, using the data collected during the course of the project, we have an ongoing study that investigates how risk taking behaviour affects fitness in different years, for which we started drafting the corresponding manuscript (Krenhardt et al. submitted)

Papers for this topic using collared flycatcher as a model species: Vaskuti et al. 2016, Laczi et al. 2017, Jablonszky et al. 2018, Nagy et al. 2019, Szász et al. 2019, Krenhardt et al. submitted, Jablonszky et al. in prep.
Papers for this topic on different organism: Garamszegi et al. 2018: *Int Comp Biol*, Soma & Garamszegi 2018, Urszán et al. 2018, Horváth et al. 2019, Canal et al. 2021 in press.

Objective 4
85% achievement.
For the genetic analyses we have started the optimization method for SNP screening that will potentially allow us to determine relatedness between individuals as well as to adopt genome-wide association approaches. We have selected a subset of 864 samples (1999-2018) for sequencing. Selection was based on the information available for the each individual to maximize the number of research questions, aside those related to this objective, that we will able to address in the future with the genomic data. DNA was extracted from whole blood and sequenced at the CNAG with an Illumina HiSeq2000 v4. Subsequently, we pre-analysed and prepared the data for further analyses. To this end, we trimmed the sequences, assembled them and performed the variant calling. We removed for further analyses individuals with a high percentage of missingness (> 20%) and run analyses of linkage by missingness. Then, for the analyses of selection, we selected autosomal SNPs i) without differences in the allelic frequencies between sexes or immigrant status (individuals born inside vs outside the study area) and ii) in Hardy Weinberg equilibrium. Finally, our sample size to analyse temporal changes in the genetic composition of the population resulting from selective processes (Tasks 4 and 5) was 809 individuals and approx.17000 SNPs. We are currently collaborating with expert bioinformatics to perform further analyses on the genetic data to calculate genotype-phenotype associations for behavioural traits, to investigate the temporal variations in the genetic profile due to climate change, to determine health condition (the load of micro-parasites) based on meta-genomic approaches.

Paper for this topic using collared flycatcher as a model species: Garamszegi et al. 2018: *Mol Ecol*.

Objective 5
90% achievement.
During the field seasons we characterised the most important, year-specific ecological parameters such as temperature, predation pressure, competition and food supply on a yearly basis that could be related to the within-year distribution of behavioural traits, and performed statistical analyses to detect long-term temporal patterns. These analyses suggested that, due to lack of power, we need to incorporate data from more years and to apply a more rigorous Bayesian statistical framework. Applying these approaches, we were able to uncover that the strength of the relationship between risk taking and survival varies across years, and this among-year variation is determined by a parallel among-year variation in predation pressure (Krenhardt et al. in prep). Some of our-long term data could also be used for an interspecific study on birds that tested similar hypotheses at a broader scale, and for which we have recently submitted a manuscript for publication: Romano et al., submitted. To extend the predictions of this objective, we are also testing for the relationship between the variance components of behaviours and environmental factors in a phylogenetic comparative context. Accordingly, we are participating in scientific collaborations, in which the predictions of the objectives are tested in the between-population or in the between-species levels. Two papers from this work have already been published (Garamszegi & Møller 2017, Samia et al. 2017).

Note that outcomes from our international collaborations focusing on another model species or on inter-specific patterns are highly relevant for this objective: Garamszegi et al. 2020: *J Evol Biol*, 33:318-328., Geffroy et al. 2020: *PLOS Biology*, 18(9): e3000818.

Papers for this topic on alternative organisms: Garamszegi & Møller 2017, Samia et al. 2017, Senar et al. 2017, Casagrande et al. 2018, Sol et al. 2018, Laczi et al. 2019, 2020, Romano et al. in press, Krenhardt et al. in prep.
Papers about general statistical topics: Garamszegi 2016, Garamszegi & de Villemereuil 2017.

Overall, we consider that the project was successful. So far, we have published more than 30 scientific papers that are indexed in Web of Science and that include K-115970 as funding ID. We still have some on-going studies in relation to ***objectives 4-5***, which are expected to lead to further publications in the near future. These future papers will appropriately acknowledge the current project as main funding.

Difficulties

During the course of the project, we experienced difficulties concerning the experiments planned for objective 2. In line of experiments, we aimed at manipulating the physiological state of the individuals (by using either Fluoxetine treatment or an artificial antigen), while in another we targeted individual experience (by applying forced territorial displacement). These manipulations were unsuccessful, as at the achieved sample size we were unable to derive statistical differences between the behaviour of control and experimental individuals. In addition, some of the predictions require large sample size within individuals meaning that one needs to obtain several measurements on behaviour in the same individuals. This is impractical in a study of a natural population of the collared flycatcher. We arrived at the general conclusion that our study species (collared flycatcher) is not an ideal model for experiments targeting within-individual changes in condition or experience. To combat this shortcoming, we established scientific collaborations, in which we could perform these experiments with higher success in more suitable, lab organisms (e.g. *Armadillidiumvulgare, Callosobruchusmaculatus, Iberolacertacyreni, Lacertaviridis, Rana dalmatina*). We believe that we could successfully solve these problems using alternative organisms to test the predictions of our hypotheses, which led to many publications (Horváth et al. 2017, 2019, Urszán et al. 2018, Canal et al. in press).
The additional difficulty appeared in 2020 was the situation that the COVID pandemic caused. We infer that this situation caused minor effects to our study, as we could perform the basic field activity allowing us to continue recording long-term data.

Training

Karis Douglas, an undergraduate student in the Faculty of Life Sciences at The University of Manchester (United Kingdom) and Katherina Kasper, an undergraduate student from the University of Köln (Germany) have successfully fulfilled a training placement of 10 and 2 months (respectively) in our research group. Within this program, the studentsparticipated in several aspects of the work associated with the current project (e.g. field work, analysis of data) under the co-supervision of László Zsolt Garamszegi and Sándor Zsebők.
Two PhD students, who worked on the project have successfully defended their dissertation and obtained doctoral degree in the last phase of the project (Eszter Szász, supervisor: Balázs Rosivall, 07.02.2019; Mónika Jablonszky, supervisor: László Zsolt Garamszegi, 20.06.2019). Two other PhD students joined the research group. Starting from 2016, Katalin Krenhardt and

Éva Vaskuti received grants from the Hungarian Government to perform PhD studies for four years. They worked under the co-supervision of László Zsolt Garamszegi, János Török and Sándor Zsebők. Katalin Krenhardt and Mónika Jablonszky were able to continue their scientific carrier, as they have recently won a young investigator position at the Institute of Ecology and Botany. We also consider this as a success in the training value of the project.
Furthermore, four Hungarian undergraduate students were also involved field work and data analyses in the course of the project.