SNN-125627 – Final report

2017 – 2021

Summary

Altogether, the Slovenian – Hungarian cooperative project had successful four years, despite the challenges we met. First, it took some time to accept the fact that long-term rearing and breeding of cave-adapted *Asellus aquaticus* (and *Niphargus* too) failed in both teams' labs. This was an unexpected obstacle considering the relative ease of laboratory rearing of surface populations. As a response, we simply shifted the main focus from within individual behavioural variation and quantitative genetic common garden experiments towards between individual behavioural variation, targeting as many functionally different behavioural traits as possible and to measure a large set of morphological traits on a large set of individuals. Further, we employed our original behavioural framework in smaller side projects, utilizing it in other model systems, typically in the form of occasional external collaborations and we also conducted a phylogenetic meta-analysis to test some of our questions in this framework. Second, the COVID pandemic made us to give up many of the originally planned 'physical' collaborations (i.e. visiting each other and working in Slovenian – Hungarian mixed groups for the different projects) and turn towards theoretical collaborations *via* numerous online discussions, shared data analyses, etc.

During this period, we published 30+ articles in international refereed journals (with impact factor) with the grant being acknowledged and there are currently seven manuscripts submitted and five in preparation. The databases being produced during the four years or being developed at the moment will provide us with material for plenty of more publications in the upcoming years. One PhD and six MSc students defended their theses on topics covered by the application, while four PhD and one MSc students joined later and are working currently on material and projects stemming from the project. Clearly, the collaboration and work did not stop by the ending of the funding period, but it is developing in a steady pace and we definitely plan on future cooperative applications (for instance, we will apply for another round of SNN in 2022). We participated in several national and international conferences, even though we would have attended more in the absence of pandemic. Our collaborations with Dr. Heather Bracken Grissom (genomics, Crustacean Genomics and Systematics Lab, Department of Biological Sciences, Florida International University, USA) and Dr. David Brankovits (stable isotope studies, Marine Chemistry and Biochemistry, Woods Hole Oceanographic Institution, USA) are continuing and will bring results in the next two years. In the first year of the funding period, we organised a scientific meeting on the evolutionary ecology of *Asellus aquaticus*, hosted by the Grupul de Explorări Subacvatice şi Speologice (GESS) in Mangala, Romania, with 11 participants from six institutions over EU and US. Below, I will briefly summarize our most interesting scientific results.

Behaviour

Movement activity

We proved that *A. aquaticus* evolve increased movement activity following the colonisation of and adaptation to the thermal Molnár János Cave, probable as a response to release from predation, and this effect is particularly strong in males who are engaged in mate searching / mate guarding activities during the reproductive periods.

In a comparison between four cave – surface population pairs, we showed various systematic shifts in activity, risk-taking ad exploration. However, thanks to the high number of repeated essays done on every study individual, we could also prove that between-individual behavioural variation is lower in caves than in surface populations resulting in higher repeatability (i.e. stronger animal personality) in the latter. This is a pioneering step in supporting the idea that directional selection results in decreased phenotypic variation compared to fluctuating selection, or with other words, fluctuating selection is a main driver of maintaining phenotypic variation within populations.

We also conducted a similar experiment to the latter, but comparing *Niphargus* species instead of *A. aquaticus* populations. We are at the stage of data analysis. This study will allow us to test the above hypothesis in another evolutionary scale.

Behavioural innovation

By studying food preference and food type innovation of *A. aquaticus* in two studies, we showed that despite of being isolated for at least 60000 years in the thermal Molnár János Cave providing only endogenous bacteria as food resource, (i) cave-adapted individuals retained the ability to identify surface food as food, (ii) actually preferred surface food over cave food and (iii) showed similar innovativeness towards unnatural but edible food items than their generalist surface conspecifics. This support that cave-adapted *A. aquaticus* remained food generalist. Further, we found that surface population almost completely avoided cave food, as well as recent colonisers (i.e. surface morphs that were found in the cave despite the strong and permanent water outflow). This suggest that the special cave food was rather an obstacle against than a trigger of cave colonisation.

Locomotion

We found that *Niphargus* morphology and locomotion show parallel patterns: lake species are large, long-legged, fast and shows a variety of locomotion patterns including upright movement resembling walking, while stream species are small, short-legged, slow and moving on their sides aided by tail flipping. The results showed that integrating locomotion patterns into the study of morphological evolution is an important step towards understanding adaptations to the subterranean environments.

Exploration

We estimated the colonisation potential (by studying exploration in a maze) of surface vs. cave *A. aquaticus*, including surface individuals found in the cave (‘colonists’). Males were more explorative than females, and surface populations were more explorative than cave individuals. Colonist individuals were more explorative than their surface conspecifics from the same population. This result suggests that caves function as dispersal traps after local adaptation takes place, and cave specialist populations lose their dispersal ability. Further, the subset of individuals in a population that are ready to colonise a markedly different habitat have higher explorativeness than the population average, supporting the personality-dispersal link. We also provided experimental evidence about surface *A. aquaticus* (that is intuitively treated as a diurnal species) moving more in the dark than in the light.

Aggregation / sheltering

We compared aggregation and sheltering in surface- (various predators) and cave-adapted (no predator) populations of the isopod *A. aquaticus*, while also testing for variation in light-induced behavioural plasticity. Variation in sheltering was explained by habitat type: cave individuals sheltered less than surface individuals. We found high between-population variation in aggregation with or without shelters and their light-induced plasticity, which was not explained by habitat type. Cave individuals decreased (habituation) whereas surface individuals increased sheltering with time (sensitization). We suggest that population variation in sheltering is driven by predation, whereas variation in aggregation must be driven by other, unaccounted environmental factors, in a similar manner to light-induced behavioural plasticity. Based on habituation/sensitization patterns, we suggest that predation-adapted populations are more sensitive to disturbance related to routine laboratory procedures.

Risk taking

We ran an experiment to see whether there is evolutionary divergence in antipredatory behaviour between cave (no predator) and surface (predator adapted) populations of *A. aquaticus*. We used olfactory stimuli from two natural predators with different foraging strategy (active *vs*. ambush), the European perch (*Perca fluviatilis*) and larvae of Southern hawker (*Aeshna cyanea*) and one introduced predator flourishing in one of the surface population’s habitat, the guppy (*Poecilia reticulata*). We finished with video analyses and are currently at the stage of statistical evaluation. So far, the preliminary results are a mixed bag: there seems to be lot of variation explained by population and sex, but habitat type does not look like an important predictor. However, due to the complex hierarchical nature of our data, further analyses are needed to reach the final results.

Meta-analysis

We ran a phylogenetic meta-analysis (19 species, 21 studies, 43 effect sizes) to reveal general trends between two major components of animal personality: behavioural type (individual mean behaviour) and behavioural predictability (within-individual behavioural variation). We found that there is no overall correlation across the studied taxa. However, the fact that the overall correlation did not differ from zero does not mean that there are no correlations. Indeed, correlations are present, but they are varying in their directions. The pattern suggests that the link between behavioural type and behavioural variation is not shaped by general constraints (physiological, genetic), but by local selective forces.

Morphology

We measured 17 functional traits of 776 individuals from six cave and nine surface *A. aquaticus* populations. We tested hypotheses about cave adaptation (troglomorphosis, elongation hypothesis), sexual dimorphism, and more importantly, sexual dimorphism in cave adaptation (i.e. sex-specific cave adaptations). 10 out of 17 traits showed habitat-dependent population divergence, likely reflecting habitat-driven changes in selection acting on sensory systems, grooming, antipredator mechanisms or feeding. We found sexual dimorphism in 15 traits, explainable by sexual and fecundity selection. In eight traits, the habitat effect was sex-specific (significant habitat*sex interaction). We conclude that cave-related morphological changes are highly trait-and function-specific and that the strength of sexual/fecundity selection strongly differs between cave and surface habitats.

We have just finished building a similar database on cave- *vs.* surface-dwelling *Niphargus* species.

We plan first to run a similar analysis on *Niphargus* to what we did with *A. aquaticus* to test the same hypothesis on a different evolutionary scale. Then, we will use both databases to test for the effects of directional (cave) *vs.* fluctuating (surface) selection of shaping the levels of morphological variations within/between populations and within/between habitats.

We are at the final stage of describing two new species from the Molnár János Cave.

Life history

Sex ratio

Many studies of subterranean species reported female-biased sex ratio, but no systematic study has yet been conducted. We tested the hypothesis that sex ratio becomes more female-biased with increased isolation from the surface.We compiled a data set of sex ratios of 35 *Niphargus* species from three distinct habitats (surface-subterranean boundary, cave streams, phreatic lakes) representing an environmental gradient of increased isolation underground. The sex ratio was female-biased in 27 of 35 species; the bias being statistically significant in 12 species. We found the predicted increase in female bias towards the deeper parts of caves. We suggest that a history of inbreeding in subterranean populations might lower inbreeding depression such that kin selection favours mating with siblings. This could select for a female-biased offspring sex ratio due to local mate competition among brothers.

Body size, sexual size dimorphism

In insular environments with simple communities, small-bodied species tend to evolve larger following a release from interspecific competition and predation (often referred as island rule). Further, body size and sexual size dimorphism (SSD) often scales allometrically across species or populations. According to Rensch's rule, male-biased SSD increases, while female-biased SSD decreases with increasing mean body size. Here, we tested the island rule and Rensch's rule in *A. aquaticus* by comparing six cave (insular) to nine surface (mainland) populations ($N_{male} = 358$; $N_{female} = 298$). We found extensive between population variation in mean body size (> 80%) and SSD (from being absent to males being 42% larger than females). However, habitat type did not explain the variation and thus we found no support for the island or Rensch's rules.

Phenology

We collected data on one cave and one surface *A. aquaticus* population monthly for a year, based on 100+ adult individuals collected, sexed and measured per sampling occasion. We are analysing the data at the moment, but preliminary results suggest that the surface populations shows the typical three reproductive peaks per year, distributed from late spring to early autumn. The cave population is completely different by not following seasonality but also showing (albeit weaker) reproductive peaks.

Genetics

Unfortunately, we invested significant amount of time and energy into finding/developing highly variable microsatellite markers that work with all our *A. aquaticus* populations (cave and surface, covering a large geographic range in Europe), but we failed. Therefore, we switched focus and went for sequencing 1500 *Niphargus* individuals covering 20+ species (including potentially new species from previously unknown habitats) for mitochondrial genes. The sequencing is under progress at the moment and the resulting database will be a highly valuable asset for future work from taxonomy to phylogeography in the future.