Investigation of environment-induced responses in an amphibian meta-population

Final Report
01.10.2013 - 31.07.2017

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The environment can directly shape organisms' traits through individuals' plastic responses to predictive cues and thus greatly contribute to the evolution and maintenance of phenotypic variation in nature. Renewed interest in phenotypic plasticity resulted in the development of numerous predictions about the conditions under which adaptive plasticity may evolve; however, consensus about the potential significance of plasticity in different traits in different life stages is still lacking. During the project I investigated the effect of predation threat, one of the most important environmental cues, on reproductive behaviour, maternal effects, embryonic and larval responses in smooth newts (*Lissotriton vulgaris*) using experimental approaches, examined genetic differentiation between sub-populations in the studied meta-population, and assessed environmental heterogeneity at the breeding habitats. I also constructed a theoretical model to explore how non-random habitat use may alter previous predictions about the evolution of plasticity in organisms characterised by multiple life stages. During the implementation of the project, 3 students took part in various phases of the conducted studies, and the main findings were presented at 3 international and 2 national conferences. Although not all results have been published yet, the manuscripts will be completed and submitted to relevant journals by the end of 2017.

In this final report I present the most important details and results of the studies that were conducted in each year of the project in separate sections, with reference to the published paper or the manuscript I plan to publish from the findings of each study. Details on these papers/manuscripts can be found in the "Publications (01.10.2013 - 31.07.2017)" section at the end of the report together with the list of conferences I attended in relation to the project and with the list of additional publications.

First year (2013-2014):

In the first year of the project I conducted one field survey and one laboratory experiment. In the field survey I aimed to estimate the environmental variation during the reproductive period in the breeding habitats of the studied smooth newt meta-population. During the reproductive season (from 9th of March to 12th of May, 2014; 10 weeks in total) I visited 8 ponds belonging to 4 groups of closely located ponds in the Pilis-Visegrád Mountain, in which ponds smooth newts were found to reproduce in previous years. Once a week, I measured temperature, pH level, conductance, total dissolved solids (TDS), and salinity using a portable CONSORT C6020T multimeter. Dissolved oxygen was measured only at the first two occasions due to the malfunction of the electrode. I collected water samples at five locations at each pond, and calculated the average values for each measurement on each week per pond. TDS and salinity strongly positively correlated with conductance in the sampled ponds (both $r_s \geq 0.78$), so only the temporal variation in pH and conductance were analysed more in detail. The fitted random-effect models (also allowing unequal variances between ponds) estimated the average pH (± SE) in the sampled ponds to be 6.5 ± 0.1 (range in the raw data: 5.6-8.3) and the average conductance to be 127.6 ± 8.8 (range: 77-235.4). The between-pond variance was approximately 4-fold compared to the within-pond variance in conductance (24.9 [95% CI: 13.6-45.5] versus 5.8 [3.5-9.4]), while the difference was negligible in the case of pH (0.5 [0.3-0.8] versus 0.6 [0.4-1]). For both measures, those models which included ‘time of sampling’ as a random slope fitted better to the data, although in the case of pH the difference between the compared models was only marginally significant (conductance: LRT=24.66, $P<0.001$; pH: LRT=6.08, $P=0.048$). I also found that the total number of eggs females laid during the experiment (see below) was negatively affected by conductance, but not influenced by pH or temperature, measured at their pond of origin 2-3 days before their capture (linear model using generalized least squares [also allowing unequal variances between ponds]; $F_{1,75}=7.54$, $P=0.008$). This may imply that conductance (or a dissolved component directly influencing conductance) has the potential to serve as an environmental cue for adults to assess the quality of the water body and/or the probability of pond desiccation during the reproductive period, which idea should be tested experimentally in the future. In such a study, conductance of the housing water would be manipulated within adequate temperature regimes and the duration and extent of females’ reproductive investment measured. Raw data on the measured water chemistry parameters was provided to the Duna-Ipoly National Park Directorate and the national authority of the Middle-Danube-Valley Inspectorate for Environmental Protection, Nature Conservation and Water Management. Heterogeneity in invertebrate predator abundance among ponds in the study area was also investigated and demonstrated as part of a field survey conducted by the Lendület Evolutionary Ecology Research Group (this latter data was a subset of a more detailed dataset on predator abundance; see Bókony et al. 2016 in the “Project-related papers and manuscripts” section).

In the experiment of the first year I investigated females’ oviposition preference and parental care in the presence of chemical cues from invertebrate predators. Gravid female smooth newts were captured by dip-net in 4 ponds in the Pilis-Visegrád Mountain between 17th of March and 14th of April, 2014, resulting in 80 females in total in 5 consecutive weeks during the reproductive season. In the experimental trials, females were put separately for overnight into a testing container (60 × 40 cm), which had two compartments at one end. In-flow at one of these compartments was clear water, whereas at the other compartment the in-flow was water mixed with chemical cues from invertebrate predators (mean ± SD of in-flow rate: 0.64 ± 0.09 mL/s). Plant for egg deposition was provided in each compartment. I collected laid eggs in each environment (i.e. compartment) in the morning, and then wrapped and unwrapped eggs were counted, and, after carefully unwrapping the wrapped eggs, photographed. Eggs from each environment were kept in approx. 100 ml water until hatching. Into the analyses only those females were included which deposited eggs at least in one of the two compartments during the trial (68 out of 80 animals). In the laboratory, Turós Brigitta, a BSc student (wildlife conservation and management) of the Szent István University, provided assistance between 1st of March and 31st of May, 2014. I found that female newts did not avoid laying eggs in the environment with predator cues; however, individuals that deposited eggs into both environments adjusted the size of the laid eggs to the perceived environment during the reproductive season. Females deposited larger eggs earlier in the season, but egg size decreased with time in the absence of predator cues, whereas individuals laid eggs of average size throughout the investigated reproductive period when predator-cues were present. Also, egg size was found to be positively related to hatching success. Individuals did not adjust their wrapping behaviour to the presence of predator cues, but females differed in the extent of egg-wrapping between ponds. Females’ body mass and tail depth were also different between ponds, whereas their body size was positively associated with egg size. According to these results, female smooth newts have the potential to exhibit activational plasticity and invest differently into eggs depending on temporal and environmental factors. The results of this experiment were published in PLoS ONE (see Tóth 2015 in the “Project-related papers and manuscripts” section).

Second year (2014-2015):
I conducted a field survey and a laboratory experiment also in the second year of the project. I collected DNA samples between 25th of March and 13th of April, 2015, in the north-eastern part of the Pilis-Visegrád Mountain, Hungary, to examine the genetic structure of the studied smooth newt meta-population. Using underwater traps and dip-net, I captured 291 male and female smooth newts from 10 ponds, at previously known breeding sites of the species. Buccal swab samples were collected from anesthetized individuals (using MS-222) on the day of capture in the laboratory, when newts were also weighted and photographed; animals were transported back to their pond of origin on the following day. Swab samples were stored at 4 Celsius degree until further processing. DNA extraction from randomly chosen 145 swabs was carried out by Botond István Heltai, a BSc student (biotechnology) of the Szent István University, between 20th and 30th of July, 2015. QiaAMP DNA Investigator Kit was used for the extraction to ensure maximum yield from the buccal swabs; however, approximately one third of the samples still had very low yield of genomic DNA (<30 ng/μL). Samples with sufficient yield of DNA (N=108) were sent to Biomi Ltd., Gödöllő, for labelling PCR and fragment analysis. Allele sizes at 15 microsatellite loci were identified from the electropherograms with the help of Fanni Mihók, a BSc student (biology) of the University of Veterinary, between 9th of March and 27th of June, 2017. After the thorough re-check of the readings, we will assess the presence of null alleles and scoring errors owing to stuttering or large allele dropout. The number of alleles, allele frequencies for each locus, tests of Hardy–Weinberg equilibrium and tests of linkage disequilibrium (LD) will also be calculated. Then, we will apply a diversity partitioning approach on the collected data to estimate genetic differences between animals originating from different ponds. We are also going to test the sensitivity of this method and compare its accuracy to conventional measures of genetic diversity using simulated data. After finishing the statistical analyses and the write-up, the manuscript will be submitted to Evolutionary Ecology (see Mihók & Tóth [in prep.] in the "Project-related papers and manuscripts" section).

In the experiment of the second year we investigated how past environmental conditions, i.e. different combinations of risky or safe adult and oviposition environments affected offspring's plastic responses to predation risk in hatching time and locomotor activity. 63-63 male and female smooth newts were collected using dip-net and underwater traps from 3 adjacent ponds in the Pilis-Visegrád Mountain on 13th of April, 2015. After capture, animals were transported to the laboratory. During the experiment, half of the egg-laying females (together with the males) were randomly allocated to the 'predator-cue' treatment group, while the other half to the 'no predator-cue' treatment group ('adult treatment'). In the former we added 80 mL of a mixture of predator cues daily to the housing water, while in the latter the same amount of cue-free water was administered. This treatment lasted for 5 days. Then, half of the pairs in each treatment group were randomly allocated to either the 'predator-cue' or the 'no predator-cue' treatment groups ('oviposition treatment'). This treatment lasted for 24 hours, after which we collected the deposited eggs. In total, 52 females laid eggs in both periods. After counting the eggs (N=427) and taking photographs of them, half of the eggs of each female was haphazardly allocated to either the 'predator-cue' or the 'no predator-cue' treatment groups ('egg and larval treatment'). In the 'predator-cue' treatment group, eggs and larvae received 2 mL of the mixture of predator cues, while in the 'no predator-cue' treatment group an equivalent amount of cue-free water was added to their container every day. The eggs of each female in each treatment group were kept together ('cohort'), and hatching events in the cohorts were checked and recorded every day. We fed larvae (N=413) with zooplankton *ad libitum* until the video trial, which took place between 13-15th May (age of the larvae ranged between 3-12 days). Survival of the offspring from egg to the recording was similar in all treatment combinations and had an overall value of 95.32%.

Video analysis of larval activity was finished by June, 2016, whereas the statistical analysis and write-up was completed by April, 2017. We found that plastic responses of developing newt larvae to the threat of predation were substantially influenced by maternal and oviposition conditions. In the absence of predator cues in these environments, larvae hatched later and showed reduced locomotor activity when developed in a risky environment compared to the control larvae. The maternal environment before oviposition, in itself, did not largely affect this pattern, but the presence of predator cues only during oviposition diminished all measured phenotypic differences among larvae developing in the presence or absence of cues on predation threat. When the past environment consistently indicated predation threat, however, predator cues in the developmental environment induced delayed hatching and triggered a tendency for higher locomotor activity in the larvae. These findings indicate that offspring phenotype is fundamentally affected by temporal variation in the presence of a predictive environmental cue and maternal effects may modulate the consequences of early environmental effects. The prepared manuscript was submitted to Oecologia and evaluated by three reviewers; we will re-submit the revised manuscript to this journal after addressing all comments and issues raised by the reviewers (see Tóth & Hettyey [under revision] in the “Project-related papers and manuscripts” section).

Third year (2015-2016) and extension (-2017):
In the third year of the project and during its extension, I was also working on the construction of a simple optimality model to investigate how a phenotype which can respond plastically to its environment in two life stages is maintained by natural selection in a population where specialist phenotypes are present and capable of exhibiting non-random habitat use. In the model, there are two life stages inhabiting separate habitats, and in each habitat there are two environments (or environmental states) occurring at constant frequencies (environment A and B in the habitat of the first life stage with the frequency of ‘r’ and ‘1 – r’, while environment X and Y at the habitat of the second life stage with the frequency of ‘q’ and ‘1 – q’, respectively). Three types of phenotypes are hypothesized to be present at both stages in these habitats: two specialists (S), one for each combination of environments in the two life stages, and one plastic type (P). Similarly to previous studies, we always assumed that in both life stages a specialist has higher fitness in its appropriate environment than in the other environment or than the specialist of another environment:

$$f(AA) > f(AB),\ f(AA) > f(BA) \text{ and } f(BB) > f(BA),\ f(BB) > f(AB),$$
$$f(XX) > f(YX),\ f(XX) > f(XY) \text{ and } f(YY) > f(YX),\ f(YY)>f(XY),$$

where ‘f(AA)’ denotes the fitness of phenotype A in the environment A, f(AB) is the fitness of phenotype A in the environment B, whereas f(BA) is the fitness of phenotype B in environment A and f(BB) is the fitness of phenotype B in environment B.

The specialists always produce the same phenotype regardless of the environmental state of their habitat, whereas the plastic type responses plastically to environmental cues while producing its phenotype, but only a given proportion of the response will be accurate (denoted by ‘a_1’ at the first and ‘a_2’ at the second life stage, respectively). Inherent cost to plasticity are also included into both life stages (‘k_1’ and ‘k_2’), because plastic types have to maintain the appropriate physiological, neurological, genetic, etc. machinery that is required to respond plastically to the environmental cues regardless whether that response is accurate or not. Because of these costs, plastic types will always have lower fitness than those specialists which are in their appropriate environmental states if $\{k_1, k_2\} < 1$. Parameter ‘g’ denotes the probability of occupancy of the optimal habitat in the first life stage, whereas parameter ‘m’ indicates probability of occupancy of the optimal habitat in the second stage.

Since the plastic phenotype can produce the optimal phenotype in both environments of each habitat, in this phenotype the probability of occupancy of each environment equals 0.5.

We computed fitness for the three phenotypes by using the arithmetic mean of the fitness gained in each environment within each life stage, and then multiplied the larval and adult fitness to calculate an overall fitness. For a phenotype that is specialist to environment A in the first life stage and to environment X in the second life stage, the fitness equations would be as follows:

$$\omega(AX)_1 = g \times r \times f(AA) + (1 - g) \times (1 - r) \times f(AB),$$
$$\omega(AX)_2 = m \times q \times f(XX) + (1 - m) \times (1 - q) \times f(XY),$$
$$W(AX) = \omega(AX)_1 \times \omega(AX)_2,$$

where ‘g’/‘1 – g’ is the proportion of larvae in the optimal/suboptimal larval environment, ‘m’/‘1 – m’ is the proportion of adults in the optimal/suboptimal adult environment. Again, ‘r’/‘1 – r’ is the frequency of environment A/B, ‘q’/‘1 – q’ is the frequency of environment X/Y.

The relative viability of the plastic phenotype is given by the equations:

$$\omega(PP)_1 = 0.5 \times r \times (a_1 \times f(AA) \times k_1 + (1 - a_1) \times f(BA) \times k_1) + 0.5 \times (1 - r) \times (a_1 \times f(BB) \times k_1 + (1 - a_1) \times f(AB) \times k_1),$$
$$\omega(PP)_2 = 0.5 \times q \times (a_2 \times f(XX) \times k_2 + (1 - a_2) \times f(YX) \times k_2) + 0.5 \times (1 - q) \times (a_2 \times f(YY) \times k_2 + (1 - a_2) \times f(YX) \times k_2),$$
$$W(PP) = \omega(PP)_1 \times \omega(PP)_2$$

where ‘0.5’ is a constant representing an even proportion of individuals in each environment (i.e. no preference), ‘r’/‘1 – r’ is the frequency of environment A/B, ‘q’/‘1 – q’ is the frequency of environment X/Y , ‘a_1’/‘$1 - a_1$’ is the frequency by which plastic phenotypes produce the appropriate phenotype in given environment in the larval stage, ‘a_2’/‘$1 - a_2$’ is the frequency by which plastic phenotypes produce the appropriate phenotype in given environment in the adult stage, and ‘k_1’/‘k_2’ is the fitness cost associated with being plastic in the first/second life stages.

We assumed that generations are separate and that in each generation the proportions of the three phenotypes change according to their relative fitness. For instance, the frequency of the plastic type in the subsequent generation is given by the following equation:

$$n(PP)' = n(PP) \times W(PP) / (n(AX) \times W(AX) + n(BY) \times W(BY) + n(PP) \times W(PP)),$$

where ‘n(PP)’ is the initial proportion of the plastic phenotype in the population. This set of equations for all five phenotypes was iterated until the changes in the proportions were very small ($<5 \times 10^{-8}$), at which point equilibrium was considered to be reached. We drew initial values for each type from a uniform distribution of pseudorandom numbers and verified that their effect was negligible. We specifically explored that part of the parameter space/those conditions under which the plastic strategy is predicted to be maintained by selection according to previous models of plasticity and examined to what extent the results of our extended model are in line with the findings of empirical studies. We found that when g(=m) equals 0.5 and $a(=a_1=a_2)$ is sufficiently high (e.g. equals 0.8), then the P type will be dominant in the majority of the frequency combinations of environments in the two habitats, but to a somewhat lesser extent compared to the predictions of those models which incorporate migration between habitats. This is not possible in our model since habitats are attributable to different life stages; coexistence of the P and one of the two S types will not be favoured for the same reason either. In accordance with previous findings, however, we also showed that the P type will not be fixed if the global cost of plasticity is >10%. The model also predicts that when both $a(=a_1=a_2)$ and g(=m) is 0.5, thus the accuracy of plastic response and habitat use is not better than random in either life stage, the P type can be fixed only if environmental variation in the two habitats favours one of the two S types in one stage and the other S type in the other stage (i.e. q=1-r). When r=q and m=g=0.5, then the model converts into a single-site model, and in accordance with previous predictions, the P type will be favoured when $a(=a_1=a_2)>r$. However, when g(=m)>0.5, P type is favoured only at maximal environmental heterogeneity and when $a(=a_1=a_2)>g(=m)$; in most of the parameter space one of the two S types will be prevalent. When q=0.5, thus environmental heterogenetity is maximum at the second life stage, the P type will be more successful than in the previous scenario and can be fixed even when there is no heterogeneity in the first life stage. Thus, maximal environmental heterogeneity in one habitat favours the persistance of the P type by relaxing the heterogeneity threshold in the other stage when g(=m)>0.5 (similarly to the effect of migration in previous studies). When q=1, thus there is no environmental heterogeneity in the second life stage (and consequently m=0.5 as non-random habitat use is not possible), we showed that all three types may be fixed at different levels of environmental heterogeneity in the first life stage and depending on the g/a_1 ratio. In this study I collaborate with Dr. Josh Van Buskirk (University of Zürich, Switzerland), and the first draft will be finalized after receiving the co-author’s comments on model construction and on the calculations of exact solutions for various boundary conditions. The completed manuscript will be submitted to Evolutionary Ecology (see Tóth & Van Buskirk [in prep.] in the “Project-related papers and manuscripts” section.

Publications (01.10.2013 - 31.07.2017):

Project-related papers and manuscripts:

Hettyey, A., **Tóth, Z.** & Van Buskirk, J. 2014. Inducible chemical defences in animals. Oikos, 123: 1025–1028.

Tóth, Z. 2015. Context-dependent plastic response during egg-laying in a widespread newt species. PLoS ONE, 10(8): e0136044.

Reuter, G., Boros, Á., **Tóth, Z.**, Phan, T.G., Delwart, E. & Pankovics, P. 2015. A highly divergent picornavirus in an amphibian, the smooth newt (*Lissotriton vulgaris*). Journal of General Virology, 96: 2607–2613.

Hettyey, A., **Tóth, Z.**, Thonhauser, K.E., Frommen, J.G., Penn, D.J. & Van Buskirk, J. 2015. The relative importance of prey-borne and predator-borne chemical cues for inducible antipredator responses in tadpoles. Oecologia, 179: 699–710.

Kurali, A., Pásztor, K., Hettyey, A. & **Tóth, Z.** 2016. Toxin depletion has no effect on antipredator responses in common toad (*Bufo bufo*) tadpoles. Biological Journal of the Linnean Society, 119: 1000–1010.

Bókony, V., Móricz, Á.M., Tóth, Zs., Gál, Z., Kurali, A., Mikó, Zs., Pásztor, P., Szederkényi, M., **Tóth, Z.**, Ujszegi, J., Üveges, B., Krüzselyi, D., Capon, R.J., Hoi, H. & Hettyey, A. 2016. Variation in Chemical Defense Among Natural Populations of Common Toad, *Bufo bufo*, Tadpoles: the Role of Environmental Factors. Journal of Chemical Ecology, 42: 329–338.

Pankovics, P., Boros, Á., **Tóth, Z.**, Phan, T.G., Delwart, E. & Reuter, G. 2017. Genetic characterization of a second novel picornavirus from an amphibian host smooth newt (*Lissotriton vulgaris*), Hungary. Archives of Virology, 162: 1043–1050.

Tóth, Z. & Hettyey, A. (under revision) Past environment modulates offspring responses to predation risk in an amphibian. Oecologia

Kurali, A., Pásztor, K., Hettyey, A. & **Tóth, Z.** (under revision) Ontogenetic changes in tadpole behaviour: the effects of food limitation and predation threat. Behavioral Ecology and Sociobiology

Tóth, Z. & Van Buskirk, J. (in preparation) Non-random habitat use limits the evolutionary maintenance of phenotypic plasticity in multiple life stages.

Mihók, F. & **Tóth, Z.** (in preparation) Application of a complexity-as-diversity approach to assess genetic differentiation between sub-populations in an amphibian.

Project-related conference participations:

Tóth, Z. 2014. Lack of plasticity in reproductive behaviour of female smooth newts. VII. European Conference on Behavioural Biology, 17-20 July 2014, Prague. Book of Abstracts, pp. 221-222. (poster)

Tóth, Z. 2014. Ragadozó-indukált plaszticitás vizsgálata pettyes gőte nőstényeknél. XVI. Congress of the Hungarian Ethological Society, 28-30 November 2014, Tihany. Book of Abstracts, p. 55. (poster)

Tóth, Z. 2015. Context-dependent plastic response to the presence of predator cues in a widespread newt species. XV. Congress of the European Society for Evolutionary Biology, 10-14 August 2015, Lausanne. Book of Abstracts, p. 443. (poster)

Tóth, Z. & Hettyey, A. 2015. Anyai és embrionális környezet együttes hatása pettyes gőtéknél. XVII. Congress of the Hungarian Ethological Society, 27-29 November 2015, Dobogókő. Book of Abstracts, p. 66. (poster)

Tóth, Z. & Hettyey, A. 2016. Maternal effects influence larval phenotype in the smooth newt. 16th Congress of the International Society for Behavioural Ecology, 28 July - 04 August, 2016, Exeter, UK. ISBE Talk Abstract Book, p. 151. (oral presentation)

Additional publications during the project:

Beleznai, O., Dreyer, J. **Tóth, Z.** & Samu, F. 2017. Natural enemies partially compensate for warming induced excess herbivory in an organic growth system. Scientific Reports 7: 7266.

Tóth, Z., Tuliozi, B., Baldan, D., Hoi, H. & Griggio, M. 2017. Social facilitation limits the exploitation of multiple hidden food patches in a bird species. Scientific Reports, 7: 816.

Molnár, B.P., **Tóth, Z.**, Kárpáti, Z. 2017. Synthetic blend of larval frass volatiles deter oviposition in the invasive box tree moth (*Cydalima perspectalis*). Journal of Pest Science, 90: 873–885.

Tóth, Z., Baldan, D., Albert, C., Hoi, H. & Griggio, M. 2016. Effect of ornament manipulations on following relations in male bearded reedlings. Ethology Ecology & Evolution, 28: 175–187.

Molnár*, B.P., **Tóth*, Z.**, Fejes-Tóth*, A., Dekker, T. & Kárpáti, Z. 2015. Electrophysiologically active maize volatiles attract European corn borer (*Ostrinia nubilalis*) gravid females. Journal of Chemical Ecology, 41: 997–1005. (*co-first authors)

Beleznai*, O., Tholt*, G., **Tóth*, Z.**, Rákóczi, A., Marczali, Z. & Samu, F. 2015. Cool headed individuals are better survivors: non-consumptive and consumptive effects in a spider-virus vector leafhopper system. PLoS ONE, 10: e0135954. (*co-first authors)

Tóth, Z., Baldan, D., Hoi, H. & Griggio, M. 2014. Food reduction has a limited effect on following relations in house sparrow flocks. Animal Behaviour, 90: 91–100.

Additional manuscripts under review:

Gyuris, E., Szép, E., Kontschán, J., Hettyey, A. & **Tóth, Z.** (under review) Efficiency against the two-spotted spider mite *Tetranychus urticae* and prey-age-related choice of three predatory mites. Acta Zoologica Academiae Scientiarum Hungarica

Gyuris, E., Szép, E., Kontschán, J., Hettyey, A. & **Tóth, Z.** (under review) Behavioural responses of two-spotted spider mites induced by predator-borneand prey-borne cues. Behavioural Processes

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