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Phenotypic plasticity of *Rana dalmatina* larvae: ontogenetic variation in anti-predator responses

verfasst von

Iris Martina Kofler, BSc

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Betreut von:	Univ. Doz. Dr. Günter Gollmann

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Abstract

Although anuran larvae are well studied model organisms for phenotypic plasticity in general, little is known about ontogenetic variation in predator induced plastic responses. To conduct an exploratory study on this topic, we performed an outdoor mesocosm experiment, confronting *Rana dalmatina* tadpoles with the presence of dragonfly predators (Aeshnidae) at five different times in their ontogenetic development. The tadpoles which experienced predator presence for the first time one week after the beginning of the experiment (approximately 9-10 days after hatching) showed the strongest plastic morphological responses, as well as retarded growth and delayed time of metamorphosis. The morphological responses were mainly deeper tail fins and a changed body-to-tail-length ratio. As the treatment which experienced predator contact in the second week of the experiment significantly differed from all other treatments and no linear relationship between time spent with predator and plastic responses was found, we can assume that developmental windows and developmental constraints are underlying our findings.

Zusammenfassung

Obwohl Kaulquappen bereits sehr gut untersuchte Modellorganismen für phänotypische Plastizität im Allgemeinen sind, ist bei ihnen noch wenig über ontogentische Variabilität in durch Kairomone induzierter phänotypischer Plastizität bekannt. In einer explorativen Studie wurden Springfrosch Kaulquappen (*Rana dalmatina*), die in Mesokosmos-Behältern unter freiem Himmel gehalten wurden, zu 5 unterschiedlichen Zeitpunkten in ihrer Larvalentwicklung erstmals mit der Anwesenheit von Großlibellenlarven (Aeshnidae) als Räuber konfrontiert. Jene Kaulquappen, die mit der Anwesenheit des Prädators erstmals in der zweiten Woche des Experiments (etwa 9-10 Tage nach dem Schlüpfen) konfrontiert waren, zeigten die stärksten morphologischen Veränderungen im Sinne von höherem Flossensaum, sowie auch verzögertes Wachstum und einen späteren Metamorphosezeitpunkt. Indem die Veränderungen spezifisch jene Kaulquappen betrafen, die ab der zweiten Woche mit der Anwesenheit eines Räubers konfrontiert waren und sich auch kein linearer Zusammenhang zwischen der Gesamtdauer des Prädatoreneinflusses und des Ausmaßes der Veränderungen herstellen ließ, ist davon auszugehen, dass unseren Ergebnissen Entwicklungsfenster, sowie Einschränkungen resultierend aus der ontogentischen Entwicklung, zugrunde liegen.

1 Introduction

Phenotypic plasticity is defined as the ability of a genotype to generate different phenotypes as a response to different environmental conditions (Tollrian and Harvell 1999). Plastic changes do not necessarily have an adaptive value (Debat and David 2001). If these different phenotypes provide relative fitness gains in the environments where they occur, this phenomenon is called adaptive phenotypic plasticity (DeWitt et al. 1998). Adaptive phenotypic plasticity by definition increases an organism's fitness (Gabriel 2005) and is therefore maintained by natural selection (Miner et al. 2006).

Evolving plastic traits can be beneficial for species or populations which are exposed to variable environments. Under those conditions there is not one single phenotype which provides the largest fitness gain and is therefore selected for and becomes genetically fixed, but several phenotypes exist which are all relatively fit (Auld et al. 2010). Relatively fit means that none of the phenotypes can be considered the absolute fittest under all possible conditions, but each of them provides a fitness advantage in one of the alternative environments (DeWitt et al. 1998). This can be experimentally tested by exposing the different phenotypes to those alternative environments and measuring their fitness (Debat and David 2001). Under those predictions one would expect that species or populations that occur in highly variable habitats should show more adaptive phenotypic plasticity than those living in very constant environments (Van Buskirk and Relyea 1998).

Both biotic and abiotic factors can induce plastic responses. Mainly two important biotic factors are known to induce phenotypic plasticity: competition and predation. There is a large number of abiotic factors which can induce plastic responses, such as temperature, light and nutrients (Relyea 2001).

Competition can often induce enhanced foraging behaviour and changes in food preference, whereas predation can lead to reduced activity, for example less time spent on foraging, as well as to morphological adaptions. The before mentioned morphological adaptions can increase the survival chances when direct predator contact occurs (Lemcke 2005).

Morphological anti-predator responses have received much attention and have been studied and documented extensively in many animal species from arthropods to vertebrates. Specimens of *Daphnia pulex*, a well-studied model organism for phenotypic plasticity, are known to develop so-called neck-teeth, when they detect kairomones of *Chaoborus*-larvae in their environment during their juvenile stage (Tollrian 1995, Krueger and Dodson 1981). Specimens with neck-teeth show a significantly lower mortality when they live in the same habitat with invertebrate predators, because the neck-teeth make the handling more difficult

for the predators (Krueger and Dodson 1981). *Heliosoma trivolvis*, a freshwater snail, develops altered shell shapes, which reduces their vulnerability to attacks of predatory water bugs (Hoverman and Relyea 2007).

Amphibian larvae show diverse plastic responses to different environmental factors such as food availability, competition and predation (Van Buskirk and Relyea 1998). Several studies had been conducted on predator induced phenotypic plasticity in tadpoles with dragonfly larvae as predators. Plastic anti-predator responses in anuran larvae are induced by kairomones. These are chemicals cues which predators release into the water and which can be sensed by the tadpoles (Relyea 2002). These cues are reliable indicators for the presence of hazardous predators in the habitat (Harvell 1990).

Predator diet has an important effect on the induction of phenotypic plasticity in tadpoles (Laurila et al. 1997, Chivers and Mirza 2001). If dragonfly larvae, which are common predators feeding on tadpoles, are fed with tadpoles from a different species, significantly smaller plastic responses were induced in *Hyla arborea* tadpoles (Lemcke 2005). The reasons for this phenomenon are not yet completely understood. One hypothesis is that predators could be specializing on different prey types and therefore predator diet together with predator presence might convey important information for the tadpoles (Chivers and Mirza 2001).

Various studies have shown that predator presence can induce both behavioural and morphological alterations in tadpoles (Miner et al. 2005). Predator-induced traits include reduced foraging activity, deeper tail fins and a changed body to tail size ratio in favour of the tail (Lemcke 2005, Relyea 2002). The relatively larger tail is considered to lure predators and is less vulnerable to attacks or at least strikes against the tail are generally mostly not lethal in contrast to attacks against the body (Miner et al. 2005). Moreover the changed tail morphology is reported to improve swimming performance (Wilson et al. 2005). The induction of the predator induced phenotype in tadpoles is mediated through the neuroendocrine stress axis, using corticosterone as regulatory hormone (Maher et al. 2013).

As a consequence of plasticity-associated costs, induced tadpoles can show retarded growth and development and a lower final body mass (Relyea 2002). In other studies performed with common frog (*Rana temporaria*) tadpoles, odonate predators have been shown to delay the tadpoles' development, which can lead to a retarded metamorphosis and higher body mass at metamorphosis, when growth rate itself is not affected (Laurila and Kujasalo 1999).

In experiments with direct predator contact, induced tadpoles showed a significantly higher survival rate (Lemcke 2005). Due to the fact that frogs lose body mass over the hibernation period and the time between metamorphosis and the onset of hibernation is limited, especially

in higher latitudes, a higher body mass at metamorphosis can be expected to increase fitness (Laurila et al. 2002).

Inducible plastic anti-predator mechanisms are associated with both direct and indirect costs for the individual. The term indirect costs refers to fitness reductions resulting from the altered phenotype (Merilä et al. 2004).

Costs of plasticity can be further subclassified into:

1) Production costs: costs for developing the altered phenotype
2) Maintenance costs: costs for maintaining the plastic genotype
3) Information acquisition costs: costs for detecting predator presence and for developing and maintaining appropriate sensory structures
4) Genetic costs: genes coding for plasticity might be linked with genes associated with low fitness values and therefore passed on to the next generation together with them. (DeWitt et al 1998)

If the development and maintenance of inducible defense mechanisms would not go along with costs for the individual, there would be no point in them to be inducible and they should be always present (Van Buskirk 2000, Tollrian and Harvell 1999, Schmidt et al. 2006). As a consequence of these costs, it should be advantageous only to display plastic anti-predator mechanisms under circumstances when the benefits resulting from them outbalance the costs, which means, when there is a positive trade-off for the individual (Callahan et al. 2008).

As predation risk, like other environmental parameters, can vary over the average life span of prey organisms and the maintenance of anti-predator adjustments is likely associated with costs, the potential to reverse predator induced plastic responses can be advantageous under certain circumstances (Orizaola et al. 2012). When the periods in which the predator poses a threat are short in comparison to the average life span of the individual, genotypes with the ability to revert the plastic morphological alterations should be favoured by evolution (Gabriel 2005). As dragonfly larvae, which are among the most important predators for anuran larvae, can appear at any point in the ontogenetic development of the tadpoles and stop preying on them as they complete their own larval development, one can assume that the potential to reduce both behavioural and morphological alterations would increase the tadpoles' fitness (Relyea 2003). Both behavioural and morphological plastic anti-predator responses are reversible in tadpoles (Relyea 2003, Orizaola et al. 2012), but for morphological traits in contrast to behavioural traits the reversion takes significantly more time than the induction (Orizaola 2012).

As different predators show different types of foraging techniques, it can be beneficial for the prey organism to show predator specific plastic responses (Wilson et a. 2005, Hoverman and Relyea 2008). Odonates are sit-and-wait-predators, which consume their prey bit by bit, whereas fish pursue their prey by swimming after if or use fast swim-starts and swallow their prey as a whole (Wilson et al. 2005). If combinations of functionally different predators are present, mechanisms against the one which poses the highest mortality risk may be induced (Lakowitz et al. 2008).

Although many studies have been conducted on phenotypic plasticity, most of them have just focused on one single ontogenetic stage. Recording plastic responses over the entire larval development allows us to track changes with regards to which traits are particularly affected at which stage and which changes are inducible at which point in development. Not all anti-predator responses may be of the same use to the prey organism at all stages and there might be limited developmental windows in which the different plastic responses can be induced. As amphibian larvae are very well studied model organisms for phenotypic plasticity in general, they are well-suited to study developmental windows of plasticity (Relyea 2003).

In this study we exposed *Rana dalmatina* tadpoles to caged dragonfly predators in a series of treatments through larval development to detect differences in plastic responses between ontogenetic stages. The aim of our present work was to detect developmental windows for phenotypic plasticity in anuran ontogenetic development and to identify differences in plastic anti-predator traits depending on the stage they were induced in. We assessed morphological traits using geometric morphometry and we also focused on the costs of plasticity. *Rana dalmatina* tadpoles are known to show reduced activity and higher refuge use and as a result lower growth and development rates, when they can sense predatory fish in their environment (Teplitsky et al. 2003). For this purpose we measured time of metamorphosis and body mass after tail reduction, as they are important fitness factors (Abrams and Rowe 1996).

2 Material and Methods

2.1 *Rana dalmatina* (BONAPARTE 1840)

The agile frog *Rana dalmatina* is spread over wide parts of middle and southern Europe, except most of the Iberian peninsula, where it only occurs in Northern Spain (Kwet 2010). In Austria, which lies in the center of the agile frog's distribution area, *Rana dalmatina* occurs only in extra-alpine regions with a primary distribution below 400m above sea level (Cabela and Grillitsch 2001). The spawning season of *Rana dalmatina* starts very early (at the end of February) and can last until May with a maximum in April, dependent on water temperature, which has to be above 6.5°C (Kuhn and Rohrbach 1998, Cabela and Grillitsch 2001). In large parts of Europe, the agile frog occurs sympatrically with the common frog *Rana temporaria*, which tolerates lower temperature and therefore also inhabits regions in higher latitudes and altitudes. In Vienna and the surrounding areas both species can be found syntopically and at the same spawning sites (Baumgartner et al. 1996).

2.2 Experimental setup

The experiment was conducted on an open terrace under natural middle European climate conditions, in a 3 x 6 randomized block design, with 6 treatments differing in the point of time when the predator was first added.

To create outdoor mesocosms, 18 opaque black plastic tanks (39 x 74 x 29cm) were put up on the terrace on 22 April 2013 (see Figure 1), filled with tap water and covered with fly screens in order to protect the tanks from invasion of other insect predators and other disturbing influences. The tanks were divided into 3 blocks (A,B,C; see table 1), each of them containing 1 replicate of each treatment.

table 1: treatments used in the experiment and assigned tanks;
0: no dragonfly larvae present in the respective tanks
1: dragonfly larva present

treatment	Week 1	Week 2	Week 3	Week 4	Week5	Assigned tanks		
						A	B	C
Treatment 1	1	1	1	1	1	A3	B3	C5
Treatment 2	0	1	1	1	1	A6	B2	C4
Treatment 3	0	0	1	1	1	A5	B5	C1
Treatment 4	0	0	0	1	1	A2	B4	C2
Treatment 5	0	0	0	0	1	A1	B1	C6
Treatment 0 (control group)	0	0	0	0	0	A4	B6	C3

Leaf litter was collected next to Teuflteich, a pond on Danube island in Vienna and a natural spawning site of *Rana dalmatina*, and equally distributed over all of the 18 tanks on April 20 2013 in order to serve as food source for the tadpoles. Each tank received approximately 60g of leaf litter. Moreover all 18 tanks were inoculated with pond water from Teuflteich in order to establish a natural microbial community. As heavy rainfalls occurred during the experiment, some litres of water had to be removed from all tanks several times in order to hold the water levels constant and prevent overflowing.

Figure 1: Setup of tanks on terrace

We used dragonfly larvae from the Aeshnidae family as predators, which were caught on 14 and 21 April at Mühlberg in Vienna. A clutch of *Rana dalmatina* spawn was taken from a pond at Kolbeterberg on 14 April 2013. All sites from which material and animals were taken are located in Vienna and the surrounding area. All tadpoles used in the experiment were taken from one clutch in order to achieve a high level of genetic homogeneity.

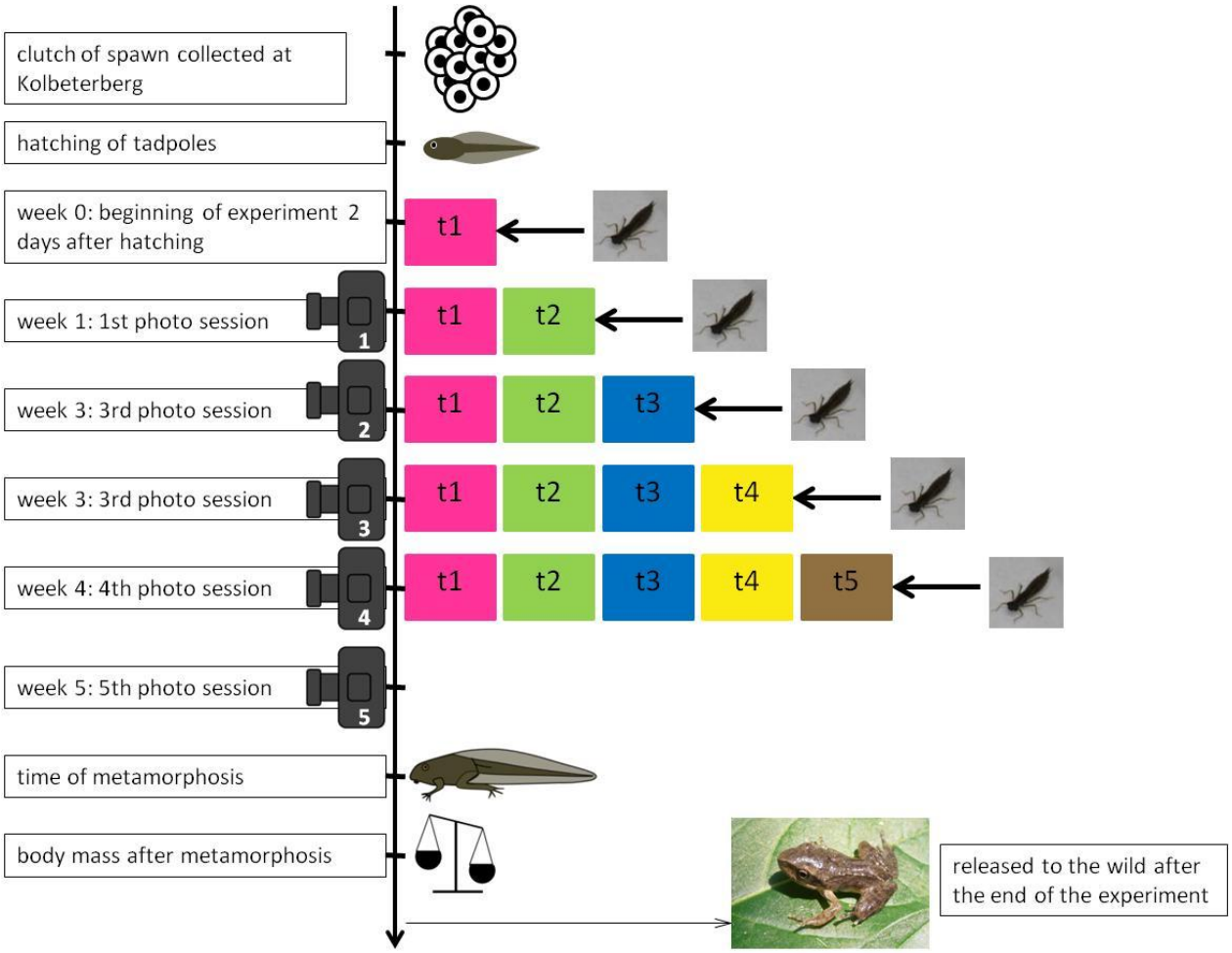


Figure 2: Chronological course of the test procedure; the vertical time axis shows the time schedule of our experiment, beginning with collecting the spawn and ending with the release of the young frogs after completely finishing metamorphosis; the dragonfly symbols on the right side show, which tanks contained a caged dragonfly in the respective weeks; the camera symbols on the left side show, when photo sessions were conducted

On 26 April 2013, about two days after hatching, tadpoles were haphazardly distributed over the 18 tanks and 15 tadpoles were assigned to each tank. The remaining tadpoles were kept in an additional tank, which was not used in the experiment and some of them served as food for the dragonfly larvae.

On the same day all tanks assigned to treatment 1 additionally received a dragonfly larva in a plastic cage (see fig. 3). Empty plastic cages were placed in all the other tanks to keep the environmental conditions as constant as possible apart from predator presence/absence.

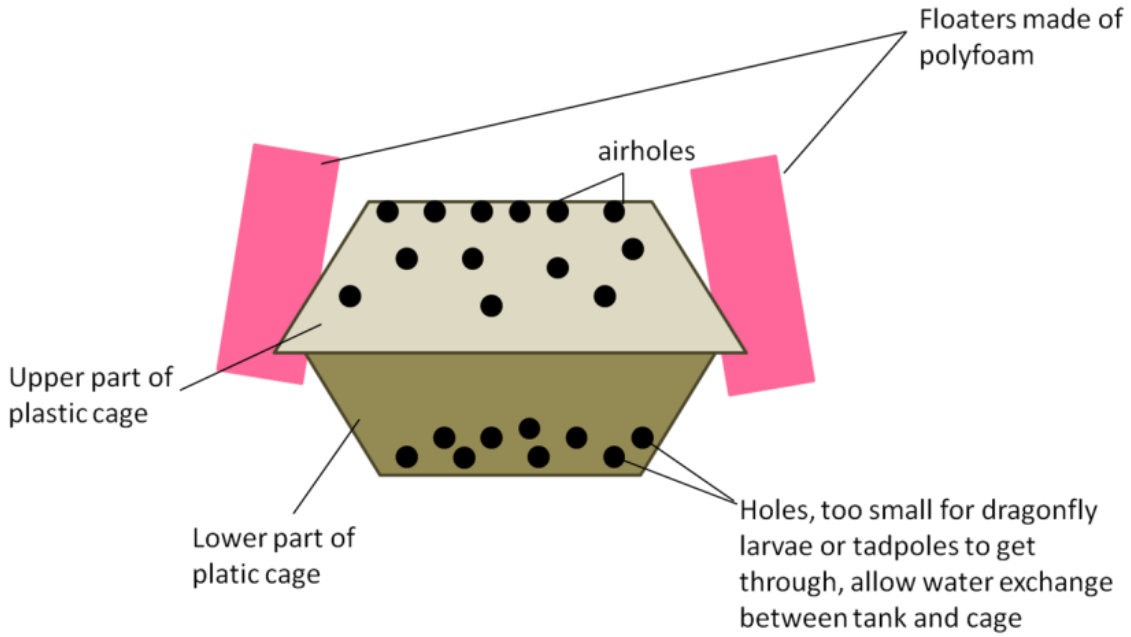


Figure 3: Schematic drawing of the plastic cages used for dragonfly larvae

One week after putting the dragonfly larvae into the tanks assigned to treatment 1, the first photo session took place and the number of tadpoles was reduced from 15 to 12 in each tank, randomly choosing 12 out of 15 tadpoles which stayed in the experiment. The photo sessions were always conducted on the same days of each week. The tadpoles were caught and then put into a photo-cuvette one by one (see fig. 4). They were always kept in water taken from their own tank and never got in contact with water from any of the other tanks.

Pictures were taken, using a digital single-lens reflex camera Olympus E3. A scale bar was always positioned on the front side of the photo-cuvette, enabling later calibration of the pictures.

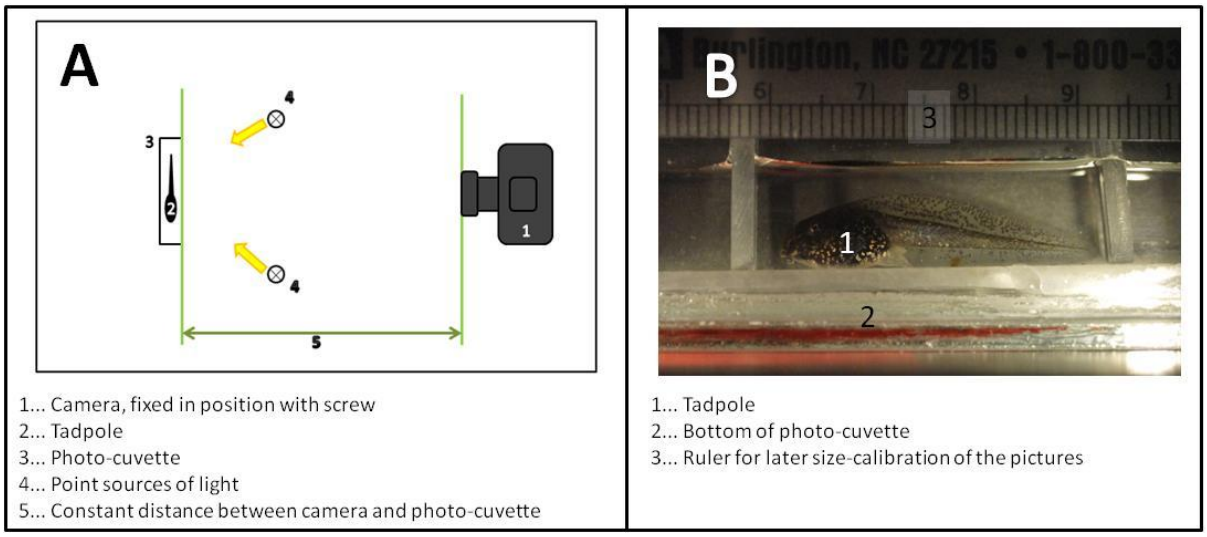


Figure 4: Photo set up; A: technical set up; B: typical picture of a tadpole in the photo-cuvette

During the photo sessions the dragonfly larvae were taken out of their cages, fed with some of the surplus tadpoles and afterwards put back into the cages and into the tanks. The dragonfly larvae were assigned to the tanks randomly after each photo session, which means that they were swapped between the tanks every week. Only dragonfly larvae which had actually fed on tadpoles were used.

Altogether five photo sessions in five subsequent weeks were conducted (see table 1).

Additionally, time of metamorphosis and body mass after finishing metamorphosis, which are indicators for fitness, were assessed. To determine time of metamorphosis, Gosner stage 42 (Gosner 1960), which is characterized by the breakthrough of the front legs, was used. This stage is easy to assess and is considered to mark the climax of metamorphosis. The day when the individuals reached Gosner stage 42 was recorded, counting from day 1 which was chosen as the day when the first tadpole in the experiment reached this stage (4 June 2013).

Body mass was measured after the absorption of the tail to a length of <1mm, using a Mettler Toledo XS204 Delta Range laboratory scale.

After the end of the experiment the little frogs were released to the wild at the location where leaf litter and pond water had been taken from.

2.3 Landmarks

Landmarks were set using TpsDig2 (Rohlf 2008). Altogether 60 landmarks were used, consisting of 11 fixed landmarks and 49 semi-landmarks (see fig. 5). The 11 fixed landmarks can be further subclassified into 5 type 1 landmarks (1, 4, 9, 11), 2 type 2 landmarks (1 and 3) and 4 type 3 landmarks (5, 6, 7, 8). Type 1 landmarks are defined as points at discrete juxtaposition of tissues (i.e. sutures of the skull), type 2 landmarks represent curvature maxima and type 3 landmarks extremal points (Bookstein 1991).

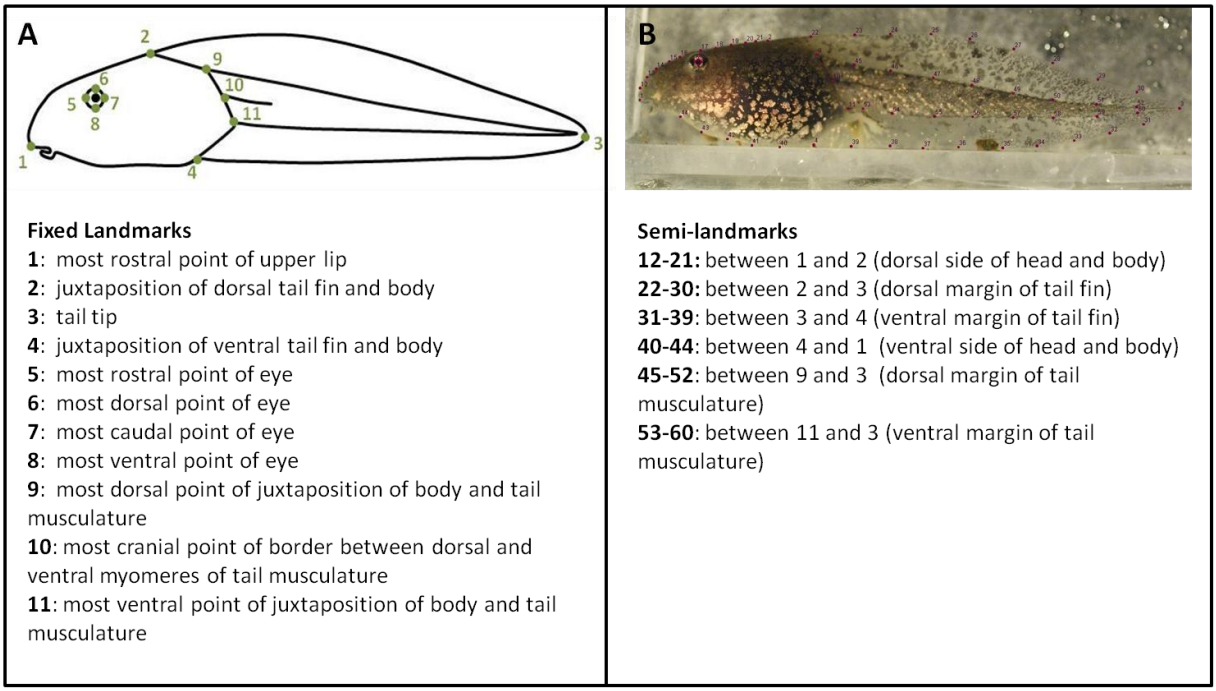


Figure 5: A: schematic drawing of tadpole with the 11 fixed landmarks and their definitions B) picture of tadpole with all real landmarks and semi-landmarks, including a description of the localization of all semi-landmarks

2.3 Statistical analysis

The semi-landmarks were aligned by sliding them along the outline curve (Adams et al. 2004).

As raw landmark coordinates still include information about size, position and orientation, they have to be freed from this source of variance as we are only interested in variation in shape (Adams et al. 2004). A frequently-used way to obtain shape coordinates, which are invariant to changes in size, position and orientation, is performing Procrustes superimposition. To obtain procrustes coordinates, individuals are first scaled to unit centroid size, which makes the coordinates invariant to individual size. Individuals are then superposed to dispose of information about position and in a last step coordinates are rotated, until the sum of squared distances between corresponding landmark coordinates is minimized, which produces coordinates that are invariant to orientation (Bookstein 1996, Mitteroecker and Gunz 2009).

A principal component analysis was performed of the procrustes coordinates of all individuals and all time points and plotted in PCA plots in order to visualize both the average shape of all tadpoles and the variability of all landmarks and semi-landmarks over the whole time of the experiment (see fig. 6). A scree plot was created to visualize the variance explained by each principle component (see fig. 7). Deformation grids were added to visualize the main shape changes along the respective principle components (see fig 8-10). As the largest shape differences between the treatments were found at time week 4 a PCA plot of procrustes coordinates of all individuals at that time was created (see fig. 10).The scores on PC2 at week 4 were further plotted in histograms per treatment and an ANOVA was performed on them (see fig. 11).

In order to detect growth patterns, weekly treatment means of procrustes coordinates were calculated and plotted in a PCA graph (see fig. 13).

As size measures total length and body length of the tadpoles, represented by distances between raw landmarks were used. For total length we used the distance between landmark 1 (upper lip, which is equal to the snout tip) and 3 (tail tip) and for body length the distance between landmark 1 and 11 (most cranial point of ventral margin of tail musculature which is nearly equivalent to the location of the anus). Group means of those distances were plotted by treatment and time (see fig. 14 and 15) in order to obtain growth curves for the treatments. This procedure was meant to enable us to detect underlying general growth patterns and compare them between the treatments. As t2 showed on average a reduced slope after being confronted with the predator, a Kruskal-Wallis-rank-sum-test was performed on the slopes of

all treatments in the respective week. As the Kruskal Wallis test turned out significant, a Wilcoxon-U-test was performed comparing the tank means of t2 to the pooled tank means of all other treatments.

Time of metamorphosis was plotted per treatment in histograms to compare mean and variability of this variable between the treatments. After plotting the time of metamorphosis data, statistical tests were performed to check for significant differences between the treatments concerning this parameter. A Shapiro test on normal distribution and a Bartlett test on homogeneity of variances were performed to assess the criteria for a parametric Analysis of variance. As those criteria were violated, a non-parametric Kruskal-Wallis rank sum test was performed. For pairwise testing, the Wilcoxon-Mann-Whitney-U-test was used and a Bonferroni correction was applied on the resulting p-values.

As a second measure of plasticity associated costs, body mass after metamorphosis was plotted in histograms and boxplots to visualize location and dispersion characteristics of this parameter and to compare them between the treatments. Afterwards the following statistical procedures were applied to detect significant differences between the treatments concerning body mass after metamorphosis. As the criteria for a parametric Analysis of variance were not violated in this case, an ANOVA was performed. Afterwards pairwise t-tests were run and as p-adjustment technique again Bonferroni correction was used.

For both time of metamorphosis and body mass after metamorphosis a simple linear regression analysis was performed to detect potential linear dependencies between them and the time spent with the predator.

Both total length (snout tip to tail tip) and body length (without tail) were assessed as size measures. As the predator-induced phenotype is characterized by an increased tail length to body length ratio (Miner et al. 2005, Maher et al. 2013), assessing and comparing both size measures seems adaequate.

Statistical analysis was conducted in R. For the analysis of landmark and semi-landmark data the geomorph package (Adams and Otarola-Castillo 2013) in R was used.

3 Results

Geometric Morphometry

Figure 6 shows the procrustes coordinates of all treatments and all weeks after semi-landmark alignment. The semi-landmarks were aligned. Therefore they vary only perpendicular to the outline in contrary to the fixed landmarks which vary in all directions. As the plot (see fig. 6) shows, not all landmarks underlie the same amount of variance. The semi-landmarks in the middle region of the tail musculature (49, 50, 57 and 58) show the least amount of variance, while those in the cranial part of the tail fin are highly variable within the data set.

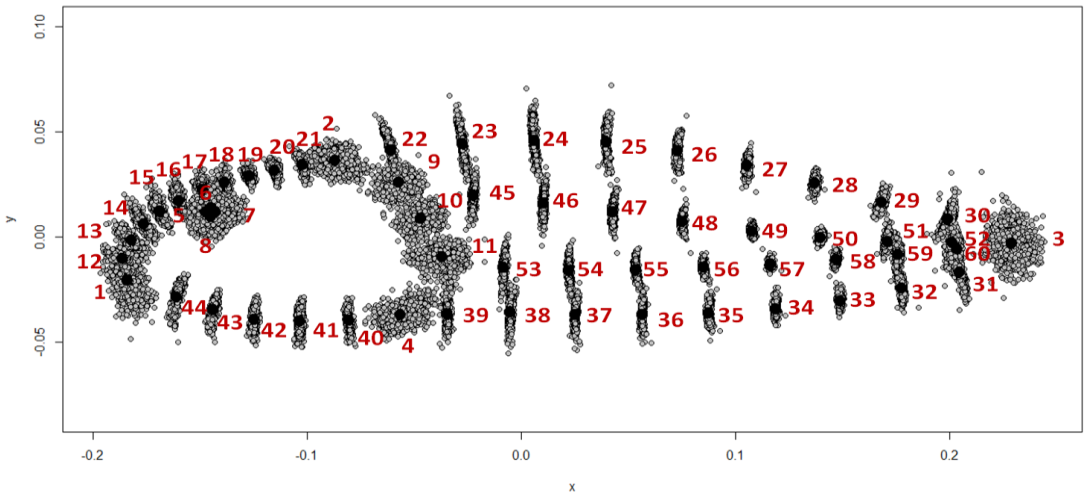
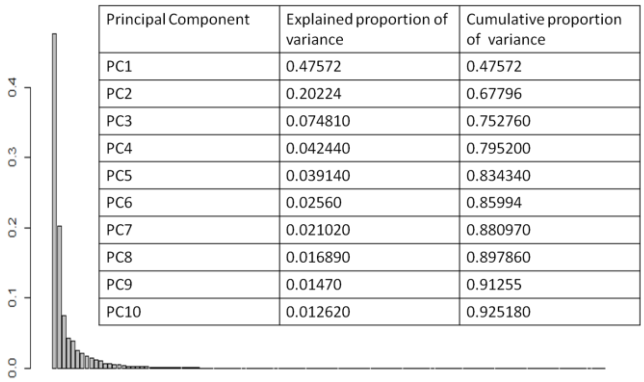


Figure 6: Procrustes coordinates of all treatments and all weeks after semi-landmark alignment

The performed principal component analysis (PCA) over all landmarks resulted in 120 principal components (PCs), out of which the first three principal components together explain 75% of the total variance in the dataset (see fig. 7).



Principal Component	Explained proportion of variance	Cumulative proportion of variance
PC1	0.47572	0.47572
PC2	0.20224	0.67796
PC3	0.074810	0.752760
PC4	0.042440	0.795200
PC5	0.039140	0.834340
PC6	0.02560	0.85994
PC7	0.021020	0.880970
PC8	0.016890	0.897860
PC9	0.01470	0.91255
PC10	0.012620	0.925180

Figure 7: Scree plot of principle components visualizing the amount of variance explained by the respective principle components

The first two PCs which are the perpendicular axes that explain the first and second largest fraction of variance in our data, are shown in figure 8. The first PC describes to a large degree the dorso- and ventroflexion of the tadpoles in the cuvette, while the second PC only relates to tadpole shape, varying between short individuals with high tail fins and elongated ones with long and low tails. As it can be seen in the deformation grids (see fig. 8), PC1 does not only contain noise in the sense of dorso- and ventroflexion of the tadpoles, but also shape information.

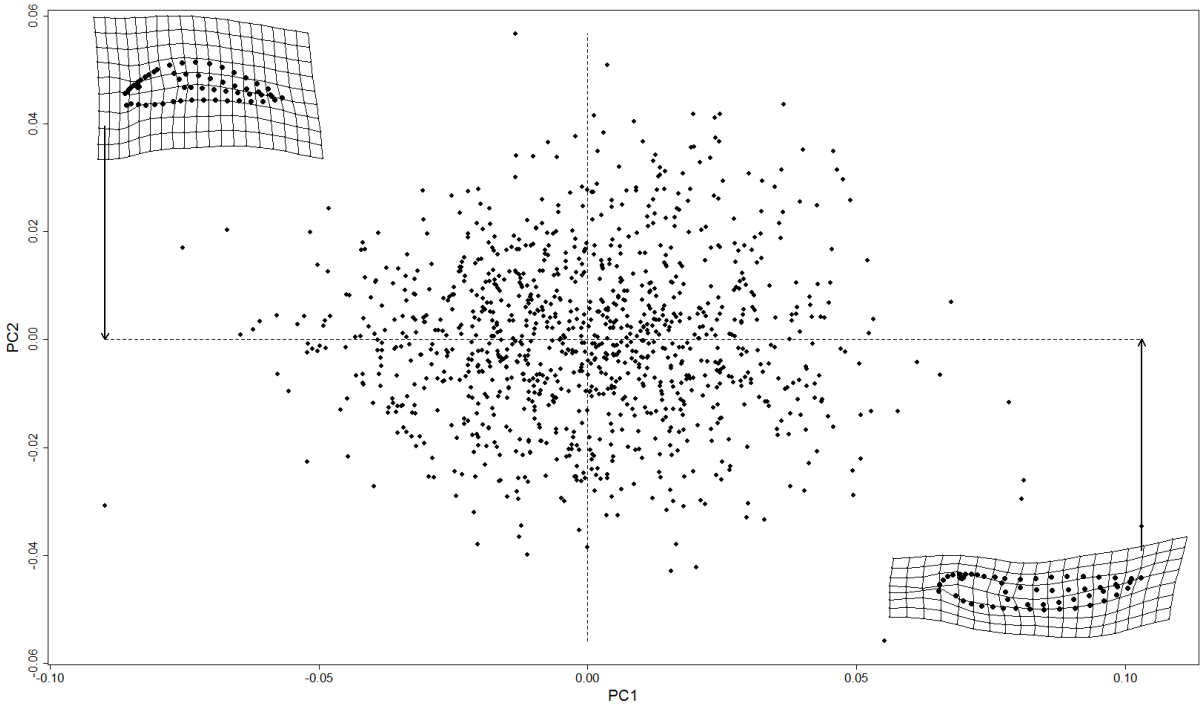


Figure 8: PCA plot of PC1 (48% of total variance) and PC2 (20% of total variance) of all treatments and all weeks; the added deformation grids visualize the major changes along PC1

As PC 1 contains a lot of information on bending artefacts (dorso- and ventroflexion of the tadpoles), PC2 identifies the major axis of shape differences in our data (see fig. 9), separating short tadpoles with deep tail fins from elongated ones with very low tail fins.

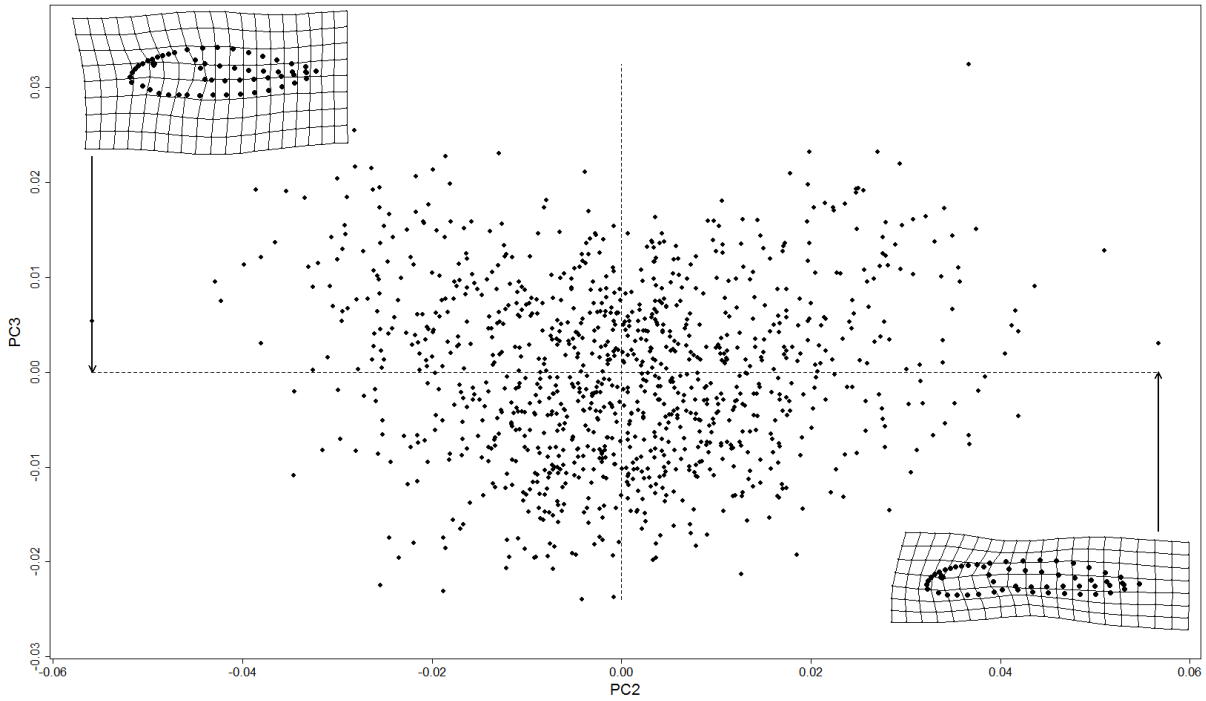


Figure 9: PCA plot of PC2 (20% of total variance) and PC3 (7% of total variance) of all treatments and all weeks; the added deformation grids visualize the major changes PC2

In week 4 the most obvious shape differences between the treatments were found. PC2 in week 4 characterizes shape differences between elongated tadpoles with low tail fins and relatively short bodies and short tadpoles with deep tailfins and a body to tail ratio shifted towards lager bodies and shorter tails (deformation grids in fig. 10). Individuals from t2 show on average relatively low scores on PC2. They are short and have deeper tail fins and a body to tail ratio shifted towards the body (see fig. 10).

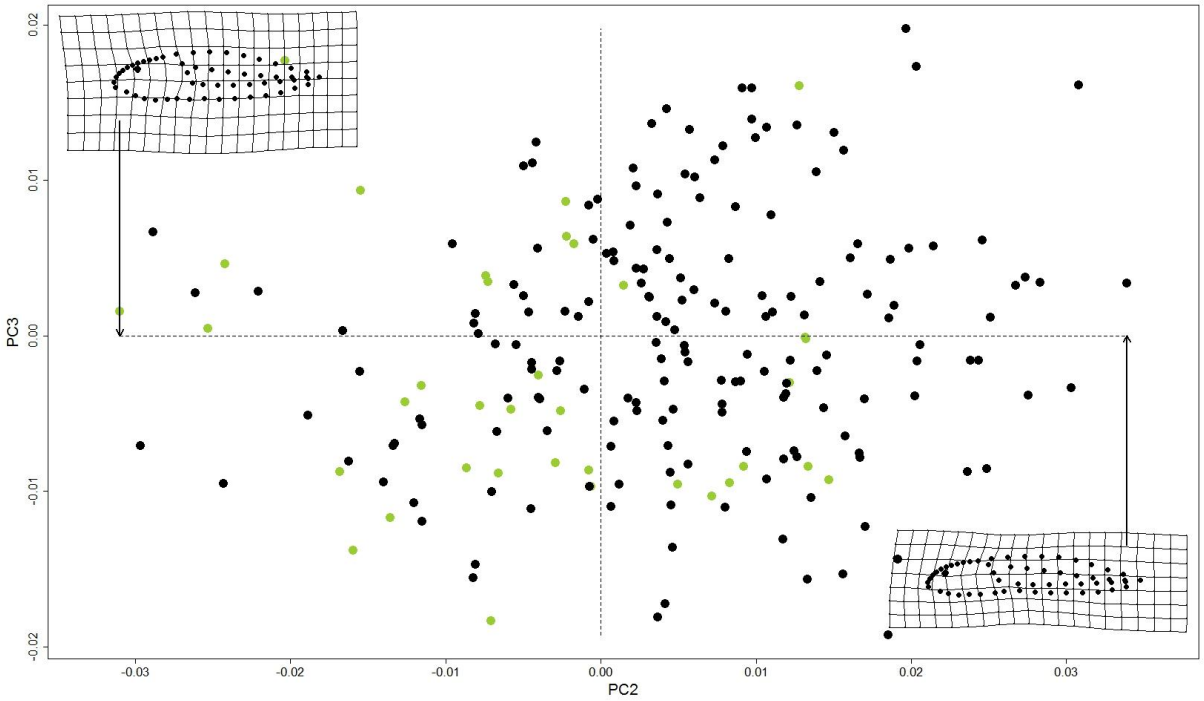


Figure 10: PCA plot and deformation grids of procrustes shape coordinates at week 4, PC2 and PC3, treatment 2 in green, other treatments represented with black dots

When comparing the distributions of PC-scores on PC2 at week 4 between the treatments, it is noticeable that t2 is the only treatment that has its central tendency in the lower half of the total range of scores on PC2 (see. fig. 11). The difference of PC2-scores per treatment at week score was not significant in the ANOVA ($p=0.2996$, $F_1=1.0811$).

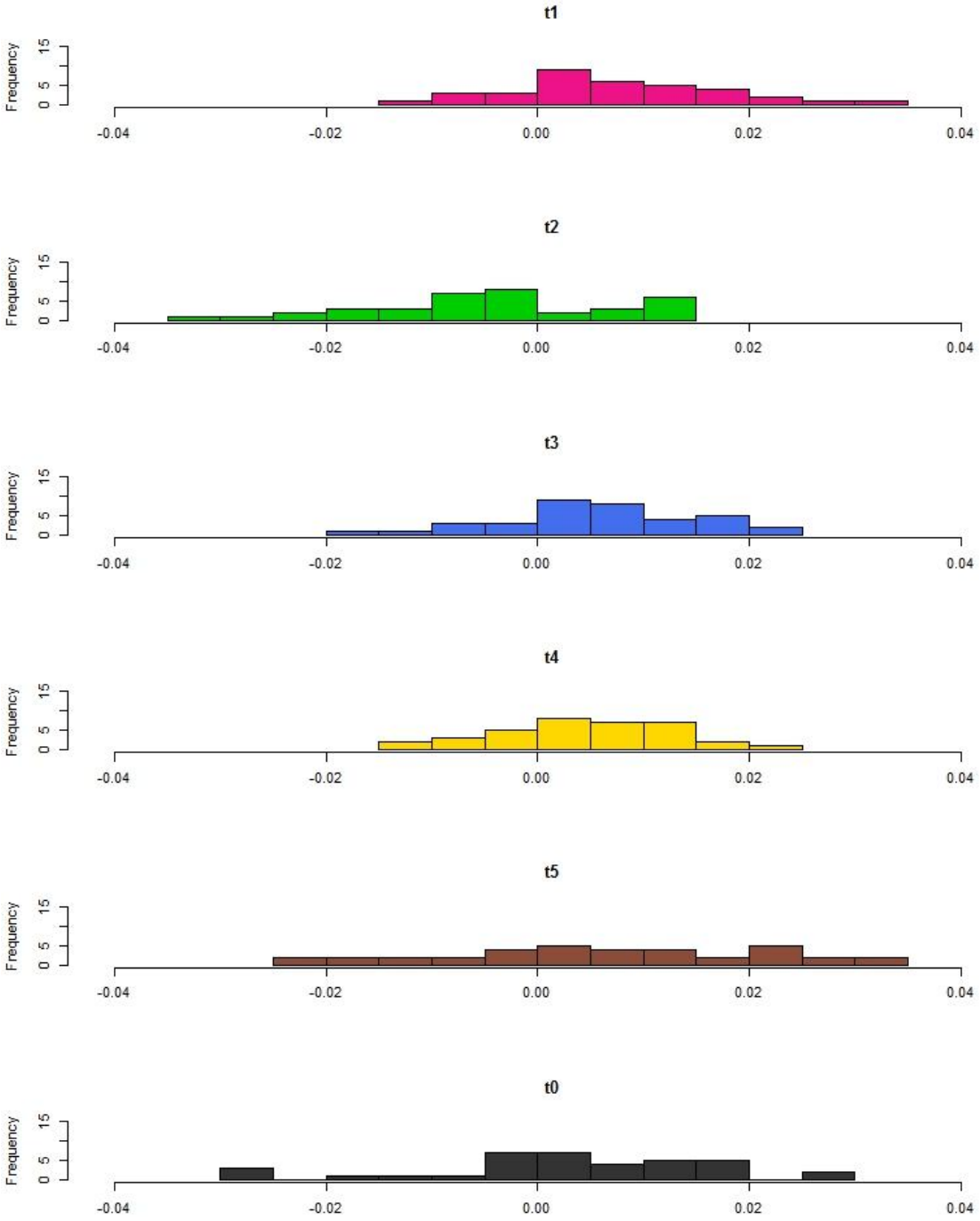


Figure 11: Histograms of PCA-scores per treatment at week 4

The average shape per treatment at week 4 (see fig. 12) indicates that the individuals from t2 had on average deeper tail fins and larger bodies. Not all landmarks and semi-landmarks show

the same amount of variation. The semi-landmarks in the middle of the tail musculature vary least. The assessed landmarks in the dataset underlie different amounts of variance (see fig. 6). Those in the cranial part of the tail obviously show a much larger variability than those assigned to the middle and caudal part of the tail. As this first graph contains all shape coordinates of the whole dataset, the variance of the landmark coordinates can be seen as a result of both growth and development and shape changes due to predator induced phenotypic plasticity.

From the PCA-plots and deformation grids over the whole dataset, we can conclude that the main shape differences in the dataset affect elongation and tail shape.

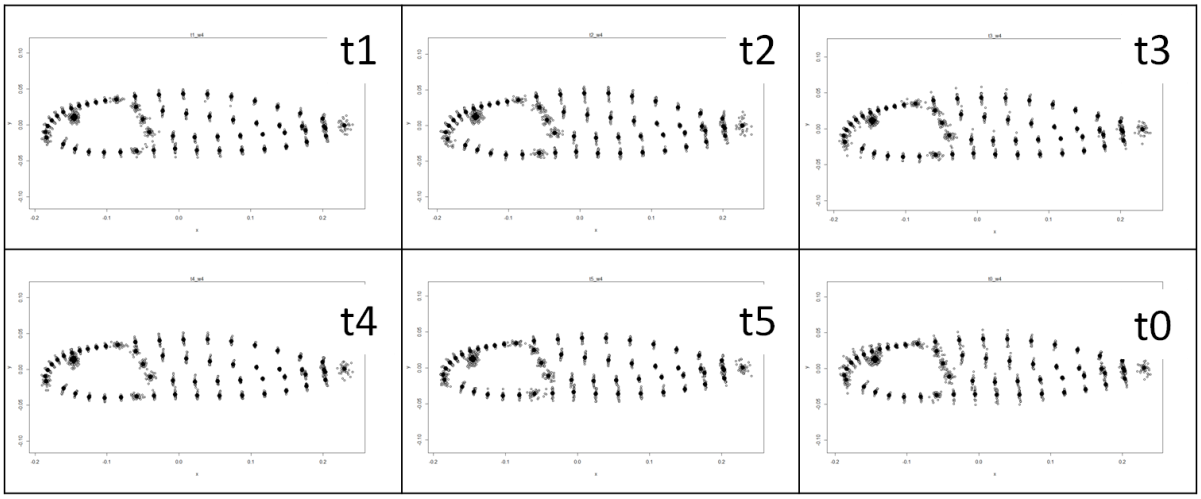


Figure 12: Procrustes shape coordinates after semi-landmark alignment at week 4 per treatment; average shape in bold dots

The weekly group means per treatment start with low scores on PC1 in week 1 (see fig. 13). In all treatments the scores on PC1 increase from week to week. The scores on PC2 do not follow such a continuous growth, but increase, decrease and increase again, but not in the exact same way for all treatments. All treatments, apart from t2 end with very similar scores on PC1. T2 ends with a markedly lower score on PC1 than the others. Apart from that, the scores from treatment 2 on PC1 are located within a narrower range than those of the other treatments.

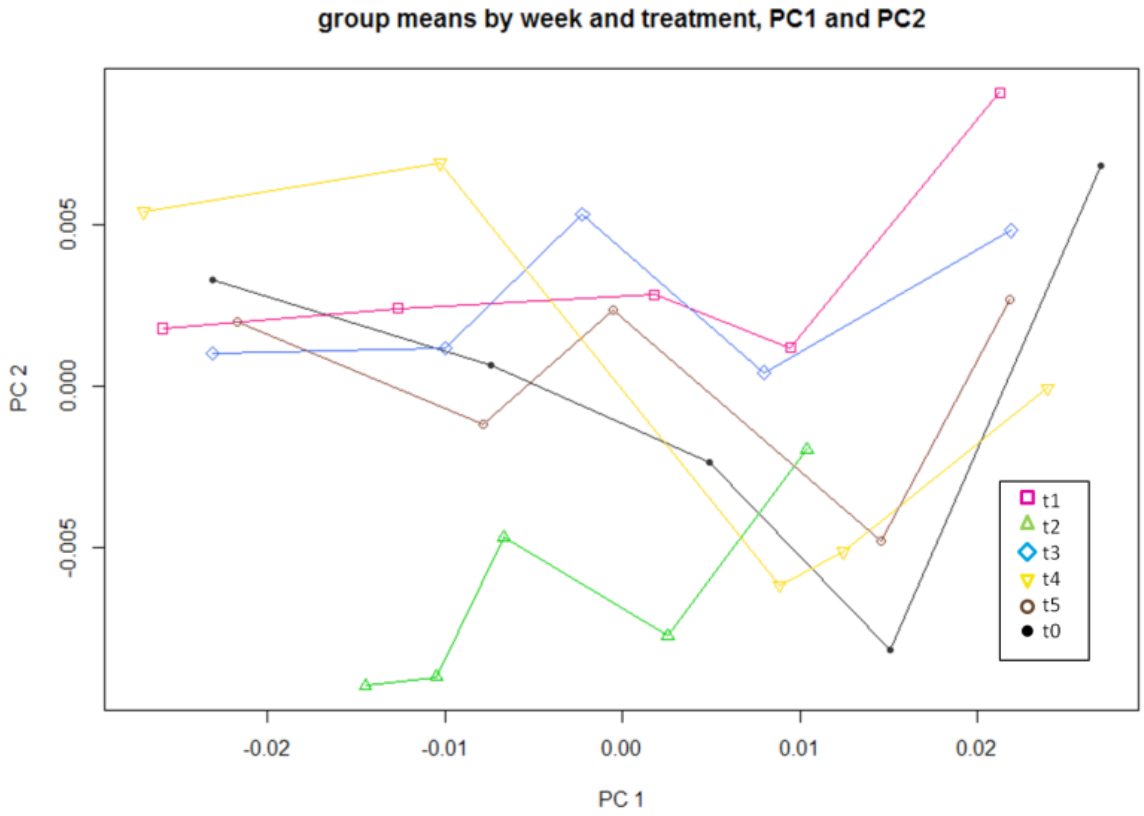


Figure 13: PCA plot of group means of procrustes shape coordinates per week and treatment; weekly group means belonging to one treatment connected with lines

There was already a rather large shape variability at time week 1 in the dataset and especially the tadpoles in treatment 2 differed on average clearly from all other treatments (see fig. 13).

To assess overall growth across treatments, we calculated the distance between landmarks 1 and 3 as an overall measure of body length. The development of the distance between landmark 1 and landmark 3, which corresponds to the distance between snout tip and tail tip and could also be referred to as total length of the individual, is shown in figure 14. The slopes of these growth curves are steepest between the first and the third photo session. After week 3, growth slows down. While the slopes of the growth curves of all other treatments but t2 are similarly steep between the first and the third photo session, growth in t2 is slower in

this time interval (see fig. 14). T2 has a slightly steeper slope than the other treatments between the third and the fourth photo session, where the other curves are already stagnating or even slightly decreasing.

The individuals from t2 were shorter than the individuals from all other treatments at the end of the experiment.

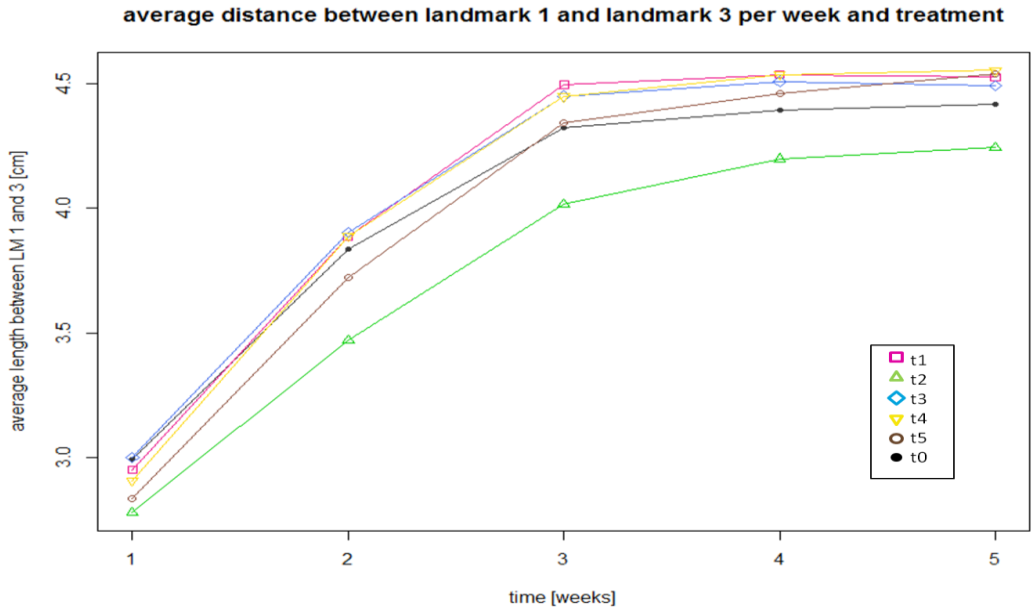


Figure 14: Average distance between landmark 1 and landmark 3 (representing the distance between snout tip and tail tip) per week and treatment

The largest shape differences between treatments were found at week 4 (see fig. 10), suggesting that on the one hand it takes some time for the plastic changes to develop and on the other hand, as metamorphosis approaches, shape changes due to metamorphosis get dominant. The main shape differences that could be found at this time were the differences between elongated tadpoles with long, low tails and short ones with deep tails. As the individuals from t2 showed lower scores for PC2, they had shorter and deeper tails. The process of shape changes in larval development lead to similar curve characteristics of mean PCA-scores of procrustes coordinates (see fig. 13). The curve of t2 looks compressed and ends at lower values of both PC1 and PC2 in comparison to the other treatment curves, indicating that the tadpoles in t2 experienced both retarded growth and development.

In the case of body length (see fig. 15) we also found a steep increase until week 3. Again we found a lower slope in treatment 2 from week 1 to week 2. A Kruskal-Wallis rank sum test

showed, that there was a highly significant ($p=2.846*10^{-7}$) difference between the treatments concerning the slope from week 1 to week 2. A Wilcoxon U-test showed, that t2 highly significantly differed from the pooled other treatments. From week 3 to week 4, body length was decreasing in t1, t3, t5 and t3 and still increasing, but with clearly reduced slope in t2 and t5. From week 4 to week 5 we found a decrease in body length in all treatments.

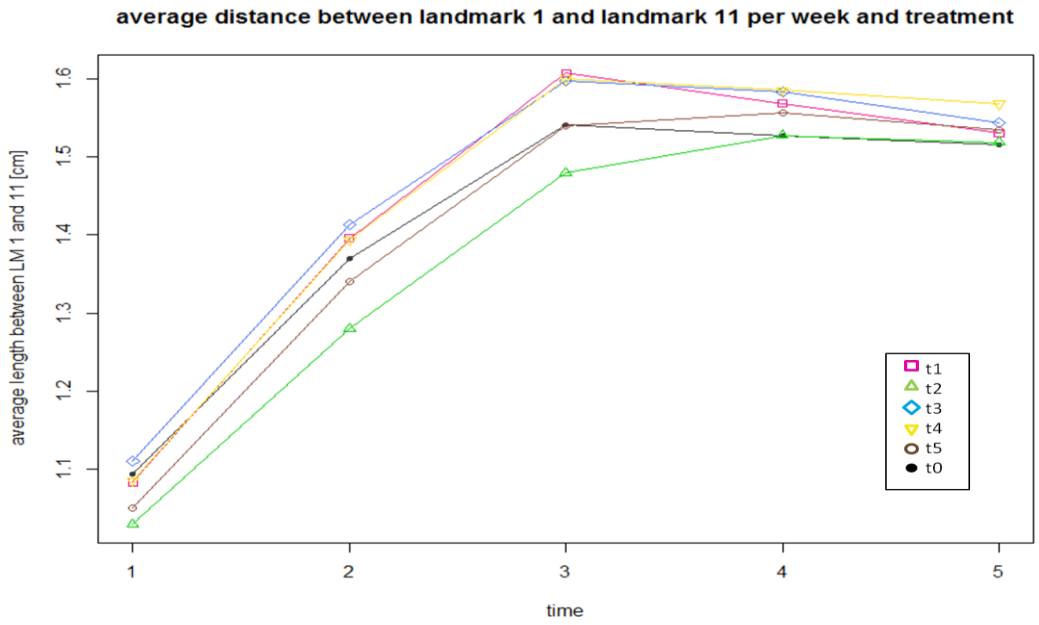


Figure 15: Average distance between landmark 1 and landmark 11 (representing body length without tail) per week and treatment

Already in the first photo session the individuals from t2 were on average shorter than those from the other treatments (fig. 14, 15). Due to the fact that the first photo session took place one week after the beginning of the experiment, it is not possible to make any statements about the size and shape variation at the beginning of the experiment. Therefore it is not possible to explain the source of this variability.

Time of Metamorphosis

The time span between the first und the last individual in the experiment reaching Gosner´s stage 42 was 24 days (see fig. 16). The first tadpoles that reached metamorphosis were from treatment t5. The individuals in treatments t2 and t3 were last to start metamorphosis, five days later than treatment t5. The last of all tadpoles reaching metamorphosis was from t2.

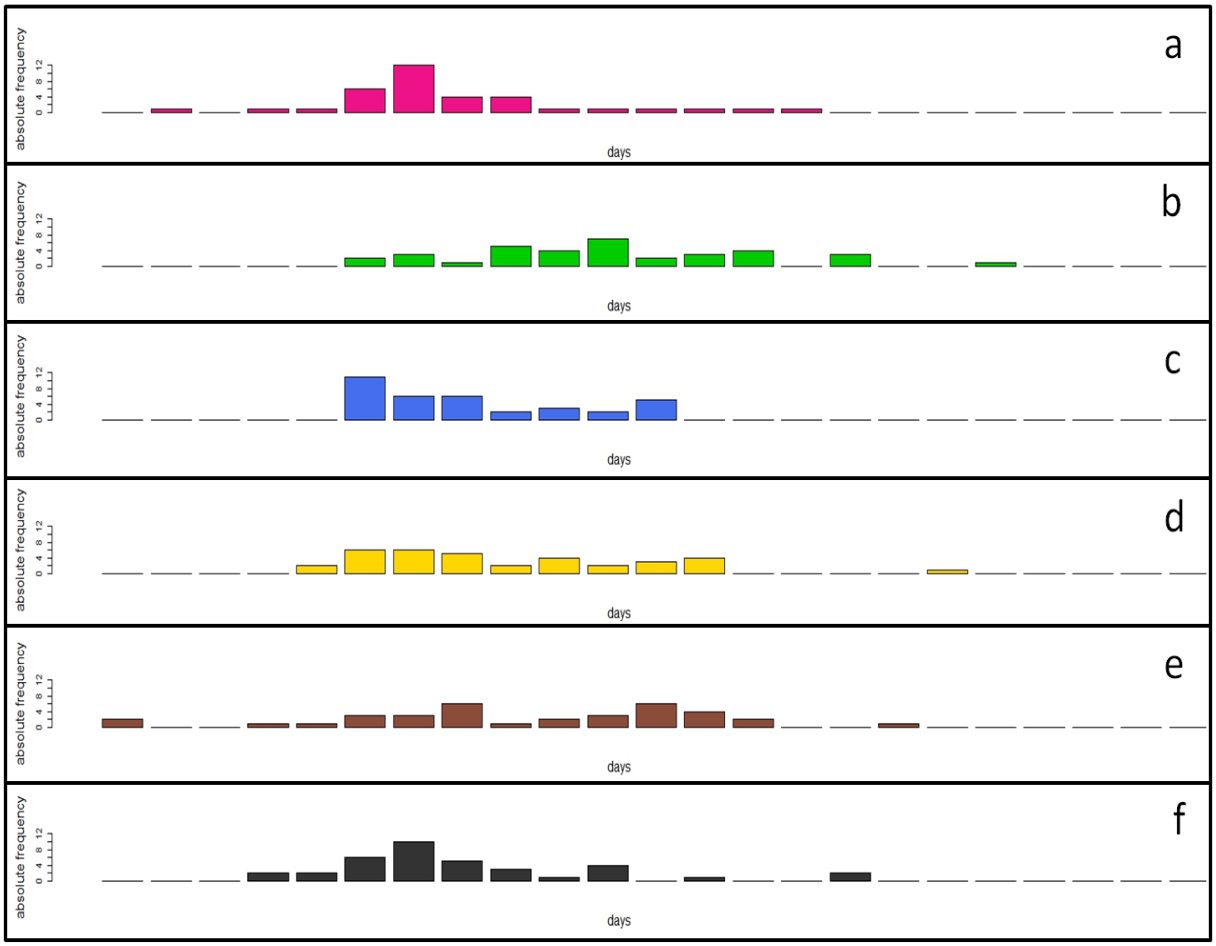


Figure 16: Time of metamorphosis in absolute numbers of tadpoles per treatment and day, beginning to count from day one, with one being the day on which the first of all tadpoles in the experiment reached Gosner stage 43 a: t1, b: t2, c: t3, d: t4, e: t5, f: t0

The distributions of time of metamorphosis are not normally distributed and the variances are non-homogenous (see fig. 16). These observations were confirmed by a significant result of the Shapiro test (only t5 can be assumed to be normally distributed, the other distributions cannot) and a significant result of the Bartlett test (variances are non-homogenous) (p=0.02035). The distributions differ between the treatments concerning curtosis, skewness and width (see fig. 16). In t1 and t0 a clear peak can be seen, while t5 shows a bimodal distribution. The lowest width of the distribution can be found in t3, where all tadpoles in the tanks reached the climax of metamorphosis within seven subsequent days, whereas in t2 21 days passed by from the first tadpole entering Gosner´s stage 42 until the last (see fig. 16). The non-parametric Kruskal-Wallis rank sum test showed a highly significant result ($p=1.48*10^{-5}$), indicating that at least one treatment significantly differs from at least one other. Pairwise Wilcoxon rank sum tests with Bonferroni adjusted p-values showed that t2 highly significantly differs from t0 (p= 0.00030), t1 (p= 0.00012), t3 (p= 0.00055) and significantly from t4 (p=0.03416).

Body mass after Metamorphosis

The lightest individuals were found in t2 and the heaviest in t1 (see fig. 17). The ranges of body mass differ between the treatments.

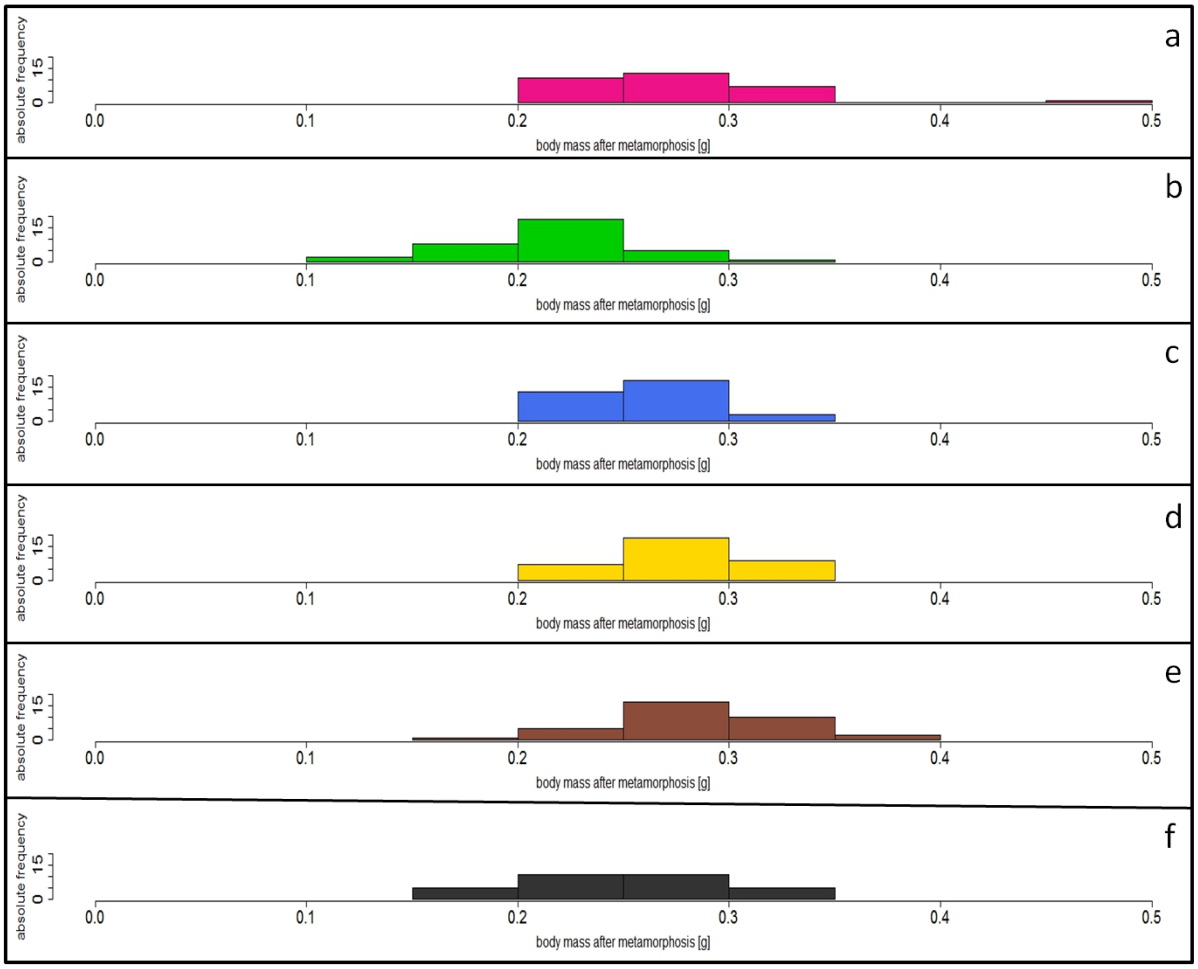


Figure 17: Histograms of body mass after metamorphosis in g; a: t1, b: t2, c: t3, d: t4, e: t5, f: t0

The treatments differ among each other concerning central tendency, range and skewness (see fig. 18). The median of t2 lies below 0.25g and therefore below the lower box limits of t3, t4 and t5 and close to the lower box limit of t1. In t0 the largest interquartile range of body mass was found, followed by t1.

The lightest of all individuals was from t2 and showed a body mass below 0.15g, whereas the heaviest was from t1 and had a body mass of more than 0.45g.

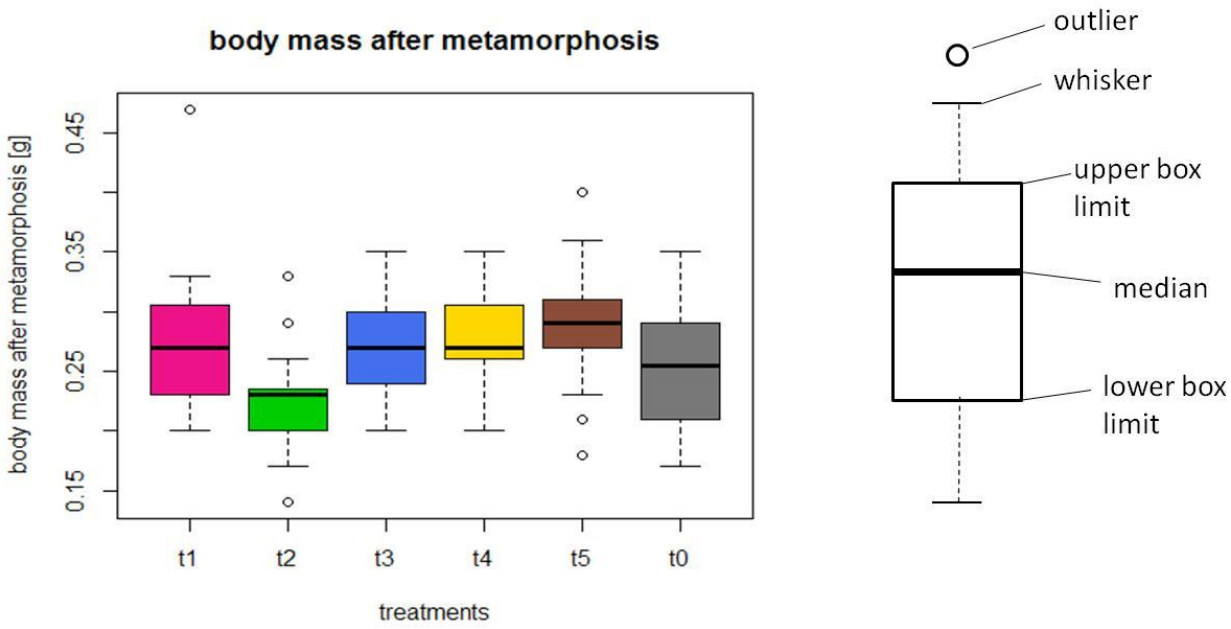


Figure 18: Boxplots of body mass after metamorphosis; lower box limit: 1st quartile, upper box limit: 3rd quartile, whiskers: smallest/largest observation that lies within a distance of 1.5 times the box size from the nearest box limit, outliers: data points beyond a distance of 1.5 times the box size from the nearest box limit

As the Shapiro test for normal distribution was only significant for t1, the normal distribution criterion for Analysis of variance was only violated for one out of six treatments and the Bartlett Test for homogeneity of variances showed an insignificant result, an ANOVA could be conducted. The ANOVA showed a highly significant result with a p-value of p=0.0003802, meaning that at least one treatment significantly differs from at least one other. Bonferroni corrected pairwise t-tests showed that t2 highly significantly differs from t1 (p=0.00017), t3 (p=0.00186), t4 ($p=2.2*10^{-5}$) and t5 ($p=4.1*10^{-8}$) and t5 differs significantly from t0 (p=0.01108).

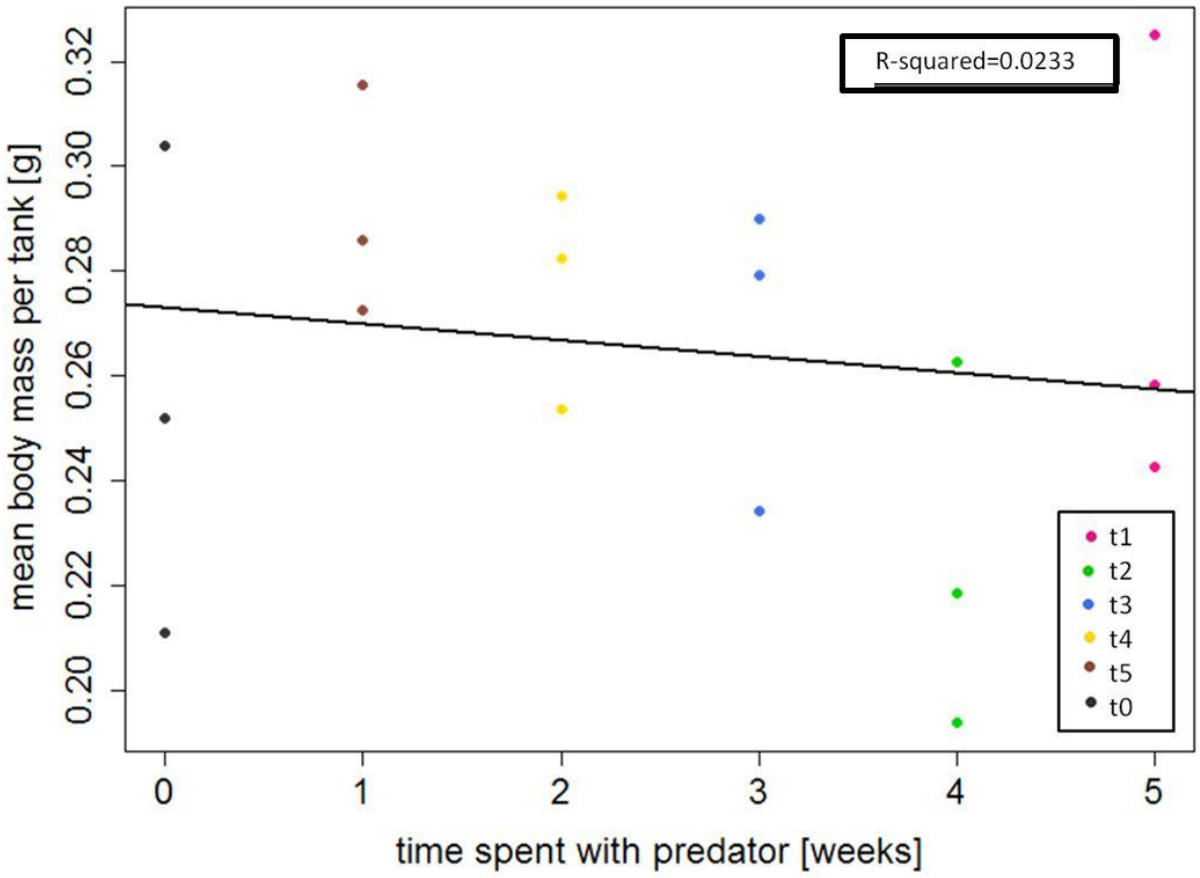


Figure 19: Regression of tank means of body mass after metamorphosis and time spent with predator

No significant (p=0.5454) linear dependency of body mass after metamorphosis on time spent with predator was found (see fig. 19).

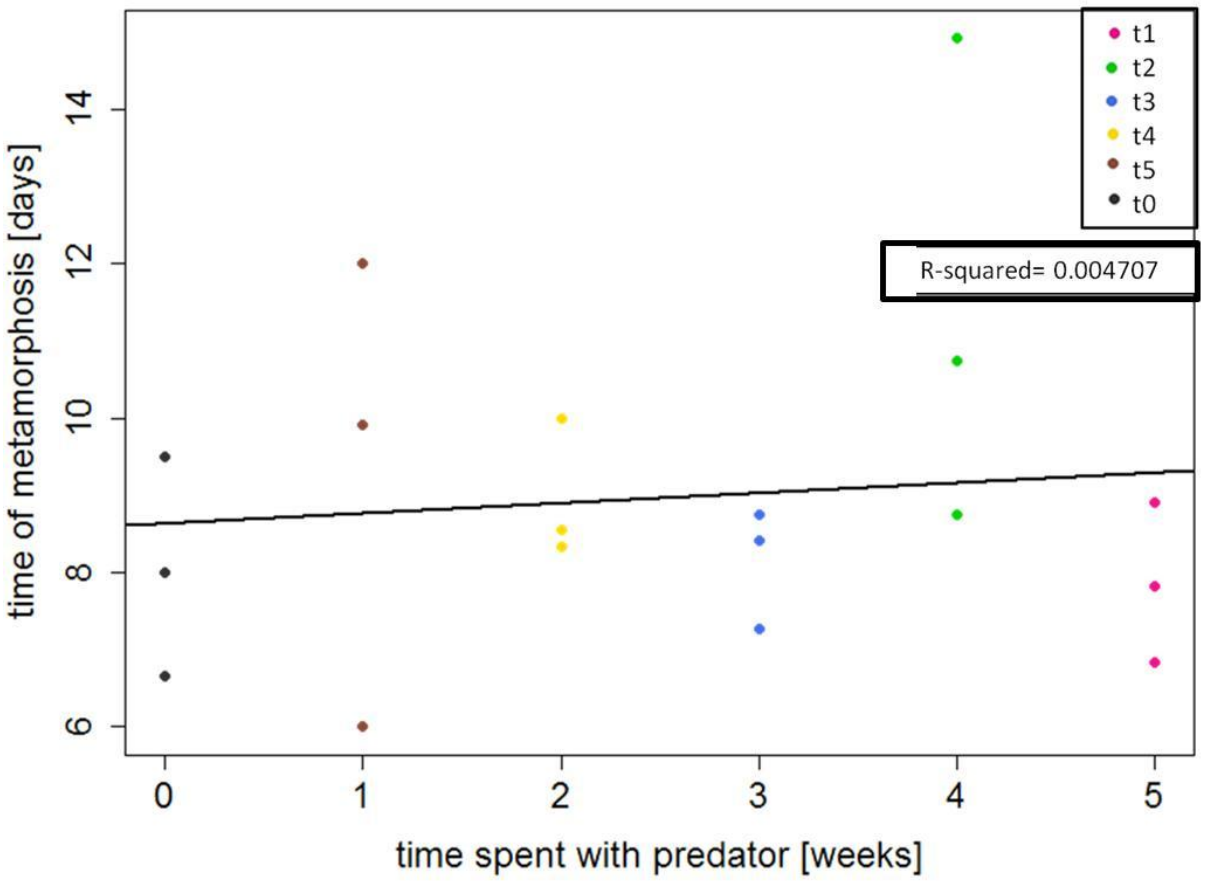


Figure 20: Regression plot of time of metamorphosis and time spent with predator

No significant correlation (p=0.5454) between time of metamorphosis and time spent with predator exists (see fig. 20).

4 Discussion

Our outdoor mescosm experiment on ontogenetic variation in predator induced phenotypic plasticity in *Rana dalmatina* tadpoles showed that the tadpoles in t2, which had their first predator contact in the second week of the experiment, about 10 days after hatching, developed the strongest plastic responses. As our growth curves show (see fig. 14 and 15), these tadpoles received the caged predator at that time in their development when the growth curves had the steepest slopes. In contrast to all other treatments, the growth curves of t2 showed a flattened slope after getting in contact with the predator. Therefore we can assume that ontogentic development constrained the qualitative and quantitative occurrence of predator induced phenotypic plasticity, meaning that the largest morphological alterations occur, when the tadpoles first get exposed to predators at a time of their development, when they experience the steepest part of their growth curves. When the expression of phenotypic plasticity is constrained by development, the potential of inducing morphological changes varies of ontogeny or is even restricted to certain developmental windows (Hovermann and Relyea 2007a). A linear dependency of predator induced plasticity on time spent with the predator was not supported by our empirical results (see fig. 19 and 20) and therefore cannot be considered as a possible explanation for any of our results.

The shape changes found in t2 in contrast to the other treatments correspond to the predator-induced phenotype, reported by various other studies (Lemcke 2005, Relyea 2002, Orizaola 2012). Concerning total length and body length, t2 differed from the other treatments and showed lower values for both parameters, indicating that the individuals from t2 were both on average shorter and had shorter bodies. The slope of their growth curves is less steep from the time on when they are first confronted with predator presence. A similar effect could not be found in any of the other treatments. Such a sharp decline of growth rate in tadpoles, which have to face predator presence early in their development, had already been reported for *Rana sylvatica* tadpoles by Van Buskirk and Yurewicz in 1998 (Van Buskirk and Yurewicz 1998), which might explain why treatments that had their first predator contact later in their larval development, did not show any similar reaction.

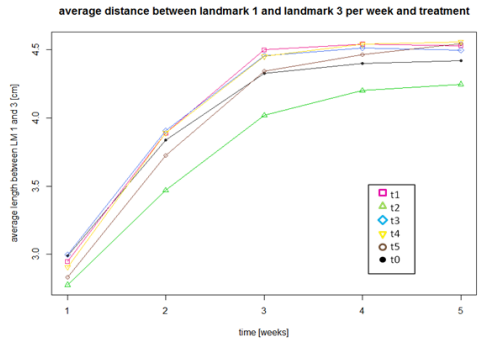
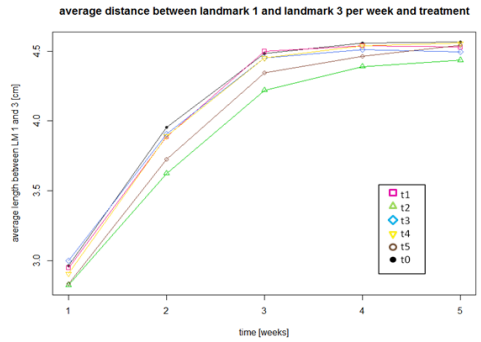
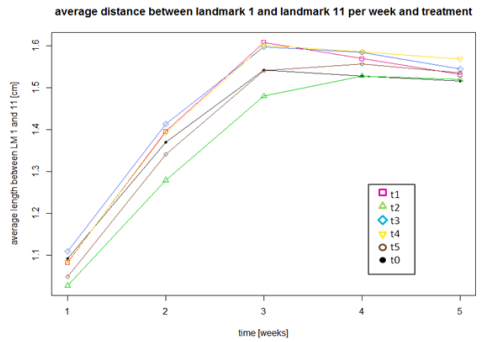
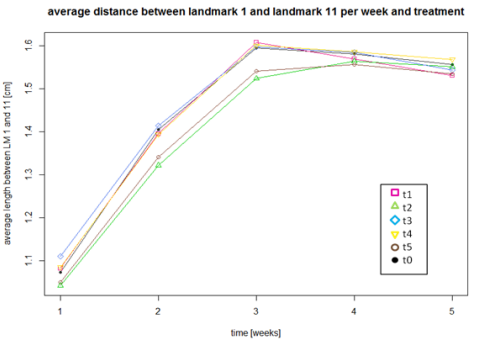
The shape differences we found were rather subtle compared to those reported in several other studies (Lemcke 2005, Orizaola 2012), which might be an effect of the experimental conditions. We kept the tadpoles in outdoor mesocosms with leaf litter, which might have created a higher heterogeneity of conditions causing other effects on the phenotype which masked the predator effect to some extent. Additionally the dragonfly larvae in our

experiment were only fed with tadpoles once a week, whereas Orizaola et al. (2012) fed them every day and Lemcke (2005) every second day, which might have caused stronger chemical cues. In our mesocosm experiment the tadpoles fed on the leaf litter, which was added to the tanks before the beginning on the experiment as well as on the biofilm that developed on the leaves. Other authors fed the tadpoles in their experiments on predator induced plasticity ad libitum with boiled spinach (Orizaola 2012) or stinging nettle (Lemcke 2005), to exclude food limitation.

Moreover, chemical cues from predators are not the only source of information about predation, which tadpoles can respond to. In other studies (Van Buskirk and Yurewicz 1998), tadpoles were constantly removed over the time of the experiment, in order to simulate a constant loss rate. This affected the daily growth rate especially in the second half of larval development. In contrast to this, the number of tadpoles per tank stayed constant over the entire duration of our experiment.

In two tanks, C3 and C4, which were assigned to t0 and t2, the water turned turbid at the end of the experiment. This could be both the result of and reason for retarded growth. On the one hand, reduced foraging activity could have lead to a higher concentration of remaining nutrients and therefore enhanced bacterial growth, while on the other hand it is also possible that bacterial communities were established which turned the water murky and affected tadpole development negatively. To control for the possible bias caused by these two tanks, we redid our statistical analysis, omitting the tadpoles from these two tanks from our dataset. Including vs. excluding these two tanks reduces the size of our observed effect, but the effect is still there (see Appendix). For further studies with a similar experimental setup higher number of replicates would be advisable.

Appendix

	with C3 and C4	without C3 and C4
Distance 1-3		
Distance 1-11		

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CURRICULUM VITAE

Persönliche Daten

Vorname: Iris Martina

Nachname: Kofler

Akademischer Titel: BSc

Geburtsdatum: 09.10.1985

Geburtsort: Innsbruck, Austria

Adresse: Brigittenauer Lände 18/9, 1200 Wien, Austria

Schulausbildung

1992 – 1996 Volksschule der Barmherzigen Schwestern in Innsbruck

1996 – 2000 Wirtschaftskundliches Realgymnasium der Ursulinen in Innsbruck

2000 – 2004 BRG Sillgasse in Innsbruck, Matura mit Auszeichnung

Studienverlauf

10/2004 – 06/2005 Bakkalaureatsstudium Biologie an der Universität Innsbruck

10/2005 – laufend Diplomstudium Veterinärmedizin an der Veterinärmedizinischen Universität Wien

10/2009 – 04/2011 Bachelorstudium Biologie an der Universität Wien

04/2011 Abschluss des Bachelorstudiums Biologie an der Universtität Wien

04/2011 – 02/2014 Masterstudium Zoologie an der Universität Wien

Studienschwerpunkte

- Amphibienökologie
- Gewässerökologie
- Wirbeltiermorphologie

Titel der Masterarbeit

Phenotypic plasticity of *Rana dalmatina* larvae: ontogenetic variation in anti-predator responses

Berufstätigkeit

WS 2011/12 Tutorin in der Lehrveranstaltung Diversität und Organisation der Pflanzen und Pilze

WS 2012/13 Tutorin in der Lehrveranstaltung Diversität und Organisation der Pflanzen und Pilze

WS 2013/14 Tutorin in der Lehrveranstaltung PP Vertebratenpraktikum

Sprachen

- Deutsch (Muttersprache)
- Englisch (gut)
- Spanisch (gut)