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Effects of copper on development and behavior of green toad tadpoles

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Abstract

The increasing heavy metal pollution caused by human activities poses a growing threat to aquatic ecosystems. While some adverse effects of the heavy metal copper, including increased mortalities, morphological deformations, and behavioral alterations, have been documented in certain amphibian species, its effects on developing green toad tadpoles remain underexplored. This work aims to address this gap by investigating the morphological and behavioral effects of copper exposure on green toad tadpoles. The findings reveal significant morphological alterations induced by copper exposure, notably a reduction in the width of the tadpoles' rumps and a decrease in tail fin depth. Furthermore, higher copper levels led to an increase in total length and a decrease in the body to tail ratio of the tadpoles. Additionally, a correlation between elevated copper levels and increased mortality rates among the tadpoles was found. Copper was also found to have significant effects on the behavior of green toad tadpoles, such as on their activity, distance from the arena wall and on the total movement duration and total movement length after external stimulation. This study sheds light on the effects copper has on *Bufotes viridis* larvae and emphasizes the urgency of reducing heavy metal pollution in aquatic habitats.

Keywords: Copper, amphibians, *Bufotes viridis*, geometric morphometrics, behavior

Zusammenfassung: Die zunehmende Schwermetallverschmutzung aufgrund menschlicher Aktivitäten stellt eine wachsende Bedrohung für aquatische Ökosysteme dar. Während einige negative Effekte des Schwermetalls Kupfer einschließlich erhöhter Sterberaten, morphologischer Deformationen und Verhaltensänderungen bei bestimmten Amphibienarten dokumentiert wurden, sind die Auswirkungen auf sich entwickelnde Kaulquappen der Wechselkröte noch nicht ausreichend erforscht. Diese Arbeit zielt darauf ab, diese Lücke zu schließen, indem die morphologischen und verhaltensbezogenen Auswirkungen einer Kupferexposition auf die Kaulquappen der Wechselkröte untersucht werden. Die Ergebnisse zeigen signifikante morphologische Veränderungen, die durch die Kupferexposition hervorgerufen werden, insbesondere eine Verringerung der Breite des Rumpfes der Kaulquappen und eine Abnahme der Schwanzflossentiefe. Höhere Kupferwerte führten außerdem zu einer Zunahme der Gesamtlänge und zu einer Abnahme des Verhältnisses von Körper zu Schwanz bei den Kaulquappen. Darüber hinaus wurde eine Korrelation zwischen erhöhten Kupferwerten und der erhöhten Sterblichkeitsrate der Kaulquappen festgestellt. Es wurde auch nachgewiesen, dass Kupfer signifikante Auswirkungen auf das Verhalten der Kaulquappen hat, wie etwa auf ihre Aktivität, den Abstand von der Arenawand sowie auf die Gesamtbewegungsdauer und die Gesamtbewegungslänge nach externer Stimulation. Diese Studie wirft ein Licht auf die Auswirkungen von Kupfer auf die Larven von *Bufotes viridis* und unterstreicht die Dringlichkeit, die Schwermetallverschmutzung in aquatischen Lebensräumen zu reduzieren.

Schlagwörter: Kupfer, Amphibien, *Bufotes viridis*, Geometrische Morphometrie, Verhalten

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Introduction

Amphibian populations are declining worldwide (McCallum, 2007) with some of the main reasons being habitat destruction, climate change, disease and the introduction of invasive species (Blaustein & Kiesecker, 2002). Another major threat to amphibians and biodiversity as a whole are pollutants such as heavy metals released by human activities (Beasley & Kneale, 2002). One important pollutant contributing to this amphibian decline is the heavy metal copper (Blaustein et al., 2003), which can be found in a variety of chemical compounds, such as different pesticides (Chiari et al., 2015). The metal industry also uses copper for the production of different alloys, for plating of other metals and for the manufacture of wires and pipes (Beasley & Kneale, 2002). The copper in these products is mostly immobilized, but some release can still occur and add to heavy metal pollution (Beasley & Kneale, 2002). Copper based nanoparticles (NPs) like copper oxide (CuO), which are also suitable for coating applications, are produced on a large commercial scale and can therefore also find ways into aquatic systems (Kent & Vikesland, 2016; Nations et al., 2015). An additional contributor to copper pollution in water bodies is road runoff, which contains dissolved and metallic copper particles from different vehicle-related sources such as tyres and brakes (Beasley & Kneale, 2002) and is primarily affecting aquatic environments adjacent to roads (Dorchin & Shanas, 2010). Stormwater ponds for example, which help with the retention of polluted road runoff, can serve as a breeding habitat for different amphibian species. However, analyses of various storm water ponds have shown significantly higher copper concentrations, which are associated with higher tadpole mortality in species such as the European green toad (Conan et al., 2023). The European green toad, *Bufotes viridis* (Laurenti, 1768), is regarded as a synanthropic species in Central Europe (Stöck et al., 2008) and is therefore more likely to be exposed to heavy metals. Some of the most prominent recent occurrences in Vienna include the agricultural area Donaufeld, gardening areas in Simmeringer Haide and Rudolf-Bednar Park close to the city center (Csarmann E, 2012; Sistani et al., 2021; Staufer et al., 2023). The focus of this work lies on the morphological and behavioral effects of chronic copper exposure on *B. viridis* larvae.

Most relevant studies focus on the effects of dissolved copper salts on amphibians and their larvae. For example, in a study from Gürkan and Hayretdağ (2012), larvae of *B. viridis* were exposed to different concentrations of copper sulfate (10 µg/L,50 µg/L and 100 µg/L), an often used fungicide, for a time span of 120 hours. The exposure resulted in morphological abnormalities as well as reduced reaction to stimuli, immobility, and shorter swimming distances. These results are similar to a study from E. García-Muñoz et al. (2009), where *Epidalea calamita* larvae were exposed to copper sulfate solutions (ranging from 0.05 to 0.40 mg/L) for 96 hours. Individuals exposed to copper sulfate concentrations of more than 0.2 mg/L grew to only half the size compared to those in the control group. The copper sulfate exposure also altered the escape behavior and the larvae showed shorter escape distances.

It is important to note that copper sulfate forms Cu^{2+} ions in aqueous solutions, which is considered the most toxic form for aquatic life (Hall, 1998). However, copper in aquatic habitats

occurs not only in its dissolved forms, but also in the form of small metallic particles that can exert direct toxic effects apart from the toxicity due to ion release (Kent & Vikesland, 2016).

But only few studies include the effects of metallic copper on *B. viridis* larvae. For instance, a study from Dorchin and Shanas (2010), were the effects of road run-off water on green toad tadpoles were tested. The runoff water, which not only contained dissolved copper but also metallic copper in the sediment, had a negative effect on the development and morphology *of B. viridis* tadpoles. The body length of tadpoles exposed to the midseason runoff (copper concentrations of 9.4 ± 3.6 µg/L and 11 µg/L) and to the synthetic solutions (copper concentrations of 180 µg/L and 25 µg/L) showed a significant reduction in body length after day 7 of the exposure. Larvae treated with midseason runoff and synthetic solutions also showed elevated rates of body, tail, mouth part and eye deformities. While the run-off and (synthetic) run-off-like solutions contained copper, they also contained lead, nickel, and other potential pollutants. It is therefore unclear, which of the components may have caused the effects they reported. In a recent study from Vaissi et al. (2024), the effects of CuO nanoparticles on green toad tadpoles were investigated. Above concentrations of 10 mg/L, the nanoparticles caused hepatic lesions and moderate vascular congestion in the internal gills of the larvae. Also, hypoactivity was observed in larvae treated with low concentrations (10 mg/L) of CuONPs at ambient temperature. Conversely, larvae at low (10 mg/L) CuONPs concentrations and high temperatures showed hyperactivity compared to the controls. Higher concentrations (30 mg/L; 90 mg/L) of CuONPs also led to hyperactivity in *B. viridis* larvae.

Due to the lack of studies using non-oxidized metallic copper on *B. viridis* tadpoles, copper salts were substituted with metallic copper powder (Cu) in this study. This should represent a realistic approach, as the larvae are exposed to such particles in their natural habitats through sources such as tyre and brake abrasions (Beasley & Kneale, 2002). Although it is anticipated that a large proportion of the metallic copper will dissolve in the test water and form copper ions (Kent & Vikesland, 2016), metallic copper particles are still expected to be present due to the larger grain size used (38 µm).

Based on the existing literature it was expected that the tadpoles would most likely show similar morphological and behavioral changes due to the influence of dissolved copper. This would be consistent with the studies mentioned above that used copper-containing compounds such as copper sulfate ($CuSO_4$), copper chloride ($CuCl_2$) and copper oxide (CuO) (e.g. Dorchin & Shanas, 2010; E. García-Muñoz et al., 2009; Gürkan & Hayretdağ, 2012; Kent & Vikesland, 2016). In this work, tadpoles of the European green toad were reared in different concentrated copper solutions which also contained metallic copper. Changes in shape were analysed using geometric morphometry and subsequent PCA. The effects of copper on the tadpoles' survival, growth and development, as well as its impact on the behavior were investigated.

Methods & Material

Climate chambers

For the duration of larval development, the tadpoles were raised in plastic containers inside two different climate chambers. Temperature and light settings of the climate chambers were programmed to simulate day and night. The duration of the day and night cycles was 12 hours each. The temperature of day cycles was set to 20°C, while the temperature of night cycles was set to 10°C.

Preparation of the treatments

The different treatment solutions were prepared and stored in 25-litre canisters by persons who were not involved in the remainder of the experiment. The canisters were filled in advance with aged tap water and, depending on the treatment level, a different amount of metallic copper (D95 38 µm) was added. The canisters containing the metallic copper were stirred occasionally to even the distribution of the copper particles inside the canisters. The maximum copper concentrations to be achieved for the different treatment levels were 10 µg/L, 70 µg/L and 140 µg/L, whereas no copper was added to the control group. The concentration range was chosen in accordance with the copper concentrations that had previously been measured in the breeding ponds of the European green toad.

During the experiment, three samples from every treatment were taken by scientific staff of the University of Natural Resources and Life Sciences in Vienna (BOKU) and sent to 'Hydrochemical Laboratories GEOtest' where the analyses were performed (Table 1).

Sampling and storage of the spawn

In April 2023, when the mating season of *B. viridis* took place, parts of 5 different strands of eggs were collected from spawning sites at Rudolf-Bednar Park. The strand fragments were then transferred to the BOKU, where the experiments were conducted. The eggs from the different clutches were separated and stored in tubs inside the climate chamber. The water in which the eggs were kept until they hatched was water brought from Rudolf-Bednar Park.

Test containers

The Larvae were held in test containers (dimensions 26 x 17 x 14 cm). Four different treatments were performed, each of which was replicated 6 times. Therefore, a total of 24 test containers were stored in the climate chambers. The test containers were filled up to 7 cm high with the different treatment solutions. For the bacterial treatment, an aquarium starter was added (approx. 0,5 ml aquarium starter per liter tap water) that contained various strains of water-purifying bacteria such as Nitrosomonas and Nitrobacter. These were added to support the breakdown of nitrogenous pollutants such as ammonia and nitrite and to create a more natural environment.

Distribution of tadpoles

On May 4th, once the larvae hatched and moved around freely, they were split up and put into the test containers. For this step, one larva from each of the 5 different clutches was taken

and then placed into the same test container. After the distribution, each of the test containers contained five larvae. The test containers were covered with a perforated lid to reduce evaporation inside the climate chambers. The test containers were arranged in a completely randomised block design within the middle and lower compartments of the two climate chambers (Figure 1). The upper compartments were not used due to another experiment. For identification purposes, the test containers in both climate chambers were marked with a number and a letter. It is important to note that the experiment was conducted in a double-blind manner. The individuals who conducted the experiment were unaware of the correlation between the labels and the treatments.

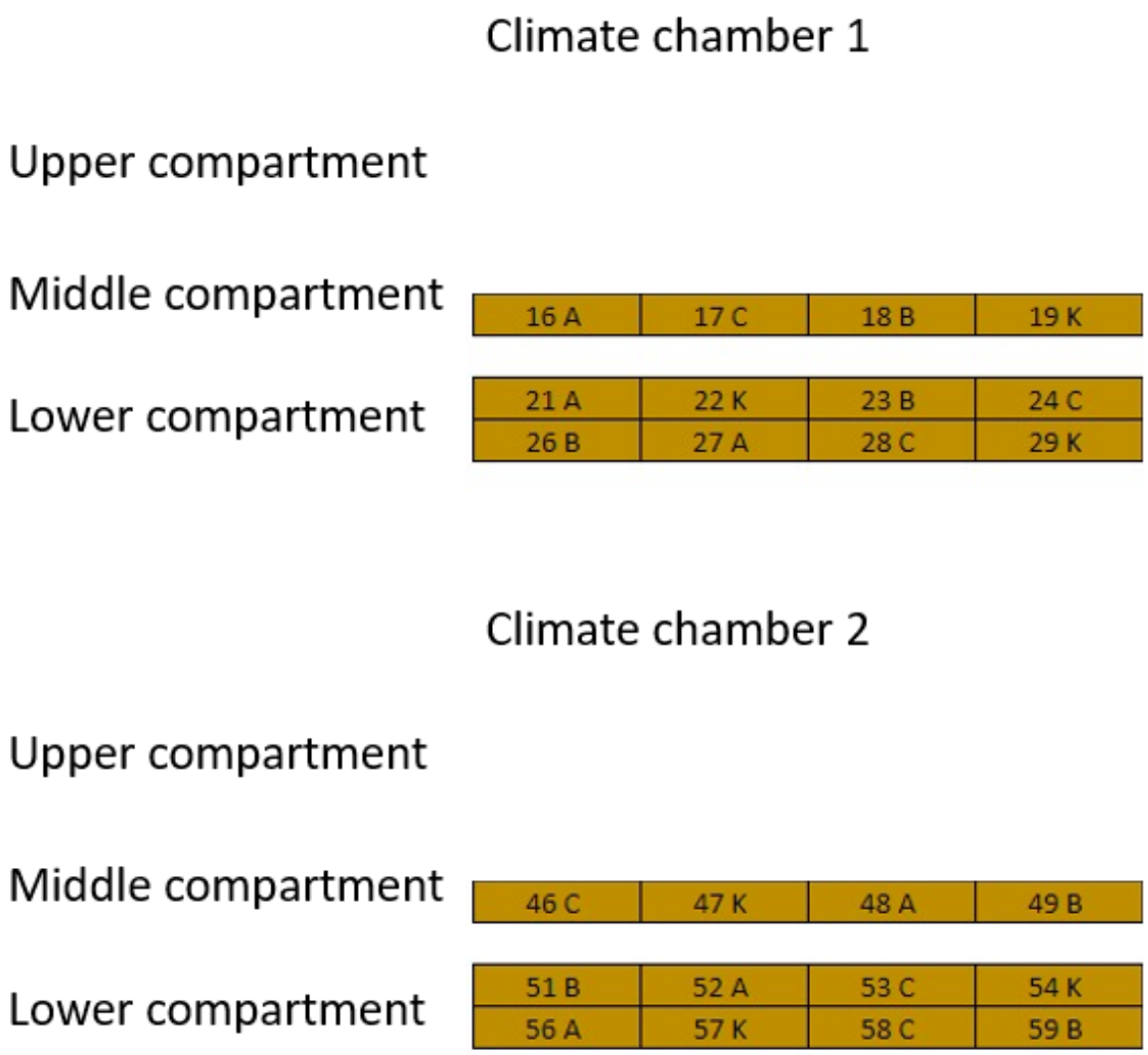

Figure 1: Distribution of the test containers within the two climate chambers. *Numbers indicate the placement within the climate chambers, while the letters indicate the corresponding treatment (K = 0 µg/l; A = 10 µg/l; B = 70 µg/l; C = 140 µg/l).*

Water changes and oxygenation

The water of the test containers was changed every 2 weeks and excrements were removed with a pipette when detected throughout. For oxygenation we used an air pump connected to air stones. The ventilation was set to the same intensity for all containers using manually adjustable valves. As the pH value was not measured during the experiment, a control experiment was carried out afterwards. For this purpose, two test containers with aged tap water were placed in each compartment of climate chamber 1. These were also mixed with bacteria and oxygenated using an air pump. The pH values and temperatures of the test waters were measured five times over a period of two weeks (Table 2).

Feeding the larvae

The larvae were fed with fish food three times a week. The amount of fish food was increased over time to adjust it to the increasing demand during growth. In the first two weeks around 0,01 g of fish food was added per larva. For the third week the quantity was increased to 0.02 g per larva and one catfish pellet (spirulina algae) was also added to each container. In the fourth week the quantity was increased to 0,03 g per larva and one catfish pellet per container. After this time, the amount was increased to 0.05 g per larva and one catfish pellet per container for the remainder of the experiment.

Growth and survival - data collection and analysis

To determine the total length of the tadpoles, dorsal photographs were taken after every 2 weeks. For this purpose, a scale was placed under the containers and the lengths were then taken from the pictures using the image processing programme ImageJ. In addition to the total length of the animals, the tadpoles' rumps and tails were measured separately for the calculation of the body to tail ratio. The centroid sizes were calculated from the different lateral and dorsal landmark configurations, which are described in more detail below. The Gosner stages were determined after 32 and 64 days from the lateral images using a table for staging (Gosner, 1960).

For the statistical analysis of growth and survival for each treatment, a Generalized Linear Mixed Model was conducted using the glmmTMB function in R (Brooks et al., 2017). For the total length as well as for the body to tail ratio of the tadpoles in each treatment, plots have been made using the ggpredict function of the ggeffects package in R (Lüdecke, 2018).

Gosner stage 42 was marked as the beginning of metamorphosis, characterized by the appearance of the forelimbs and the resorption of the tail (Gosner, 1960). The animals were then transferred to transition tanks containing both water and land components in which the animals could complete their metamorphosis. For the statistical analysis of the metamorphosis timepoints for each treatment and the mass of the animals after metamorphosis, Generalized Linear Mixed Models were conducted, again using the glmmTMB function mentioned before.

Shape - data collection and analysis

Lateral and dorsal photos of all larvae were taken after 32 days and again after 64 days after start of the experiment. For the photos, the animals were individually transferred to glass cuvettes filled with stagnant tap water and placed in a photo light box. The dorsal images of the larvae were taken with a smartphone camera, which was placed on the top opening of the photo light box (Figure 2). The distance between the smartphone camera and the glass cuvette was 23 cm. The lateral photos were taken with a Canon EOS 200D DSLR camera, using a lens with 55 mm focal length. For the lateral photos, additional light was directed onto the cuvette from the front using two swan-neck lights (Figure 3). The distance from the camera lens to the cuvette was 8.3 cm. For stabilization and reproduction purposes during the lateral photo shootings, an ESDDI tripod was used.

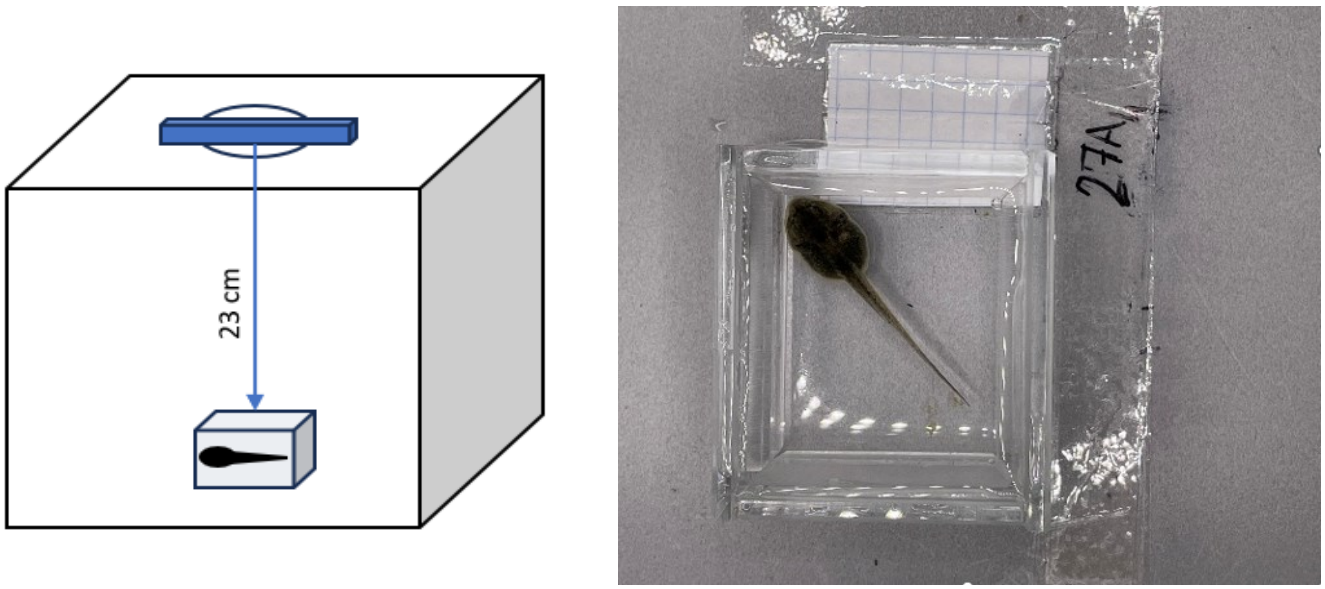

Figure 2: Setup for the dorsal images. *Left side shows the schematic setup for the dorsal images of the tadpoles. Right side shows the view of the smartphone camera through the upper opening of the photo light box.*

Figure 3: Setup for the lateral images *of the tadpoles using a photo box and swan-neck lights for illumination.*

For the analysis, the images were first randomised using the program TpsUtil and then landmarked in TpsDig. For the lateral images 9 anatomic landmarks and 51 semilandmarks were used (Figure 4). For the dorsal images 6 anatomic landmarks and 16 semilandmarks were placed (Figure 5). The files containing the 2D landmark data were then imported into the statistics programme R. There, the landmark configurations were standardised in terms of position, scale and orientation using a Generalised Procrustes Analysis (GPA) in the R package 'geomorph' (Baken et al., 2021; D. C. Adams et al., 2023). To remove shape differences during the GPA caused by the ambiguous placement of the semilandmarks, their final locations were computed by the sliding landmark algorithm. This algorithm minimizes the bending energy of the thin-plate spline between each landmark configuration and their sample average (Bookstein, 1997; Gunz & Mitteroecker, 2013).The resulting Procrustes shape coordinates then only contained information on the shape of the landmark configurations. Due to large variation in tail position, the dorsal coordinates were averaged with their mirror image. A principal component analysis (PCA) was then performed using the Procrustes shape coordinates from both perspectives and both photo sessions. The loadings obtained from the PCA were then used to perform a principal component regression (PCR) with the copper levels.

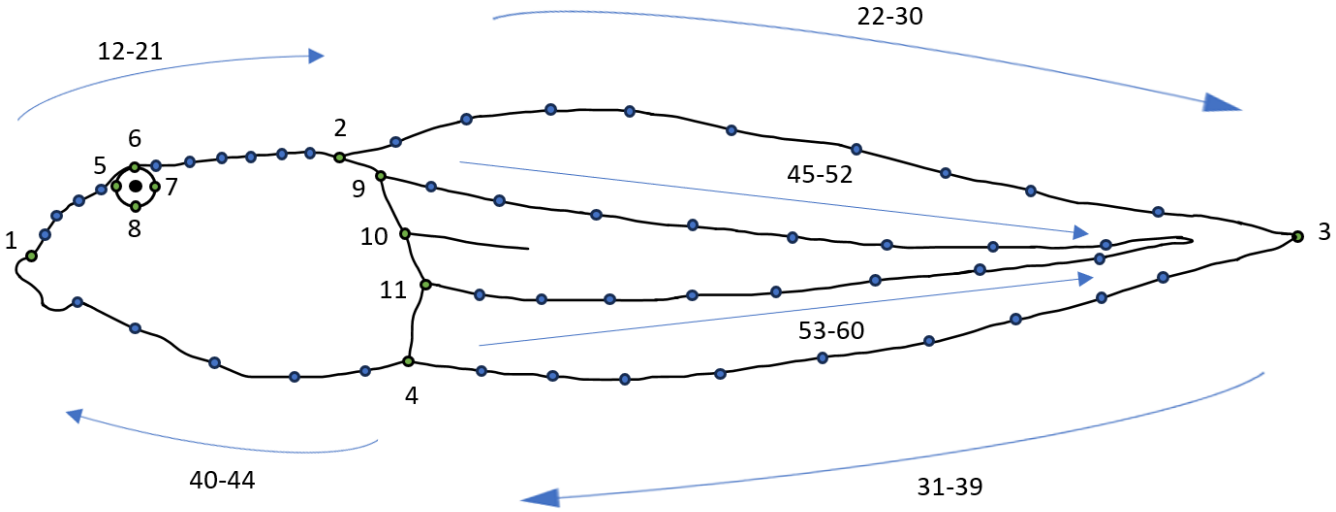

Figure 4: Landmarks (1, 3, 5–11) and semilandmarks (2, 4, 12–60) for the lateral view. *1, most rostral point of upper lip; 2, juxtaposition of dorsal tail fin and body; 3, tail tip; 4, juxtaposition of ventral tail fin and body; 5, most rostral point of the eye; 6, most dorsal point of the eye; 7, most caudal point of the eye; 8, most ventral point of the eye; 9, most dorsal point of juxtaposition of body and tail musculature; 10, most anterior point of juxtaposition between ventral and dorsal myomeres of the tail musculature; 11, most ventral point of juxtaposition of body and tail musculature. Arrows indicate the direction of numbering for the semi-landmarks. 12-21, dorsal outline of head and body; 22-30, dorsal side of tail fin; 31-39, ventral side of tail fin; 40-44, ventral outline of head and body; 45-52, dorsal margin of tail musculature; 53-60, ventral margin of tail musculature (Hagauer, n.d.).*

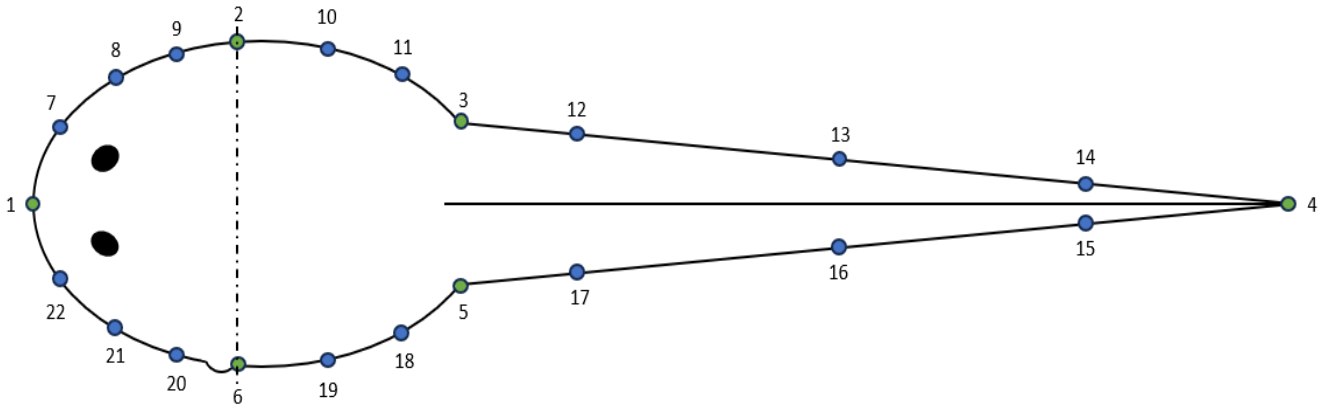

Figure 5: Landmarks (1-6) and semilandmarks (7-22) for the dorsal view. *1, most rostral point of the head; 2, on body outline opposite of 6; 3, intersection of torso and tail on the right side of the body; 4, tail tip; 5, intersection of torso and tail on the left side of the body; 6, juxtaposition of body and spiracle.*

Behavioral experiments

To investigate possible behavioral effects of copper on the larvae, the swimming and exploration behavior of the tadpoles raised in different concentrated copper solutions were observed and analysed. For this experiment, on 28th of June, a tadpole subsample was randomly selected from all treatments and transferred into test arenas. For the subsamples, one individual was taken at random from each container that still contained living tadpoles at that time. A total of 14 subsamples were therefore taken from the various containers. However, as 16 test individuals were planned for the experiment, a second tadpole was taken from two randomly selected containers (56A and 57K). The experiment then contained a total of 6 tadpoles from treatment K, 5 from treatment A, 4 from treatment B and 1 from treatment C as this was the last one to survive. Each larva of the subsample was placed and tested in a separate arena. The test arenas were cylindric plastic containers with an inner diameter of 11.3 cm and a height of 4 cm. The test arenas were filled up to 1.2 cm high with aged tap water. The water temperatures during the behavioral experiments were obtained later from the recorded room temperatures (Table 9). Eight animals were tested in each of two different runs, with eight arenas being used in parallel to speed up the test procedure (Figure 6). The test arenas were placed in a frame (Figure 6), which was located between a window and an illuminated terrarium (Figure 7). The frame was covered with black fabric to keep out light. Inside the frame, 8 LED bulbs shining through a diffuser were used for illumination (Figure 8). The recordings were made with a webcam, which was mounted on the ceiling of the frame and directed at the test arenas from above. The tadpoles were then able to explore the new environment for half an hour without any external influence. After this time, three swimming responses were elicited in succession by touching the tadpoles with a fine copper wire (e.g Wilson et al., 2005; Zambrano-Fernández et al., 2022). After each touch, the next touch was delayed until the animal stopped moving.

Figure 6: Arrangement of the test arenas within the frame for the behavioral experiments.

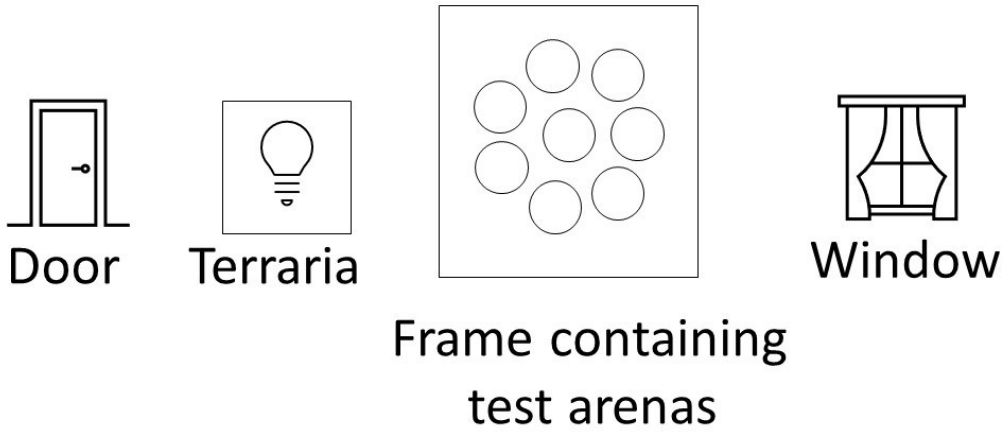

Figure 7: Schematic layout of the test room.

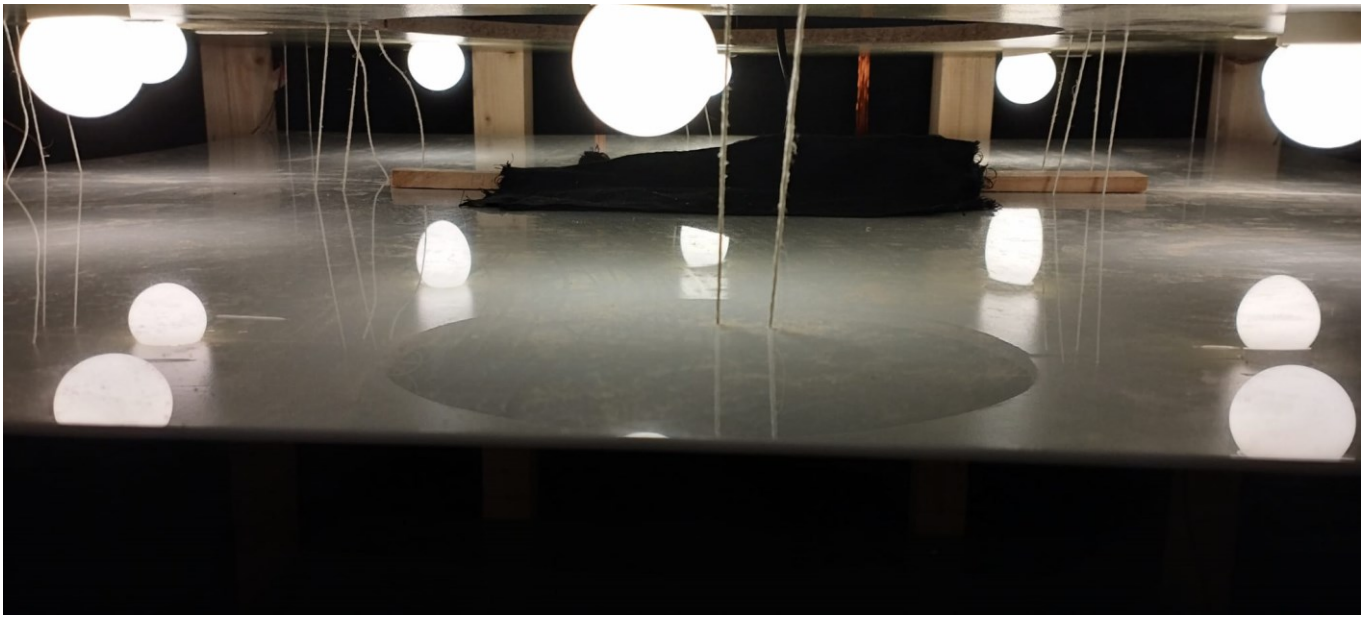

Figure 8: Lighting and diffuser (white sheet) inside the frame above the test arenas.

Data analysis for behavioral experiments

The observed swimming behavior was analysed using a customized program for tadpole movement. This program is based on the tracking software 'Alignment v4' (e.g. Landler et al., 2019). Generalized linear mixed models were used to statistically detect treatment effects on the behavioral response variables taken. The tested response variables were the activity (distance moved per 1 min interval relative to the arena radius), the tadpoles' distance to the arena wall, the total movement duration and the total movement length after eliciting escape responses. After the experiments were over, the remaining animals were kept for future research.

Results

Treatment analyses

The measured treatment concentrations were lower than the maximum achievable concentrations, as the copper did not dissolve completely (Table 1).

Table 1: Results of treatment analyses.

Treatment	Parameter	Unit	Result	Result	Result	Uncertainty
Control group (K)	Cu(copper)	µg/l	<10.0	<10.0	<10.0	±20%
Level 1 (A)	Cu(copper)	µg/l	<10.0	11.8	<10.0	±20%
Level 2 (B)	Cu(copper)	µg/l	57.0	48.7	42.5	±20%
Level 3 (C)	Cu(copper)	µg/l	104	92.8	94.6	±20%

pH values of the control experiment

All test waters remained at pH = 8.3 ± 0.1 for a two-week period during the control experiment (Table 2).

Table 2: Measured pH values and temperatures within the control experiment.

Date of measurement	**Position in climate chamber 1**	**Temperature (°C)**	**pH Value**
07.12.23	Middle left	22.5	8.4
	Middle right	22.4	8.3
	Bottom left	22.3	8.3
	Bottom right	22.3	8.3
12.12.23	Middle left	20.5	8.3
	Middle right	20.4	8.3
	Bottom left	19.4	8.3
	Bottom right	19.8	8.3
14.12.23	Middle left	20.5	8.3
	Middle right	20.5	8.3
	Bottom left	19.3	8.3
	Bottom right	19.7	8.3
19.12.23	Middle left	20.5	8.2
	Middle right	20.5	8.2
	Bottom left	20.0	8.2
	Bottom right	20.0	8.2
21.12.23	Middle left	20.3	8.3
	Middle right	20.2	8.3
	Bottom left	19.5	8.3
	Bottom right	19.3	8.3

Survival Rates

In the control group, 15 tadpoles (50 %) survived, whereas in the lowest treatment level 16 tadpoles (53.5 %) and in the medium treatment level 17 tadpoles (56.6 %) survived to the end of the experiment (Figure 9). The survival rate of the tadpoles from the highest treatment level (purple line) showed a major decline after just one day with only one surviving tadpole (3,3%) for the remainder of the experiment (Figure 9).

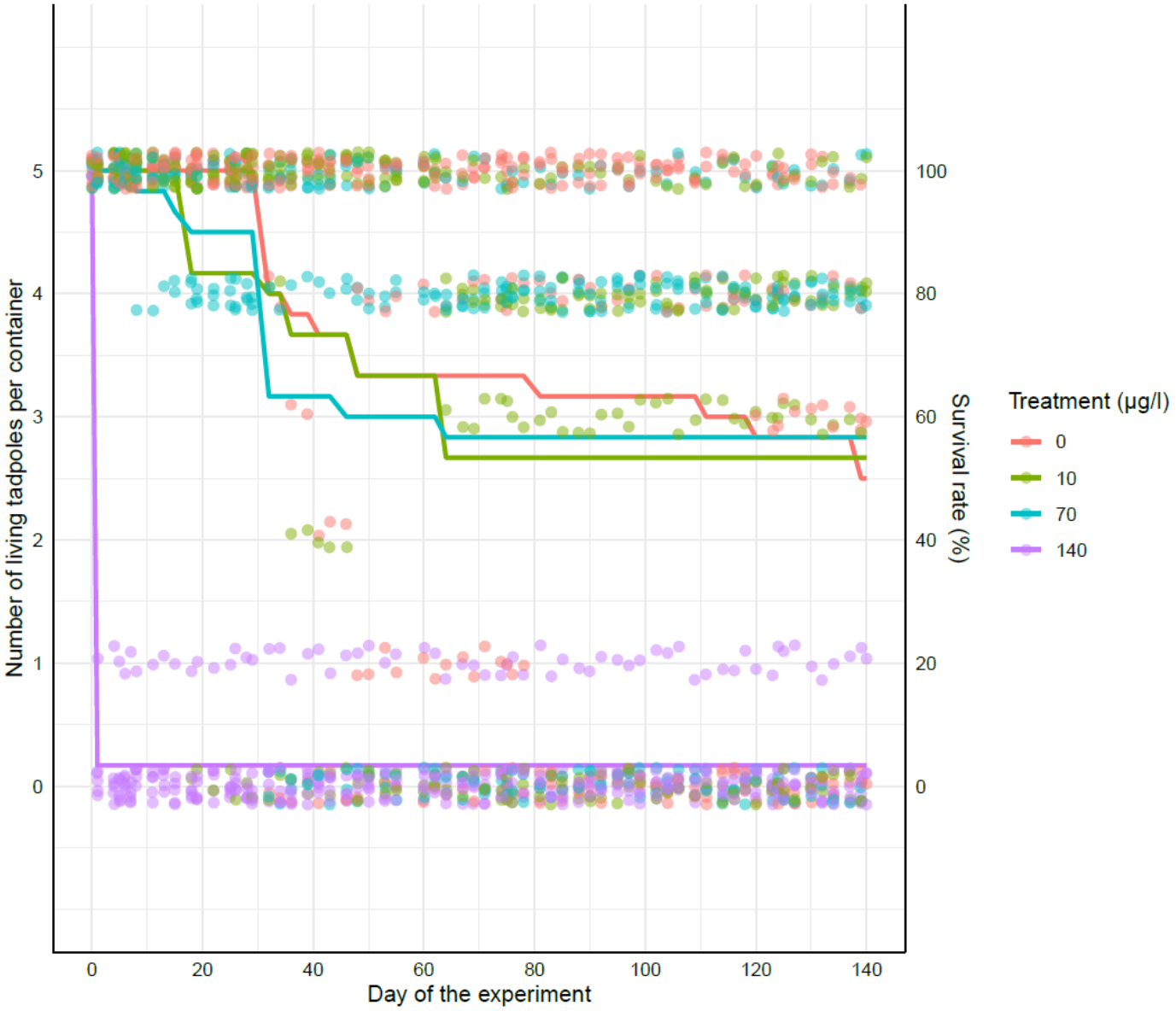

Figure 9: Survival of the tadpoles per container and for each treatment. *Dots represent the number of living tadpoles for a particular container at a specific time. The lines show the survival rate of the respective treatments.*

We then fitted a logistic mixed model (LMM) to predict the survival of the tadpoles with the treatment and the day of the experiment. The model included the container as a random effect. The analysis (Table 3) showed a statistically significant correlation between survival and the different treatment levels ($p<0.001$) and a significant interaction between treatment and

day in experiment (p<0.001). Higher copper levels led to earlier and higher rates of mortality in *B. viridis* larvae.

Table 3: Effects of the treatment on the survival of green toad tadpoles using an LMM.

Parameter	Coefficient	95% CI	z	p	Std. Coef.	Std. Coef. 95% CI	Fit
Intercept	7.73	[5.52, 9.93]	6.87	< .001	1.05	[-0.45, 2.54]	
Treatment	-0.07	[-0.10, -0.04]	-4.97	< .001	-2.73	[-4.21, -1.24]	
day in experiment	-0.06	[-0.07, -0.05]	-16.82	< .001	-1.75	[-1.88, -1.62]	
Treatment × day in experiment	0.000317	[0.00, 0.00]	5.75	< .001	0.76	[0.62, 0.89]	
R2 (conditional)							0.88
R2 (marginal)							0.40

Total length and body to tail ratio

The average total length increase per container and per treatment was measured over a period of 64 days (Figure 10).

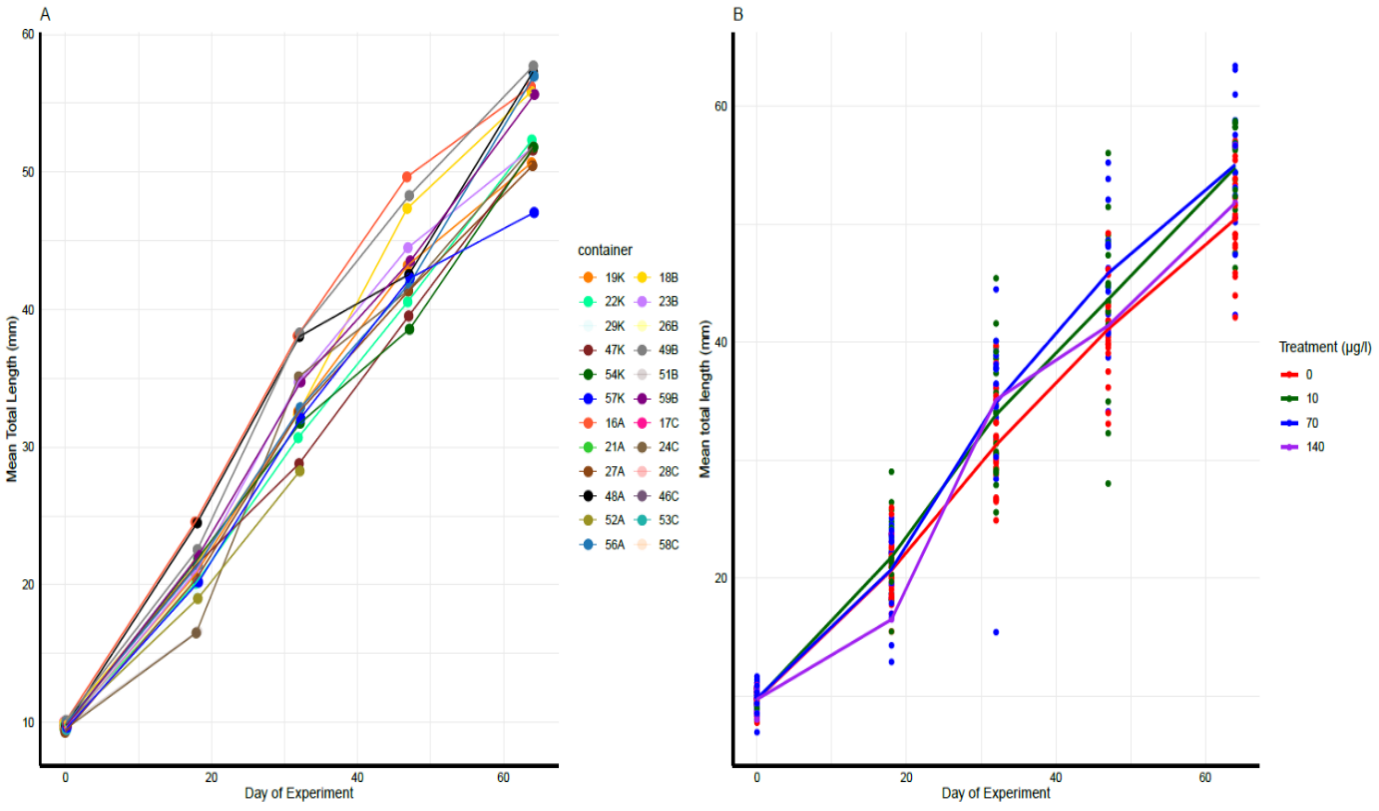

Figure 10: Total length increase of the tadpoles over time. *(A) Mean total length increase per container over time. (B) Mean total length increase per treatment over time.*

We then fitted a linear mixed model to predict the total length of the tadpoles with treatment and the day of the experiment. The model included the container as a random effect. The analysis (Table 4) showed no statistically significant correlation between the treatment levels and the total length of the tadpoles ($p>0.05$). However, there was a significant interaction between the treatments and the day of the experiment ($p=0.011$) on the total length of the tadpoles. With increasing copper concentrations, the tadpoles grew faster and longer in total length when compared to the control group (Figure 11A).

Table 4: Effects of the treatment and the day of the experiment on the total length of the tadpoles using a GLMM.

Parameter	Coefficient	95% CI	z	p	Std. Coef.	Std. Coef. 95% CI	Fit
Intercept	9.73	[8.71, 10.76]	18.55	< .001	0.00284	[-0.04, 0.04]	
Treatment	-0.0012936	[-0.02, 0.01]	-0.18	0.856	0.04	[0.00, 0.08]	
Day of experiment	0.68	[0.65, 0.70]	57.60	< .001	0.97	[0.94, 0.99]	
Treatment × Day of experiment	0.0005937	[0.00, 0.00]	2.54	0.011	0.04	[0.01, 0.06]	
R2 (conditional)							0.95
R2 (marginal)							0.94

We then also fitted a linear mixed model to predict the body to tail ratio of the tadpoles with treatment and the day of the experiment. The model included the container as random effect. The analysis (Table 5) also showed no significant correlation between treatment levels and the body to tail ratio ($p>0.05$). However, there was a highly significant interaction between treatment and day of the experiment ($p=0.00379$) on the body to tail ratio of the tadpoles. With increasing copper concentrations, the body to tail ratio of the tadpoles decreased more rapidly over a period of 64 days after the start of the experiment (Figure 11B).

Table 5: Effects of the treatment and the day of the experiment on the body to tail ratio of the tadpoles using a GLMM.

Parameter	Coefficient	95% CI	z	p	Std. Coef.	Std. Coef. 95% CI	Fit
Intercept	0.38	[0.38, 0.39]	220.93	< .001	-0.04	[-0.15, 0.07]	
Treatment	-0.0000228	[0.00, 0.00]	-0.95	0.344	-0.21	[-0.33, -0.10]	
Day of experiment	-0.000345	[0.00, 0.00]	-7.42	< .001	-0.55	[-0.64, -0.46]	
Treatment × Day of experiment	-0.00000254	[0.00, 0.00]	-2.89	0.004	-0.14	[-0.24, -0.05]	
R2 (marginal)							0.30

Therefore, the increase in length in the tadpoles (Figure 11A) is due to the increased growth of the tail rather than the body and head region and is shown strongest in the higher treatments (Figure 11B).

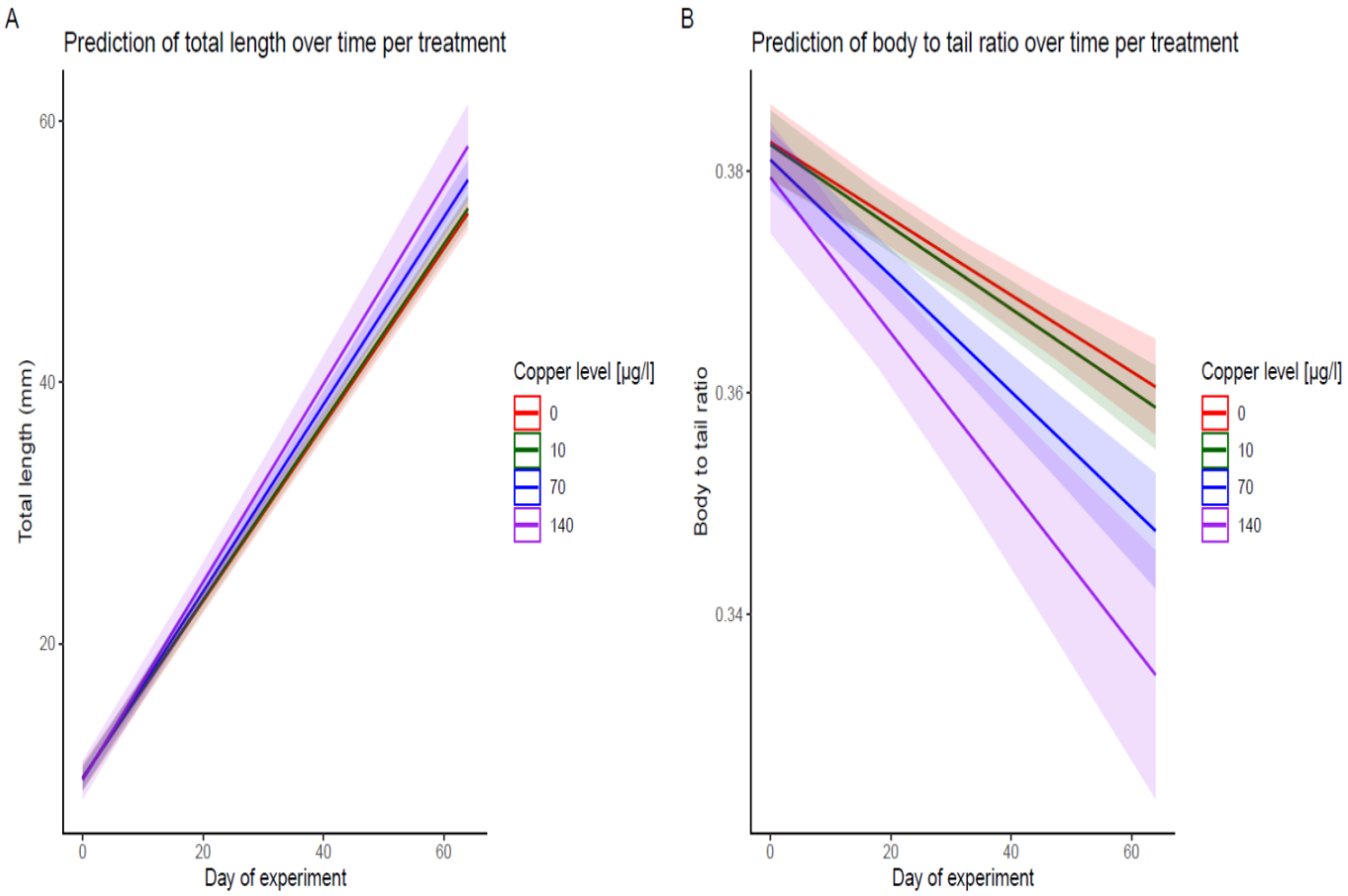

***Figure 11: Predictions of the total length (A) and body to tail ratio (B) of the tadpoles** from different treatments over the duration of 64 days.*

Centroid size

On average, the tadpoles from the lowest and medium copper treatment showed a greater increase in centroid size than those from the control group (Figure 12). The tadpoles from the lowest treatment containers (16A, 48A) and middle treatment containers (18B, 49B) showed the greatest increase in centroid size (Figure 13). These are also the tanks in which the tadpoles developed fastest (Figure 15, Figure 16).

We fitted linear mixed models to predict the tadpoles' centroid sizes from the lateral (Table 6) and dorsal (Table 7) view with the treatment and the day of the experiment. The models included the container as a random effect. Within the models, no significant treatment effects on the centroid size from the lateral view ($p = 0.600$) and on the centroid size from the dorsal view were found ($p = 0.792$).

Table 6: Effects of the treatment and the day of the experiment on the centroid size (lateral view) of the tadpoles using a LMM.

Parameter	Coefficient	95% CI	z	p	Std. Coef.	Std. Coef. 95% CI	Fit
Intercept	3.32	[2.65, 4.00]	9.59	< .001	-0.02	[-0.16, 0.12]	
Treatment	0.00423	[-0.01, 0.02]	0.52	0.600	0.08	[-0.04, 0.21]	
Day of experiment	0.12	[0.11, 0.13]	18.59	< .001	0.87	[0.80, 0.94]	
Treatment × Day of experiment	0.0000268	[0.00, 0.00]	0.18	0.855	0.006.60	[-0.06, 0.08]	
R2 (conditional)							0.85
R2 (marginal)							0.79

Table 7: Effects of the treatment and the day of the experiment on the centroid size (dorsal view) of the tadpoles using a LMM.

Parameter	Coefficient	95% CI	z	p	Std. Coef.	Std. Coef. 95% CI	Fit
Intercept	2.00	[1.57, 2.43]	9.08	< .001	-0.02	[-0.15, 0.12]	
Treatment	0.00134	[-0.01, 0.01]	0.26	0.792	0.05	[-0.07, 0.18]	
Day of experiment	0.08	[0.07, 0.09]	20.15	< .001	0.89	[0.82, 0.95]	
Treatment × Day of experiment	0.0000216	[0.00, 0.00]	0.24	0.813	0.00800	[-0.06, 0.07]	
R2 (conditional)							0.86
R2 (marginal)							0.81

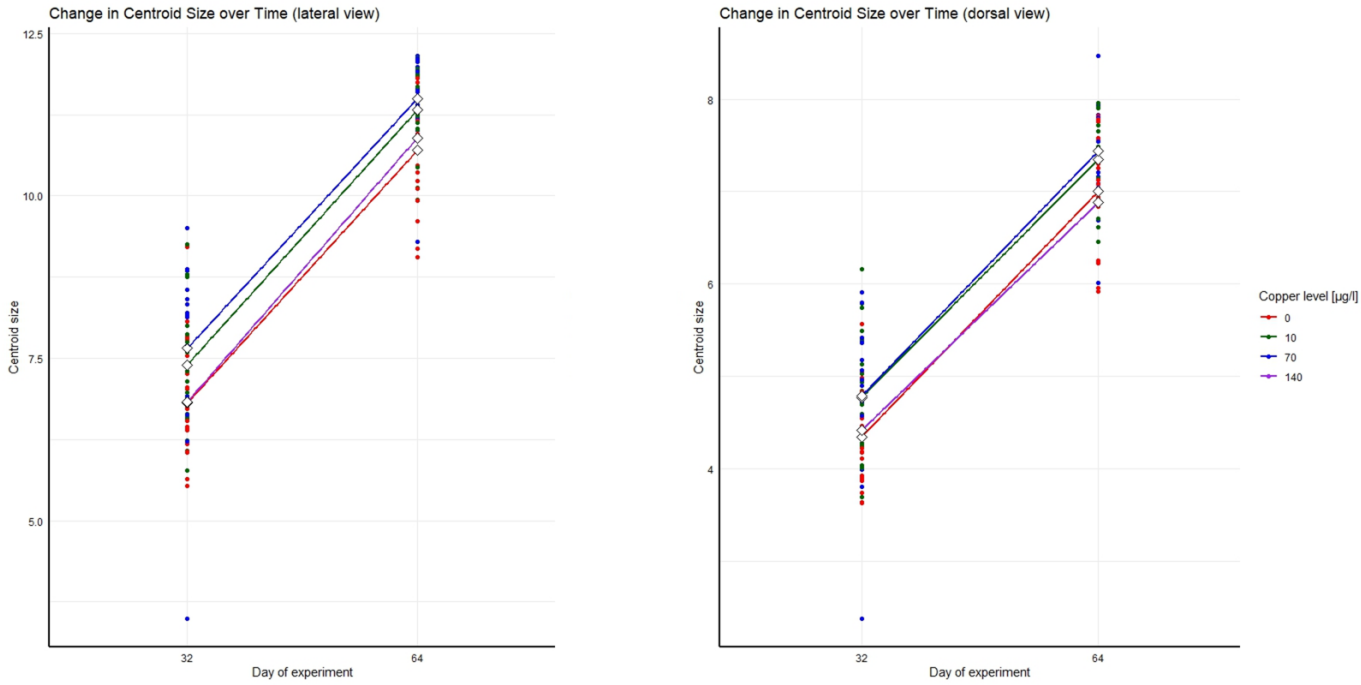

Figure 12: Mean centroid sizes for each treatment. *Left side shows the mean centroid sizes (white diamonds) for each treatment from the lateral view; Right side shows the mean centroid sizes (white diamonds) for each treatment from the dorsal view. The raw data points are shown as colored points depending on the treatment.*

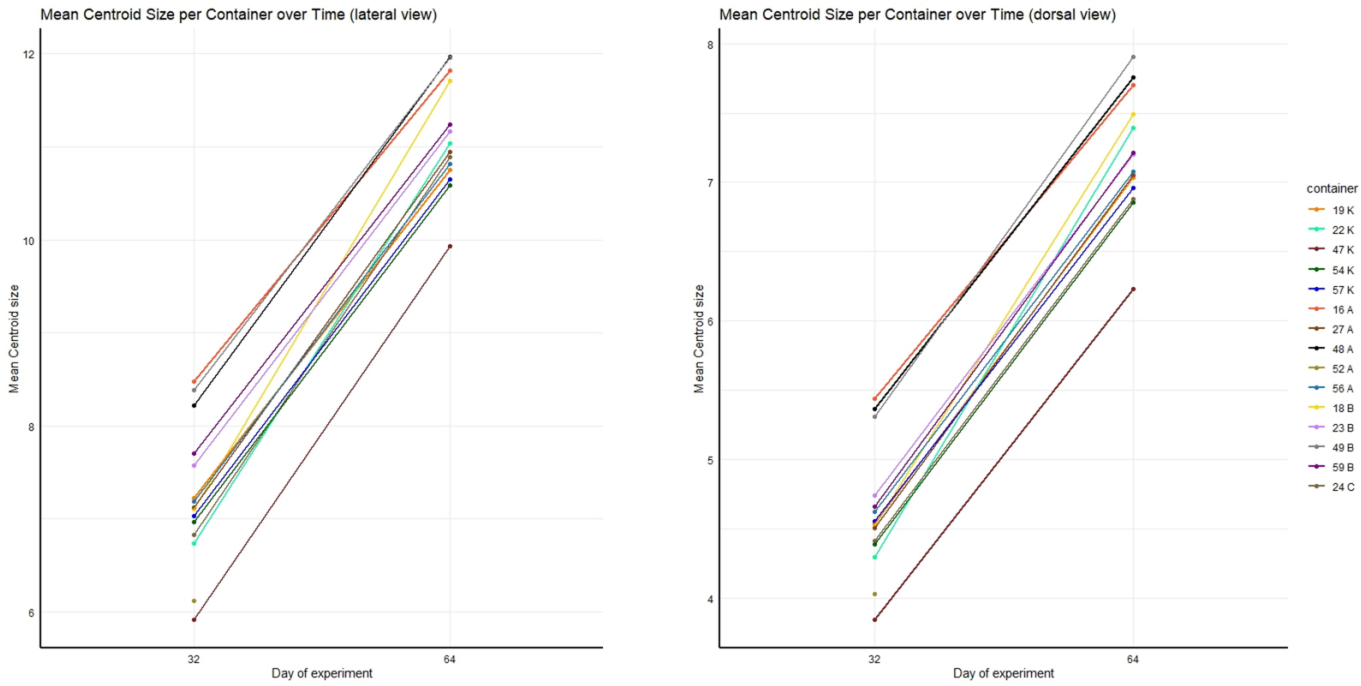

Figure 13: Mean centroid sizes for each container*. Left side shows the mean centroid size from the lateral view; Right side shows the mean centroid size from the dorsal view.*

Gosner stages

After 32 days, the tadpoles from the lowest treatment were on average more developed than those from the control group (Figure 14, Figure 15). Tadpoles from the middle treatment were again on average more developed than those from the control and lowest treatment. The only surviving tadpole from the highest treatment showed Gosner stage 31 after 32 days.

After 64 days, the tadpoles from the lowest treatment were on average more developed than those in the control group (Figure 14, Figure 15). Tadpoles from the middle treatment were on average more developed than those from the control group, but less developed than those from the lowest treatment. The only surviving tadpole from the highest treatment showed Gosner stage 36 after 64 days.

We then fitted a linear mixed model to predict the Gosner stages with the treatment and the time in the experiment. The model included the container as a random effect. Within this model (Table 8), no significant treatment main effect was found ($p = 0.402$). Additionally, no significant interaction effect between treatment and day of the experiment was found ($p = 0.648$).

Table 8: Effects of the treatment and the day of the experiment on the Gosner stages of the tadpoles using a LMM.

Parameter	Coefficient	95% CI	z	p	Std. Coef.	Std. Coef. 95% CI	Fit
Intercept	25.83	[24.98, 26.68]	59.55	< .001	0.00130	[-0.12, 0.12]	
Treatment	0.00854	[-0.01, 0.03]	0.84	0.402	0.05	[-0.06, 0.17]	
Day	0.16	[0.14, 0.17]	19.05	< .001	0.89	[0.82, 0.96]	
Treatment × Day	0.0000870	[0.00, 0.00]	-0.46	0.648	-0.02	[-0.09, 0.06]	
R2 (conditional)							0.84
R2 (marginal)							0.80

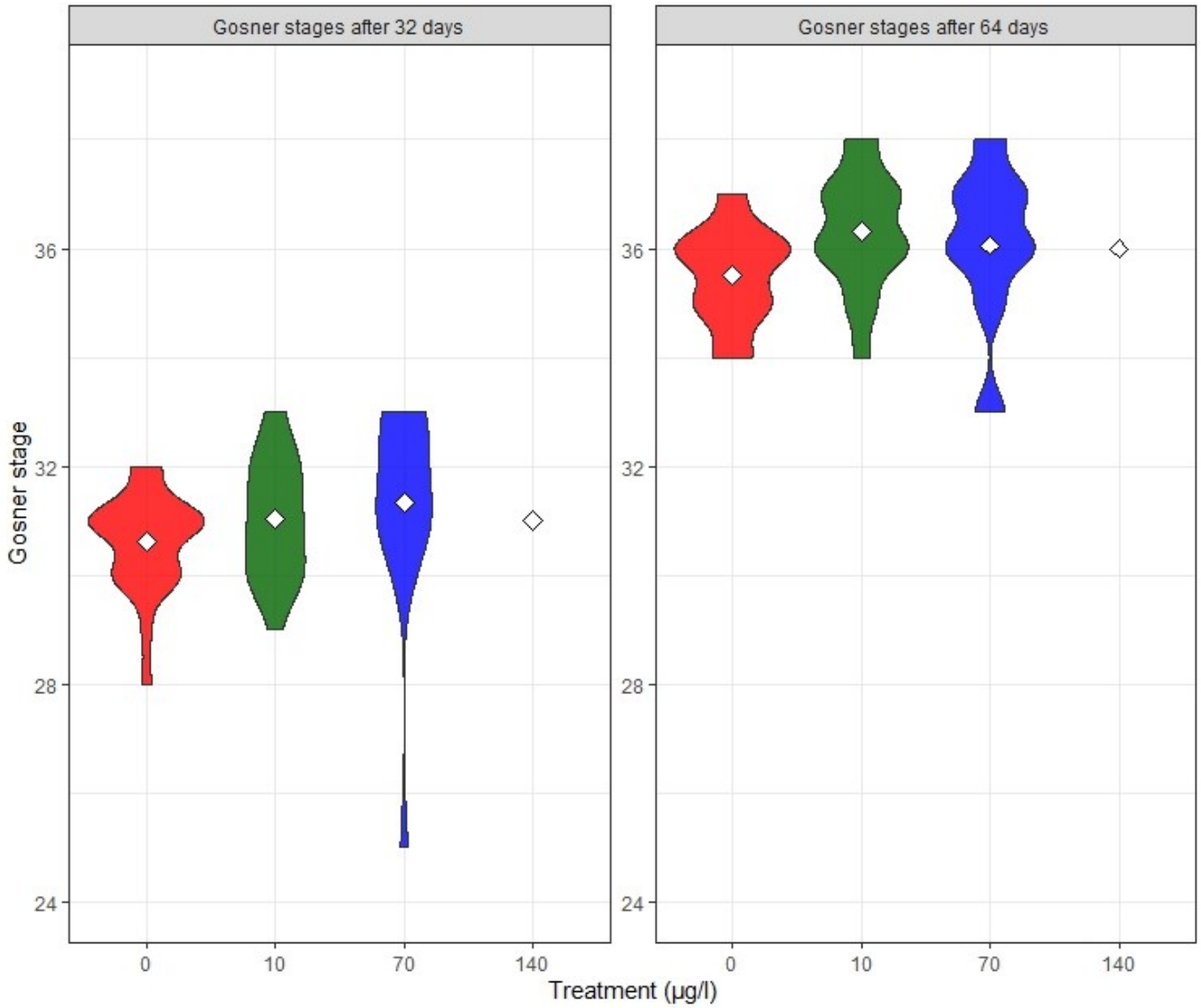

Figure 14: Distribution of the developmental stages (Gosner stages) within the treatments shown as violin plots*. The means for each treatment are shown as white diamonds.*

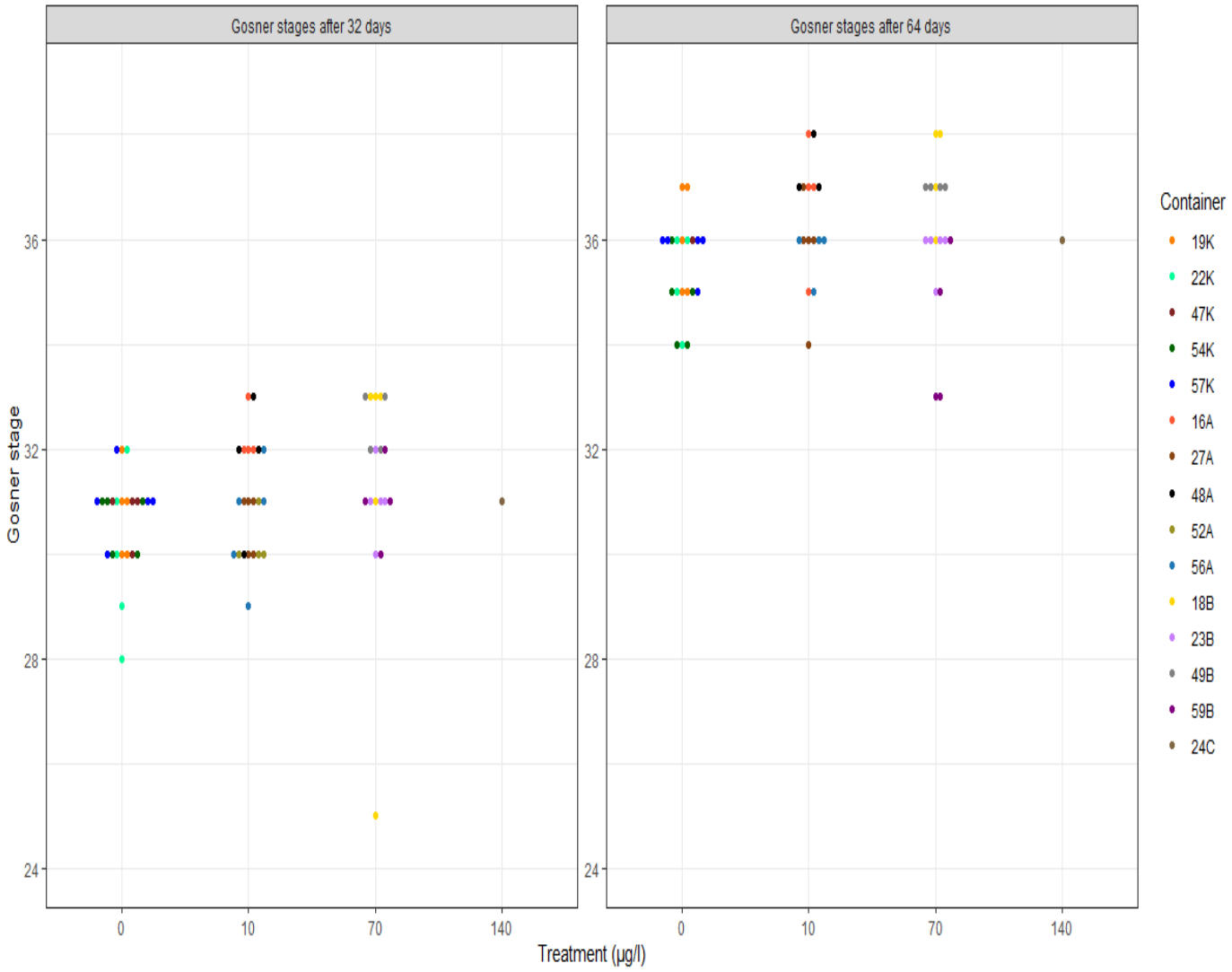

Figure 15: Detailed distribution of the developmental stages (Gosner stages) within the treatments. *Each data point represents a tadpole and the color the corresponding container.*

Time of metamorphosis

The metamorphosis time points (reaching Gosner stage 42) of a total of 49 individuals were measured (Figure 16). 29 individuals did not reach Gosner stage 42 until the end of the experiment (13 from the control group (K), 7 from the lowest treatment (A), 8 from the middle treatment (B) and 1 from the highest treatment (C).

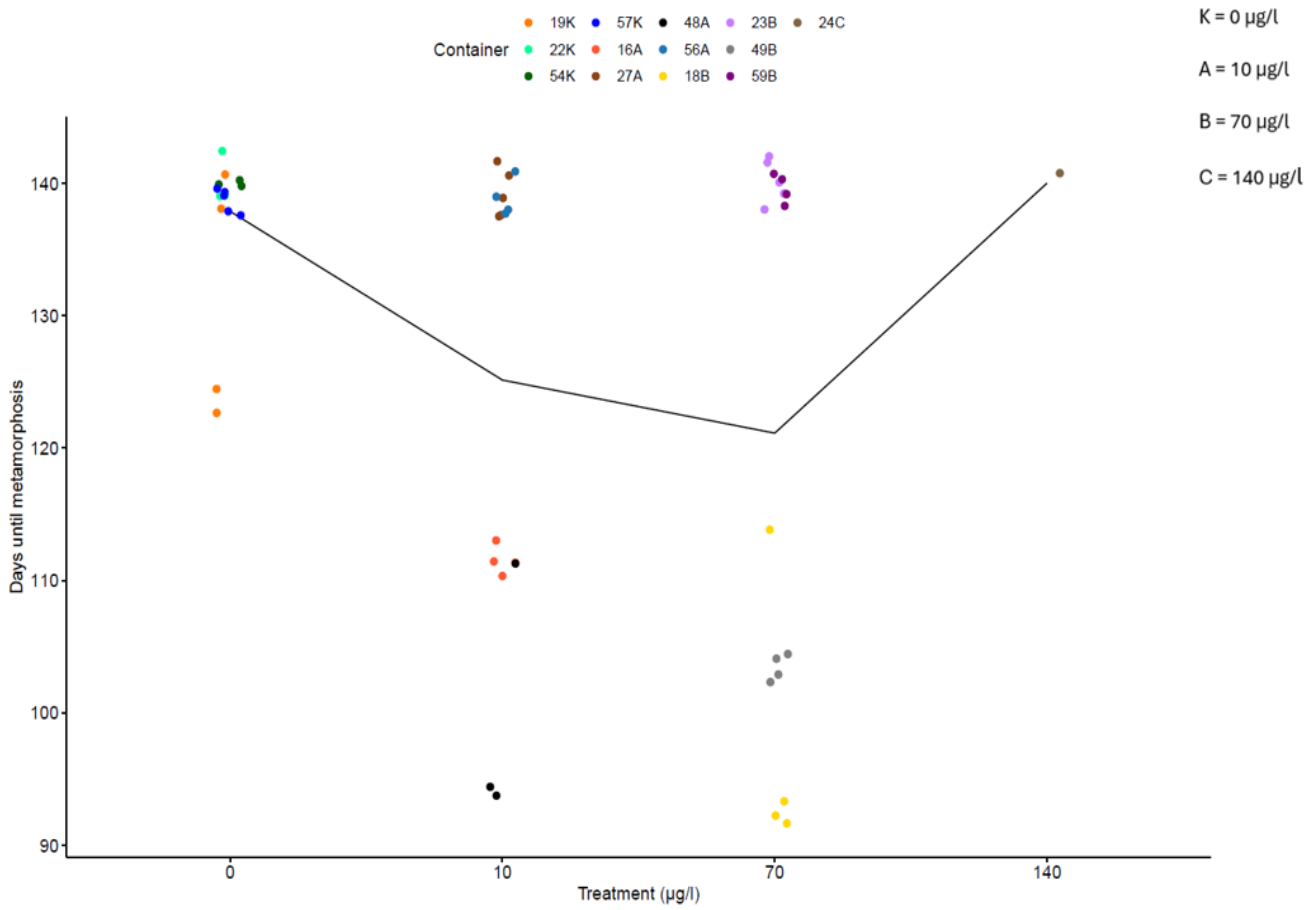

Figure 16: Time of metamorphosis in relation to the copper concentration. *The data points show the individual metamorphosis times of the tadpoles per treatment. The letters in the container label refer to the associated treatment level (K = 0 µg/l, A = 10 µg/l, B = 70 µg/l, C = 140 µg/l). Individuals from the same container are marked with the same color. The black line shows the average time of metamorphosis for every treatment.*

For potential treatment effects, a linear mixed model was fitted to predict the day of metamorphosis with the different treatments. The model included the container as a random effect. Within this model, no statistically significant correlation was found between the time of metamorphosis and the copper concentrations the tadpoles were raised in (Table 9).

Table 9: Effects of the treatment on the time of metamorphosis of green toad tadpoles using a GLMM.

Parameter	Coefficient	95% CI	z	p	Std. Coef.	Std. Coef. 95% CI	Fit
Intercept	128.76	[116.74, 140.79]	20.98	< .001	0.00381	[-0.52, 0.53]	
Treatment	-0.03	[-0.25, 0.19]	-0.28	0.778	-0.06	[-0.50, 0.38]	
R2 (conditional)							0.93
R2 (marginal)							0.00405

Mass after Metamorphosis

The mass of 30 individuals was measured after metamorphosis (Figure 17A). A linear mixed model was then fitted to predict the mass of the animals after metamorphosis with the treatments and the development time (Figure 17B). The model included the container as random effect. Within this model (Table 10), the treatment main effect (p=0.063) and the interaction between treatment and the time until metamorphosis (p=0.068) was not significant. However, a trend was observed where individuals that metamorphosed before day 134 showed a lower mass as the treatment levels increased. With later metamorphosis time points, the masses of the animals from all treatments approached and intersected each other on day 134. Individuals that metamorphosed after day 134 showed a higher mass as the treatment levels increased.

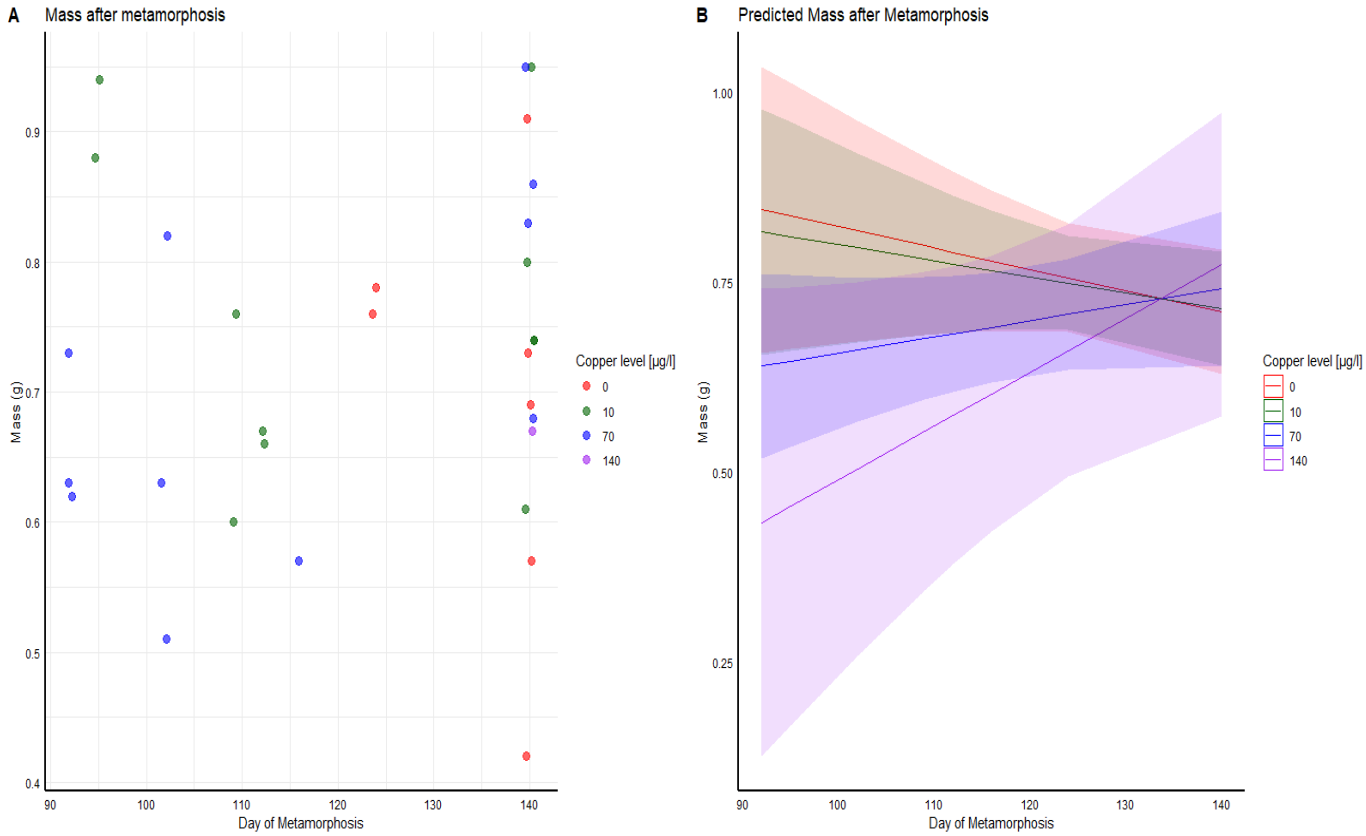

Figure 17: Mass after metamorphosis. *Mass (A) of the individual animals after metamorphosis. Data points are colored by treatment. (B) shows the prediction of the animals' mass with the day of metamorphosis for every treatment.*

Table 10: Effects of the treatment on the tadpole mass after metamorphosis using a GLMM.

Parameter	Coefficient	95% CI	z	p	Std. Coef.	Std. Coef. 95% CI	Fit
Intercept	1.10	[0.49, 1.71]	3.55	< .001	0.07	[-0.30, 0.44]	
Treatment	-0.00944	[-0.02, 0.00]	-1.86	0.063	-0.22	[-0.65, 0.21]	
develop time	-0.00280	[-0.01, 0.00]	-1.18	0.240	-0.06	[-0.53, 0.41]	
Treatment × develop time	0.0000706	[0.00, 0.00]	1.82	0.068	0.38	[-0.03, 0.79]	
R2 (conditional)							0.23
R2 (marginal)							0.13

Effects of copper on shape

Principal component analyses of the Procrustes shape coordinates were performed for the dorsal and lateral views on days 32 and 64 (Figure 18, Table 11). The first two principal components of Dorsal Day 64 were discarded, as these only showed artefacts caused by the bent tails of the tadpoles. Therefore, only the third principal component was considered.

Table 11: Summary of variances explained by the principal components after PCA of the Procrustes shape coordinates.

PCA Dorsal Day 32	PCA Lateral Day 32	PCA Dorsal Day 64	PCA Lateral Day 64
PC 1: 45.61%	PC 1: 51.18%	PC 1: 28.58% (discarded)	PC 1: 46.96%
PC 2: 20.1%	PC 2: 15.97%	PC 2: 25.1% (discarded)	PC 2: 24.05%
		PC 3: 24.13%	

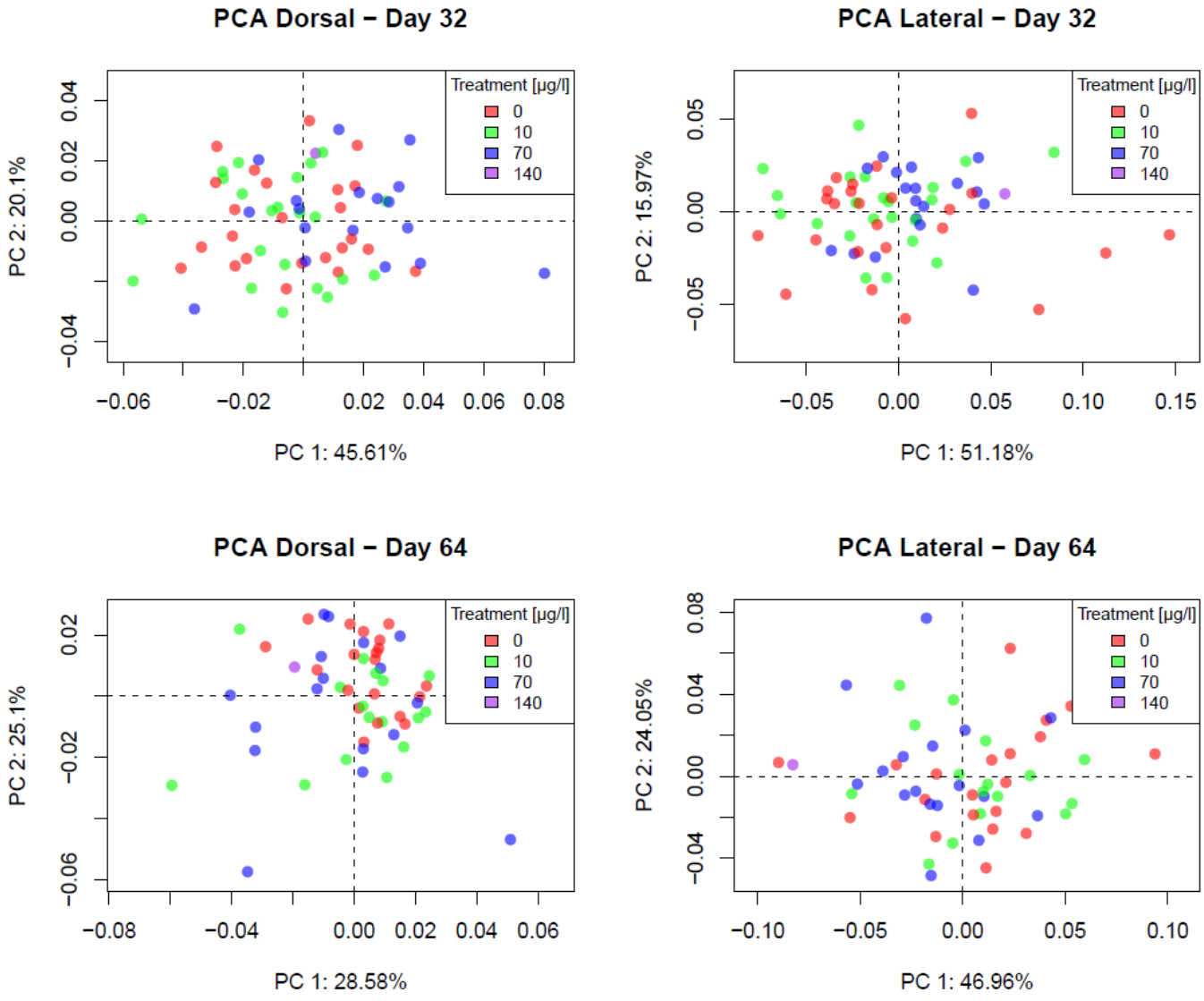

Figure 18: Principal component analysis (PCA) of the Procrustes shape coordinates *(from lateral and dorsal view, showing only PC1 and PC2).*

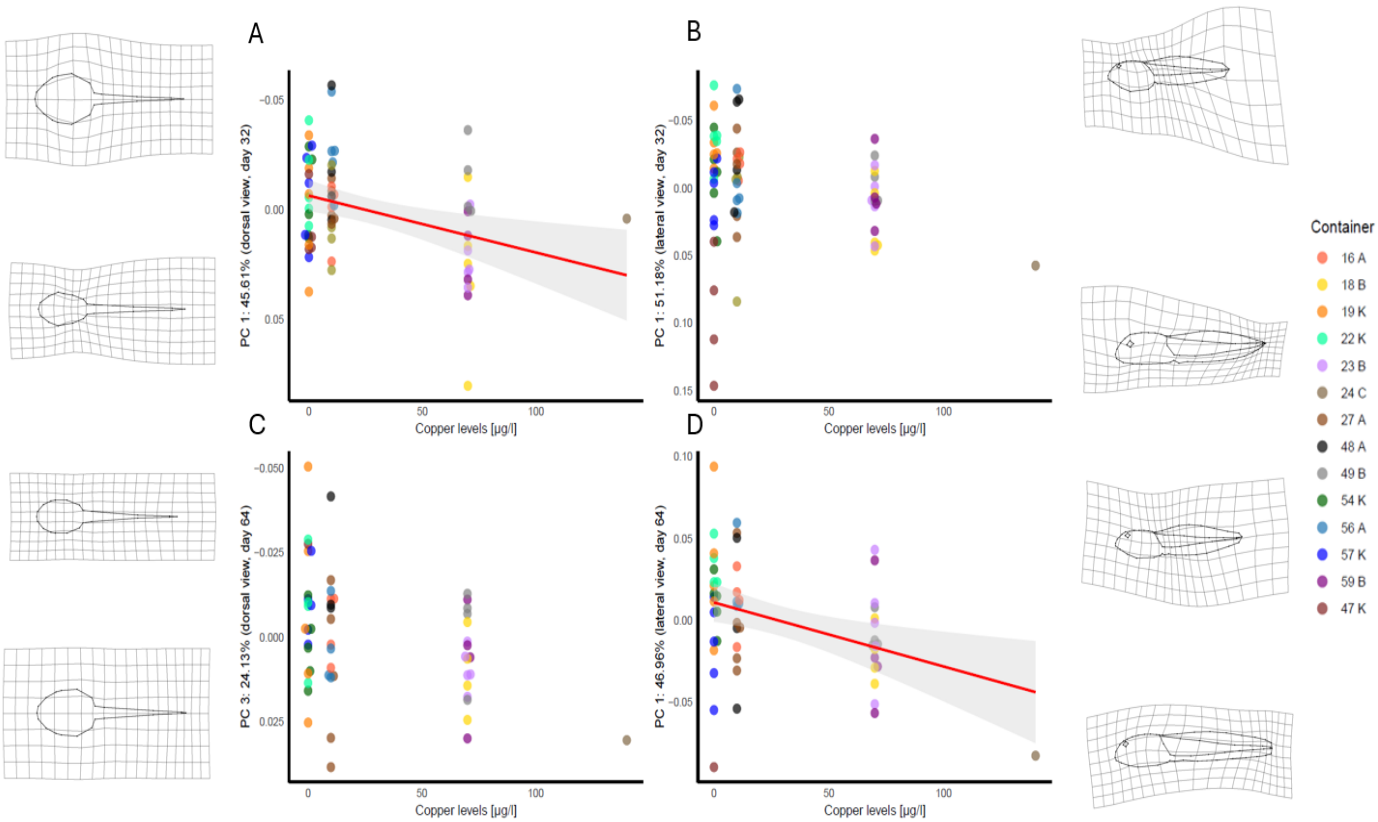

Figure 19: Correlation between copper levels and the shape (loadings) of the tadpoles using principal component regression (PCR). *The shape differences between the maximum and minimum values of the principal component loadings are visualized as deformation grids respectively. (A) shows the principal component regression between the copper levels and the loadings of PC 1 at day 32 from the dorsal view. (B) shows the principal component regression between the copper levels and the loadings of PC 1 at day 32 from the lateral view. (C) shows the principal component regression between the copper levels and the loadings of PC 3 at day 64 from the dorsal view. (D) shows the principal component regression between the copper levels and the loadings of PC 1 at day 64 from the lateral view.*

Principal component regressions (PCR) have been performed to show how increasing treatment levels influence the tadpole shapes (Figure 19). The shapes differences are illustrated as deformation grids. Linear mixed models were used to predict principal component loadings as dependent variables with treatment, and centroid size as independent variables. The model included the containers as a random variable. The first principal component for the dorsal view on day 32 (Figure 19A) accounts for over 45.61% of the observed shape variation. The rumps of the tadpoles become significantly narrower as the copper concentration increases. Also, the tails of the tadpoles become longer with higher copper levels, which results in a reduction of the body to tail ratio ($p = 0.004$) (Figure 19A).

The first principal component for the lateral view on day 64 accounts for 46.96% of the observed shape variation (Figure 19D). The tadpoles become significantly narrower with increasing copper concentrations, as the dorsal and ventral fins become smaller (Figure 19D). The torso also becomes flatter, and the mouth gets more subterminal with increasing copper

concentrations (p = 0.009) (Figure 19D). No significant shape changes in correlation to the copper levels (p>0.05) could be found for the lateral view on day 32 (Figure 19B).

For the dorsal view on day 64 (Figure 19C) the first and second principal components were discarded due to artefacts caused by the bent tails and the subsequent mirroring process. The third principal component which still accounts for 24.13% of the shape variation was therefore used, but no correlation with the copper concentration was found (p>0.05).

Behavior

Room and water temperatures during the behavioral tests

Both runs lasted a total of 40 minutes, including the 30-minute exploration phase and subsequent external stimulations. The room temperatures were recorded in 15-minute intervals. The first run lasted from 14:02 to 14:42 and the second run from 15:20 to 16:00. The temperatures at 14:02, 14:42 and 15:20 were obtained by interpolation. In the last 12 minutes of the first run, the temperature increased from 25.9 degrees Celsius to 26.2 degrees Celsius (Table 12). During the duration of the second run, the temperature increased from 26.3 degrees Celsius to 26.6 degrees Celsius (Table 12).

Table 12: Room temperatures during the first and second run of the behavioral experiments.

	Time	Temperature
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	14:00	25.9°C
Run 1 Start	14:02	25.9°C
	14:15	25.8°C
	14:30	25.9°C
Run 1 End	14:42	26.2°C
	14:45	26.3°C
	15:15	26.0°C
Run 2 Start	15:20	26.3°C
	15:30	26.4°C
	15:45	26.6°C
Run 2 End	16:00	26.6°C

Activity during 30-minute exploration phase

We fitted a linear mixed model to predict the activity of the tadpoles with the treatment, test duration (30 min), testing round and the position of the arena (x and y coordinates). The model included the different test arenas as a random effect. Within this model (Table 13), no statistically significant treatment main effect on the activity of the tadpoles was found (p>0.05). However, the test duration (p< 0.001), the testing round (p = 0.022) and the arena position (y coordinate; p = 0.001) had significant influence on the activity.

Furthermore, there was a significant interaction effect between treatment and test duration (p < 0.001), with the higher treatments leading to higher activities at the beginning of the

experiment (Figure 20). This effect disappears towards the end of the experiment (Figure 20). Additionally, there were significant interactions between the predictor variables treatment, test duration and testing round on the activity of the tadpoles ($p < 0.001$) (Table 13).

Table 13: Effects of the treatment, test duration, testing round and arena position on tadpoles' activity using a GLMM.

Parameter	Coefficient	95% CI	z	p	Std. Coef.	Std. Coef. 95% CI	Fit
Intercept	0.69	[-1.71, 3.09]	0.56	0.573	0.00853	[-0.02, 0.04]	
Treatment	-0.01	[-0.05, 0.03]	-0.61	0.541	0.02	[-0.02, 0.06]	
Duration	0.00256	[0.00, 0.00]	9.80	< .001	0.08	[0.08, 0.08]	
Testing round	1.83	[0.27, 3.40]	2.29	0.022	0.09	[0.05, 0.14]	
x	0.000964	[0.00, 0.00]	1.07	0.285	0.02	[-0.02, 0.07]	
y	-0.00272	[0.00, 0.00]	-3.25	0.001	-0.06	[-0.09, -0.02]	
Treatment × Duration	-0.0000297	[0.00, 0.00]	-5.90	< .001	-0.01	[-0.02, -0.01]	
Treatment × Testing round	0.02	[-0.01, 0.04]	1.22	0.221	0.04	[0.01, 0.08]	
Duration × Testing round	-0.0000938	[0.00, 0.00]	-0.57	0.568	0.00614	[0.00, 0.01]	
(Treatment × Duration) × Testing round	0.0000146	[0.00, 0.00]	3.88	< .001	0.01	[0.01, 0.02]	
R2 (conditional)							0.02
R2 (marginal)							0.02

Distance from the arena wall during 30-minute exploration phase

We also fitted a linear mixed model to predict the distance from the arena wall with treatment, test duration (30 min), testing round and the arena position (using x and y coordinates).

The model included the different test arenas as random effect. Within this model (Table 14), no statistically significant treatment effect was found on the distance of the tadpoles from the arena wall ($p>0.05$). However, the test duration ($p < 0.001$) had significant influence on the tadpoles' distance to the wall. There were also significant interactions between the treatments and the test duration ($p < 0.001$), with the higher treatments leading to an increased

distance from the arena wall (Figure 20). This effect decreased with increasing test duration. Additionally, there were significant interactions between the test duration and testing round ($p < 0.001$), as well as between test duration, treatment, and testing round ($p < 0.001$) (Table 14). The arena position (x coordinate) also had significant influence on the tadpoles' distance from the wall.

Table 14: Effects of the treatment, test duration, testing round and arena position on tadpoles' distance from the arena wall using a GLMM.

Parameter	Coefficient	95% CI	z	p	Std. Coef.	Std. Coef. 95% CI	Fit
Intercept	16.81	[13.77, 19.86]	10.83	< .001	0.01	[-0.03, 0.05]	
Treatment	0.02	[-0.02, 0.07]	0.98	0.326	0.03	[-0.01, 0.08]	
Duration	0.00223	[0.00, 0.00]	8.51	< .001	-0.01	[-0.02, -0.01]	
Testing round	-1.72	[-3.71, 0.26]	-1.70	0.089	-0.07	[-0.13, -0.01]	
x	0.00375	[0.00, 0.01]	3.25	0.001	0.09	[0.04, 0.14]	
y	0.000801	[0.00, 0.00]	0.75	0.453	0.02	[-0.03, 0.06]	
Treatment × Duration	-0.0000834	[0.00, 0.00]	-16.49	< .001	-0.00464	[-0.01, 0.00]	
Treatment × Testing round	-0.00582	[-0.04, 0.03]	-0.34	0.736	0.06	[0.01, 0.11]	
Duration × Testing round	-0.00167	[0.00, 0.00]	-10.11	< .001	-0.00176	[-0.01, 0.00]	
(Treatment × Duration) × Testing round	0.00005.35	[0.00, 0.00]	14.13	< .001	0.04	[0.03, 0.04]	
R2 (conditional)							0.01
R2 (marginal)							0.00823

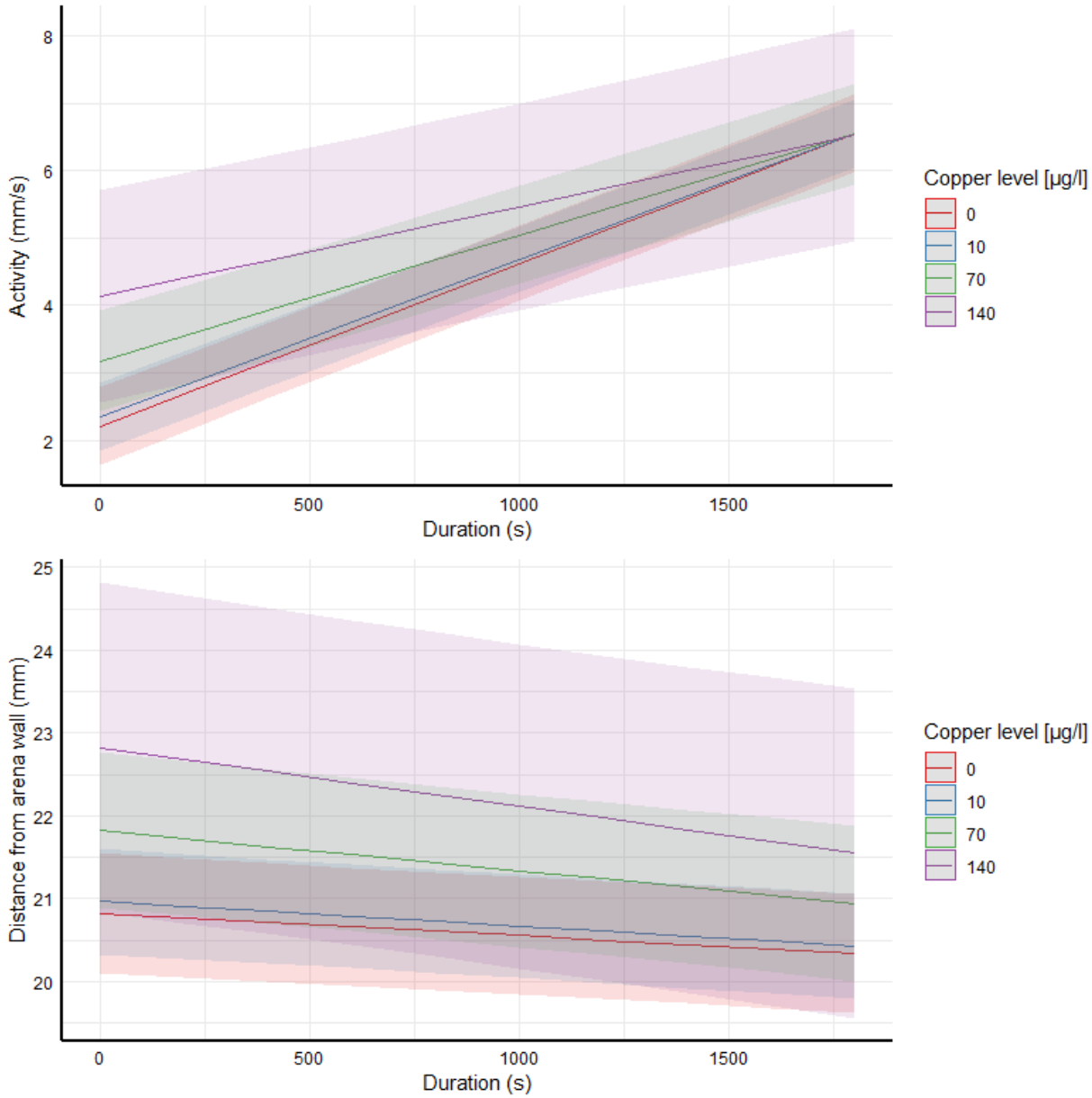

Figure 20: Predictions for the tadpole activity (top) and their distance from the arena wall (bottom) for each treatment level.

Total movement duration after external stimuli

We fitted a linear mixed model to predict the total movement duration of the tadpoles with treatment, the number of escape stimuli and the position of the arena (x and y coordinates). The model included the arena number as a random effect. The analysis (Table 15) showed no statistically significant effects of the treatment, the escape stimulus, and the arena position on the total movement duration ($p>0.05$). However, there was a significant interaction between the predictor variables treatment and escape stimulus ($p = 0.031$), with the animals from the higher treatments showing shorter total movement durations after the first stimulus, but longer total movement durations as the number of escape stimuli increased (Figure 21).

Table 15: Effects of the treatment, escape stimulus and arena position on the tadpoles' total movement duration using a GLMM.

Parameter	Coefficient	95% CI	z	p	Std. Coef.	Std. Coef. 95% CI	Fit
Intercept	3.19	[-2.02, 8.40]	1.20	0.230	0.00000174	[-0.31, 0.31]	
Treatment	-0.03	[-0.08, 0.02]	-1.26	0.209	0.20	[-0.13, 0.53]	
Escape stimulus	-0.11	[-1.11, 0.89]	-0.22	0.824	0.15	[-0.08, 0.38]	
x	-0.0000922	[0.00, 0.00]	-0.06	0.948	-0.01	[-0.34, 0.32]	
y	0.00189	[0.00, 0.01]	1.13	0.257	0.19	[-0.14, 0.53]	
Treatment × Escape stimulus	0.02	[0.00, 0.04]	2.15	0.031	0.25	[0.02, 0.49]	
R2 (conditional)							0.35
R2 (marginal)							0.15

Total movement length after external stimuli

We fitted a linear mixed model to predict total movement length of the tadpoles with treatment, the number of escape stimuli and the position of the arena (x and y coordinates). The model included the arena number as a random effect. The analysis (Table 16) showed no statistically significant effects of the treatment, the escape stimulus, and the arena position on the total movement length ($p>0.05$). However, there was a marginally significant interaction between the predictor variables treatment and escape stimulus ($p = 0.0593$), with the animals from the higher treatments showing less total movement lengths after the first stimulus, but higher total movement lengths as the number of escape stimuli increased (Figure 21).

Table 16: Effects of the treatment and escape stimulus on the tadpoles' total movement length using a GLMM.

Parameter	Coefficient	95% CI	z	p	Std. Coef.	Std. Coef. 95% CI	Fit
Intercept	533.47	[-544.61, 1611.55]	0.97	0.332	0.00000126	[-0.28, 0.28]	
Treatment	-6.92	[-17.63, 3.80]	-1.27	0.206	0.15	[-0.15, 0.44]	
Escape stimulus	45.81	[-194.09, 285.71]	0.37	0.708	0.23	[-0.01, 0.48]	
x	0.29	[-0.27, 0.85]	1.00	0.316	0.15	[-0.14, 0.45]	
y	-0.04	[-0.69, 0.62]	-0.11	0.913	-0.02	[-0.32, 0.29]	
Treatment × Escape stimulus	4.63	[-0.18, 9.45]	1.89	0.059	0.24	[-0.01, 0.49]	
R2 (conditional)							0.25
R2 (marginal)							0.16

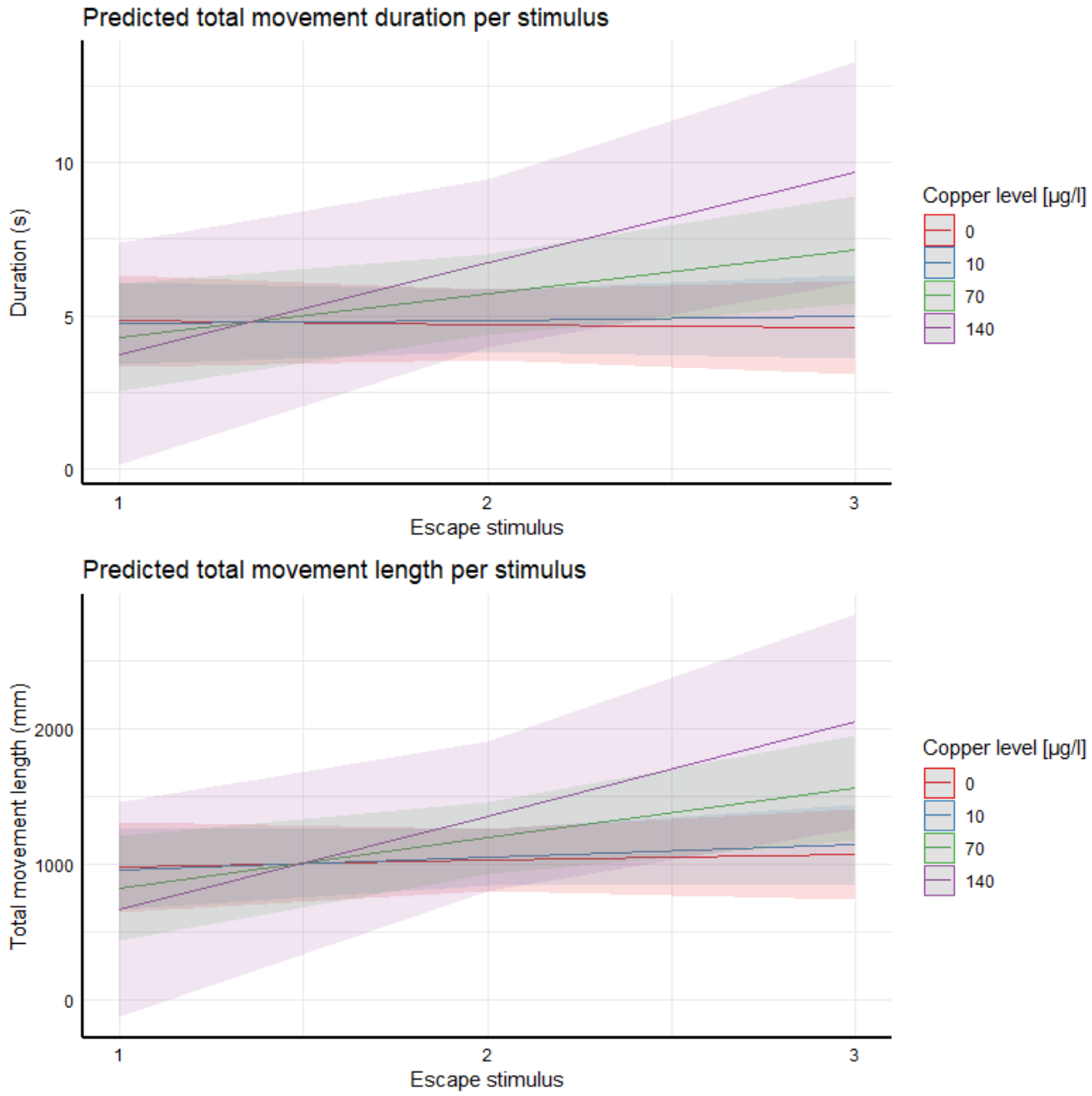

Figure 21: Predictions for the total movement duration (top) and the total movement length (bottom) of the tadpoles per stimulus for every treatment.

Observations

Over the course of the experiment, some of the individuals were unexpectedly infected with a possible fungus that spread on the tips of the tadpoles' tails (Figure 22), potentially contributing to a higher mortality rate. It is likely that this organism was introduced into the experiment with the collected spawn. However, these infections were only found in one container of the control group and in one of the lowest concentrated treatments. This was not surprising, as copper can act as a fungicide in higher concentrations.

Figure 22: Possible fungal growth on the tip of a tadpole's tail.

Discussion

The results showed that copper had a significant influence on the morphology of green toad tadpoles during their development. It was shown that there was a significant correlation between the increasing copper levels and the width of the tadpoles' rumps after 32 days (Figure 19A). With increasing copper levels, the flanks of the tadpoles tended to become narrower. This morphological change in body width could indicate a disturbed feeding behavior or metabolism (Liu et al., 2023) caused by the copper exposure. There is also a study which showed that copper disrupts the gut microbiota in *Bufo gargarizans* tadpoles, which led to impaired food digestion and energy harvest (Chai et al., 2024) and therefore to a decreased body width. Furthermore, with increasing copper levels, the tadpoles developed tail fins with decreasing depths after 64 days (Figure 19D). This change in tail morphology is associated with a reduction in the surface area of the caudal fin, which has been reported to negatively affect some components of the swimming performance of other anuran larvae (van Buskirk & McCollum, 2000). This is of course important in the presence of various predators, as a larger tail fin means better agility and manoeuvrability and therefore a higher chance of escape (Wassersug, 1989). In addition to reduced swimming performance, the decreased depth of the tail fins can also have negative consequences in other ways. A study from Caldwell (1982) suggested that a larger tail fin could serve to distract predators from the vulnerable head and body region. Larger caudal fins could therefore serve as a distraction and thus increase the tadpoles' chances of escape and survival (Caldwell, 1982). A copper-induced reduction of the caudal fin may therefore also reduce the chances of survival as the predators are less distracted from the vital body of the animals. One study from Barry (2011) has also shown that copper can inhibit the phenotypic response of *Bufo arabicus* tadpoles to predator released kairomones and therefore prevent the development of deeper tail fins and its correlated benefits.

Furthermore, it was shown that the total length of the tadpoles increased significantly with increasing copper concentrations over the duration of the experiment. At later time points in the experiment, the tadpoles from higher copper treatments showed greater total lengths when compared to those from the control group. Also, it was found that the tadpoles' body to tail ratio decreased significantly with increasing copper concentrations over the duration of the experiment. Therefore, the increase in length in the tadpoles is due to the increased growth of the tail rather than the body and head region and is shown strongest in tadpoles from higher treatments. At first glance this may appear to be a positive change in morphology, but a study investigating the relationship between swimming performance and different tail lengths in *Hyla versicolor* larvae have shown that tadpoles with longer tails do not necessary display any better swimming performances (van Buskirk & McCollum, 2000). It should be noted that large morphological variation within the individual containers could also be related to the origin of the larvae and thus to genetic or maternal influences.

After examination of the survival data, it becomes clear that the survival probabilities of the *B. viridis* larvae drop sharply with increasing copper concentrations. This result was not surprising as there are similar results from other sources (Conan et al., 2023; Gürkan & Hayretdağ, 2012). What was surprising, however, was the high number of tadpoles that died after just

one day in the highest copper treatment (59 out of 60 tadpoles). This is alarming because such copper concentrations also occur naturally in spawning waters of the green toad here in Vienna. Other heavy metals are often also present in the water, which can further increase the toxic effects of copper (Landé, 1973).

During this study, no significant correlation between the copper treatments and the time of metamorphosis was found. Some studies have already shown that copper and its interaction with other different heavy metals can delay the metamorphosis of *B. viridis* larvae (Dorchin & Shanas, 2010; Fort et al., 2022) due to its effects on the thyroid hormone action (Thambirajah et al., 2019). Although the effects of copper on the mass of the animals after metamorphosis were not significant, a trend was observed in which animals from higher treatments showed less mass after metamorphosis. This is consistent with an existing study showing that larvae of *Bufo gargarizans* show a reduction in mass at the metamorphic climax due to chronic copper exposure (C. Wang et al., 2016). Also, no significant effect of copper on the centroid size of the tadpoles was found.

During the behavioral tests, the animals from higher copper treatments also showed significantly higher activities when compared to the control group. Over the course of the experiment, however, the activities of the tadpoles from the different treatments converged. These results are similar to those from (Vaissi et al., 2024), in which high concentrations of CuONP led to hyperactivity in green toad tadpoles. However, these results contradict the study from Enrique García-Muñoz et al. (2011), where they have shown a reduction in tadpoles escape displacement distance (and therefore activity) associated with increasing copper sulfate concentrations. It is possible that these differences are due to the sulfate used in their study, as this anion can also have a toxic effect on aquatic organisms (N. Wang et al., 2020).

In this regard, we found consistent results with respect to the total movement lengths and total movement durations after external influences. Within these experiments, the animals that were reared in higher copper concentrations showed longer total movement durations and longer total movement lengths with increasing numbers of escape stimuli. A possible explanation for this could be a copper-induced learning inhibition regarding the escape stimuli. Since a reaction to an external stimulus always involves the usage of energy, it is essential for the tadpoles to be able to distinguish between dangerous and harmless stimuli (Ferrari & Chivers, 2011) and to react to them appropriately. However, some species must first learn to recognise potentially negative stimuli (Ferrari & Chivers, 2009). In our particular case, it could be possible that the tadpoles from the control and lowest copper treatments learnt that the external stimulus was not dangerous because there was no negative consequence. In the higher treatments, this learning experience may have failed to materialise due to the effects of the copper exposure.

Lastly, with increasing treatment levels, the animals showed significantly higher distances from the arena wall during the experiment. Wall following may be exhibited as a defensive behavior or as an exploratory strategy (Hänzi & Straka, 2018). Copper-induced disturbances in the wall following can therefore lead to an increased risk of predation. Also, the arena

position (x - coordinate) had a significant influence on the distance of the animals to the arena wall. This effect could be due to external influences, such as light sources outside the experiment (window and terraria next to the frame) that could shine faintly through the black fabric, or due to the different proximity of the arenas to the door and the window, through which noise could leak in and disturb the tadpoles. For future studies on this topic, the experiments should be conducted in an even more undisturbed environment. And as mentioned above, the shape of the tail fins also has an influence on the swimming performance of the tadpoles but was not considered in this study and should therefore also be included in future studies on this topic.

In summary, this study showed negative effects of metallic and dissolved copper on the development and behavior of green toad tadpoles. Hopefully this work can emphasise the urgency of reducing copper and heavy metal pollution in general.

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