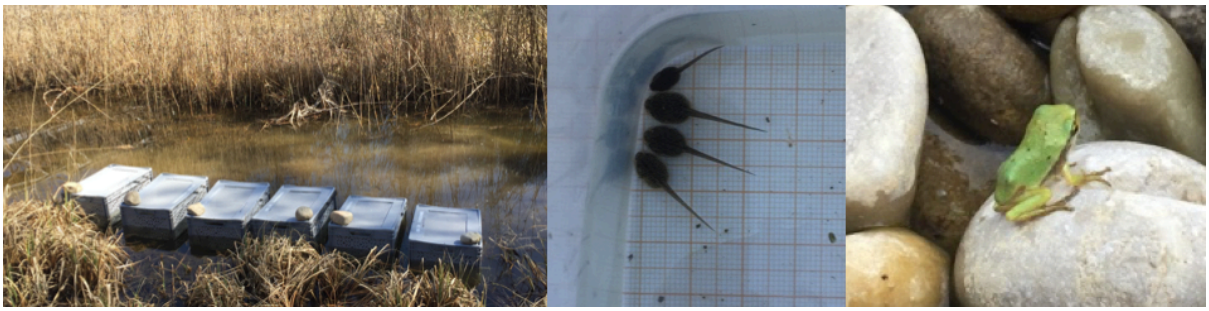
Master Thesis

Impacts of pesticide concentrations on amphibian development and survival in natural ponds



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Abstract

Worldwide, biodiversity is threatened by changes in land-use. One major driver thereof is the intensification of agriculture, leading not only to the conversion of natural ecosystems to cultivable land but also to intensive pesticide use. With pesticide use predicted to increase, concerns about the impacts of pesticides on non-target organisms are growing. A group of particular interest are amphibians since their populations are declining globally at an alarming rate. While laboratory studies reported adverse effects of pesticides on amphibians, the situation under natural conditions is largely unexplored. To test whether elevated levels of pesticides (>500 ng/L) and mixtures thereof have an impact on amphibian survival, growth, and development under natural conditions, I conducted a field experiment in the Berner Seeland, in Switzerland. There, I raised tadpoles of two frog species (*Rana temporaria* and *Hyla arborea*) in four natural ponds containing different levels of pesticides. By comparing multiple life history traits, I found that survival of *H. arborea* possibly decreased when exposed to elevated pesticide concentrations. Beyond that, I did not encounter measurable negative effects on larval life history traits (growth, size and mass at metamorphosis and length of the larval period). My results suggest that some environmental conditions with a strong effect on growth and development, or interactions thereof, can override and/or mask potential effects of pesticides under the conditions observed. Nevertheless, negative effects of pesticides on survival and development cannot be precluded. Taken together, my study shows that the effects of pesticides are not as clear-cut under natural conditions as they are in the laboratory, emphasizing the need for further research that takes realistic environmental conditions into account.

Zusammenfassung

Weltweit wird die Biodiversität durch die stetige Veränderung der Landnutzung bedroht. Eine der Hauptursachen dafür ist die starke Expansion und Intensivierung der Landwirtschaft. Diese führt nicht nur dazu, dass natürliche Ökosysteme zunehmend kultiviertem Land weichen müssen, sondern auch zu einem Anstieg des Pestizideinsatzes. Da Zukunftsprognosen einen höheren Pestizidverbrauch vorhersagen, wachsen die Bedenken um Auswirkungen auf exponierte Organismen, die nicht das Ziel des Einsatzes sind. Von besonderem Interesse sind dabei die Amphibien, da deren Bestände global alarmierend schnell abnehmen. Während Pestizide in einer Vielzahl von Laborstudien negative Effekte auf Amphibien gezeigt haben, ist jedoch noch wenig darüber bekannt, wie Pestizide Amphibien unter realistischen Bedingungen in der Natur beeinflussen. Deshalb wollte ich mithilfe eines Feldexperimentes testen, ob sich erhöhte Pestizidkonzentrationen (>500 ng/L), oder Mischungen davon, nachteilig auf das Wachstum, die Entwicklung oder das Überleben von Amphibien auswirken. Das Feldexperiment fand in vier Weihern des Berner Seelandes statt. Das Wasser dieser Weiher wies unterschiedlich hohe Pestizidkonzentrationen auf. Darin zog ich Kaulquappen zweier Froscharten auf (*Rana temporaria* und *Hyla arborea*) und erhob laufend verschiedene Parameter, die eine Aussage über die Fitness der Kaulquappen erlauben. Der Vergleich dieser Parameter zwischen den Arten und den Weihern zeigte, dass die Überlebenswahrscheinlichkeit von *H. arborea* möglicherweise abgenommen hat, wenn die Kaulquappen erhöhten Pestizidkonzentrationen ausgesetzt waren. Darüber hinaus habe ich aber keine messbaren Anzeichen eines negativen Einflusses gefunden. Meine Resultate lassen darauf schliessen, dass, unter den beobachteten Bedingungen, einige Umweltbedingungen, oder Interaktionen davon, einen stärkeren Einfluss auf das Wachstum und die Entwicklung ausüben können und so womöglich Effekte von Pestiziden aufheben und/oder überdecken. Nichtsdestotrotz können inhibierende Effekte von Pestiziden nicht mit Sicherheit ausgeschlossen werden. Insgesamt zeigt meine Studie auf, dass Effekte von Pestiziden unter natürlichen Bedingungen nicht so eindeutig sind wie in Laborstudien. Dies wiederum unterstreicht die Notwendigkeit von Experimenten, welche realistische Bedingungen berücksichtigen.

1 Introduction

Humans are dramatically altering environmental conditions, thereby triggering species extinctions and local population declines (Dirzo et al. 2014, Vitousek et al. 1997). One major anthropogenic contributor to the global loss of biodiversity is land-use change, mostly aimed at expanding cultivable land (Lambin & Meyfroidt 2011). Owing to the need to feed a growing world, agricultural area has become one of the largest terrestrial biomes on earth, occupying around 40% of the land surface (Foley et al. 2005). However, to maximize the output, these areas receive high chemical input in the form of pesticides (Tilman 1999). These biologically active substances are the most effective means to protect crops against yield losses and reduction of product quality (Damalas 2009). Worldwide, several thousand pesticide formulations are registered and about 2.3 million tons are utilized per year (Grube et al. 2011). Forecasts predict an additional 1.9- up to 4.8-fold increase in pesticide application by 2050 (Tilman et al. 2001). Despite this, it has been estimated that only around 0.1% of pesticides applied to crops reaches the target pest; the rest enters ecosystems, where it may negatively affect plants and animals through contamination of land, water, and air (Pimentel & Levitan 1986). As a consequence, concerns about adverse effects on non-target organisms are growing (Relyea 2005a).

Amphibians, as the most endangered class of vertebrates (Stuart et al. 2004), are one group of non-target organisms of particular interest (Relyea 2005b). Throughout the world, their populations are drastically declining (Houlahan et al. 2000). After habitat loss and degradation, the IUCN considers human-caused environmental pollution as the second most common threat to amphibians (as reviewed in Mann et al. 2009). Pesticides fall within the category of environmental pollution and could potentially be important contributors to the decline (Davidson et al. 2002). Via spray drift and run-off, aquatic habitats for amphibians are often receiving pesticides from agricultural areas (Brühl et al. 2013, Fryday & Thompson 2012). It is assumed that amphibians are particularly vulnerable to chemical pollutants due to the unshelled eggs and the highly permeable skin (Blaustein et al. 2003). Additionally, reproduction and critical hormone-regulated developmental changes, occurring in the aquatic environment, coincide with the application of pesticides on agricultural land (Hayes et al. 2006, Mann et al. 2009).

More and more, the question is raised if current risk assessment covers amphibians sufficiently (Ortiz-Santaliestra et al. 2018). Numerous laboratory studies have shown that pesticides can have lethal and various sublethal effects on amphibians (Ockleford et al. 2017). As an example, Berrill et al. (1993) have observed retardation of growth and disturbed locomotor behaviour in tadpoles after short term exposure to an insecticide pulse. However, the majority of these toxicological studies were performed with very high, acute doses of single chemicals, whereas few studies have focused on the more realistic effects of low concentrations, or pesticide mixtures over prolonged periods (Hayes et al. 2006). Nevertheless, exactly such studies would be crucial to obtain meaningful data for actual exposure conditions. Bernabò et al. (2011), for instance, have documented skeletal and muscular defects, as well as gill alterations, in chronic toxicity tests at ecologically relevant concentrations of an insecticide. Further, Hayes et al. (2006) have found that a mixture of nine pesticides prolonged metamorphosis of tadpoles significantly compared to individual pesticide effects. Additional complexity is added by environmental conditions, which can potentially modify or even mask the impact of contaminants (Holmstrup et al. 2010). While traditional laboratory studies ignore this variability completely, mesocosm studies make the transition to a more realistic experimental venue (Relyea 2005a). Zaga et al. (1998), for example,

have detected synergistic effects of carbaryl, a broad-spectrum insecticide, and UV-B radiation on mortality of tadpoles. In contrast, Brogan & Relyea (2013) have found that submersed plants decreased the toxicity of an insecticide on *Daphnia*. Such studies demonstrate that the integration of environmental conditions is key. Still, the majority of mesocosm studies focus on few interactions, while in reality, there are a lot. At the same time, monitoring data of pesticide levels in small, standing water bodies is scarce, such that the exposure of amphibians to pesticides under natural conditions is largely unknown (Aldrich et al. 2016). An assessment of Stoffel (2016) has shown that some ponds in the Berner Seeland exceeded the critical value of 100 ng/L, set by the federal water protection ordinance (WPO), by far and contained mixtures of at least 29 pesticides. Taken together, it appears that despite numerous studies investigating toxicity on amphibians, there is a gap of knowledge when it comes to predicting how pesticides influence amphibians under natural conditions. Besides, it seems like little is known about substances and mixtures of substances amphibians are actually exposed to and in which concentrations they occur (Aldrich et al. 2016).

Therefore, the purpose of my study was to investigate whether amphibian development is negatively affected, if larvae are exposed to elevated pesticide concentrations (>500 ng/L) and pesticide mixtures under natural conditions in ponds. To do so, I carried out a field experiment in four natural ponds containing different levels of pesticides (Stoffel 2016). In each pond, tadpoles of two frog species (*Rana temporaria* and *Hyla arborea*) were raised, all originating from the same source pond. Throughout the whole larval phase, different life history traits that are key for individual fitness were assessed. In addition, a water residue analysis for pesticides was performed and resource availability, as well as temperature, were evaluated. The combination of life history traits and environmental conditions allowed me to draw comparisons between ponds and species and test for correlations. Further, I compared patterns between years, since studies at the same locations were conducted in 2016 and 2018 (Stoffel 2016, Pittet & Zumbach 2018). Expecting to find negative impacts of pesticide exposure on tadpole development, I set up the following hypotheses: 1) Tadpoles exhibit a lower survival rate when raised in a pond with elevated pesticide concentrations. 2) Tadpoles exhibit slower growth, are therefore smaller and have lower mass at metamorphosis when raised in a pond with elevated pesticide concentrations. 3) Time to metamorphosis is prolonged in tadpoles raised in a pond with elevated pesticide concentrations. 4) Decreases in survival rates from the pre-juvenile to the juvenile stage do not translate at the population level. Investigating these hypotheses will help to gain an insight into actual exposure conditions and how they affect amphibian development under natural conditions. In conclusion, this study aims to provide recommendations for future management actions regarding amphibian conservation in agricultural areas.

2 Material and Methods

2.1 Study system

The field experiment took place in the Berner Seeland and along the river Saane. This region is characterized by intensive agriculture. In addition, information about pesticide contamination of multiple ponds in this region is available for 2016 and 2018 (Stoffel 2016, Pittet & Zumbach 2018). Based on these measurements, as well as some physical parameters like having stable water levels and sufficient space for the experimental set-up, four ponds were chosen (Figure 1). Out of these four ponds, one has had relatively high (between 5100 – 1900 ng/L) and three ponds have had relatively low (between 50 and 650 ng/L) pesticide contamination in 2016/18.

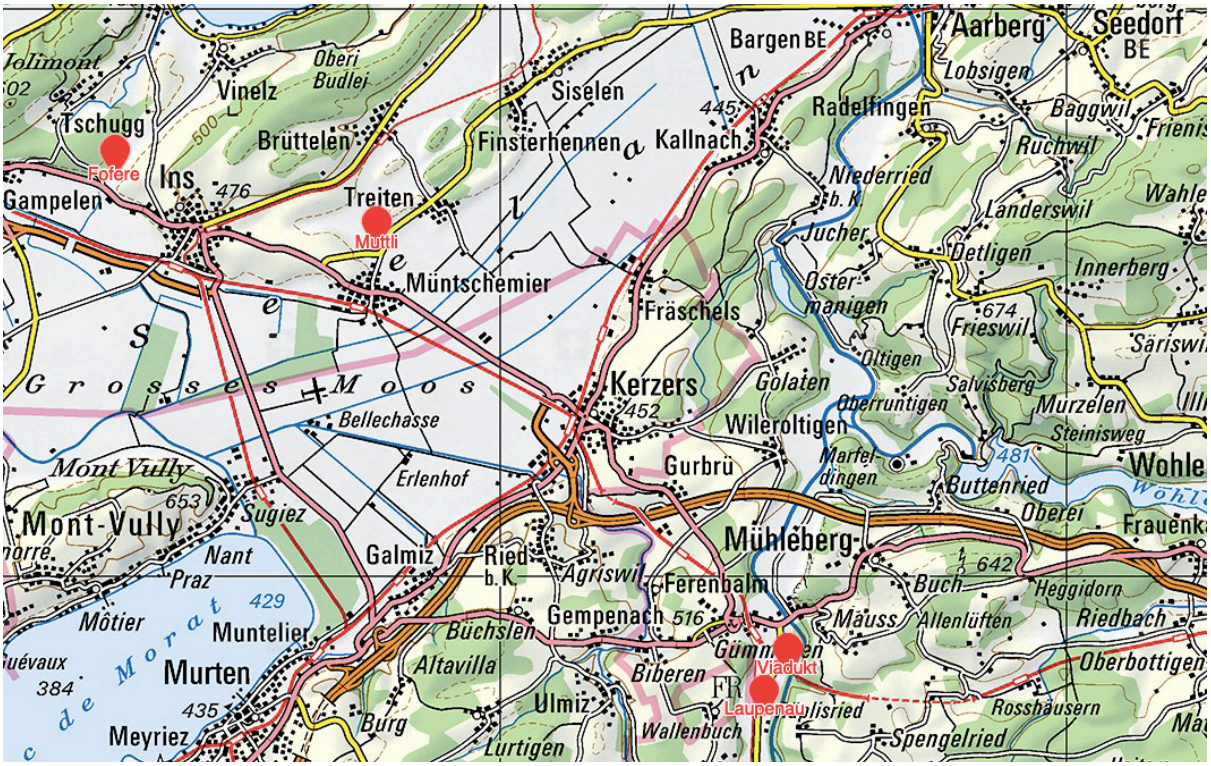

Figure 1: The red dots indicate the location of the four ponds. While Fofere and Muttli are located in the Berner Seeland, Laupenau and Viadukt were located along the river Saane. Map taken from https://map.geo.admin.ch/.

Muttli, the pond with the highest pesticide concentration in the previous years, also has the largest surface area of all four ponds. It is located adjacent to agricultural fields, which have a drainage directly in its waterbody. However, Muttli itself is surrounded by wood. The ponds Laupenau and Viadukt were built between 2001 and 2007 as part of a project to connect two isolated *Hyla arborea* populations. Both ponds are artificially discharged in autumn and filled again in spring to create temporal waters, which are highly valuable for *H. arborea* (Schmidt et al. 2015). Fofere was built in 2006 to compensate for a road project. Water is supplied by a streamlet and it possesses an artificial discharge (Stoffel 2016).

2.2 Study organisms

As study organisms served tadpoles of the two frog species *Rana temporaria* and *Hyla arborea*, which are both native inhabitants of the area. I studied wild populations to make the experiment as realistic as possible through the integration of naturally occurring phenotypic variability.

2.2.1 Common frog (*Rana temporaria*)

Rana temporaria is the most widespread anuran species in Switzerland and is classified on the Swiss Red List as well as globally on the IUCN Red List as least concern (Schmidt & Zumbach 2005). Reproduction occurs early in spring, starting from February (Meyer et al. 2009). Individual clutches comprise around 1000 eggs (Gibbons & McCarthy 1986). After eight to ten days, larvae hatch and metamorphose after seven to twelve more weeks to juveniles, depending on environmental variables like temperature (Meyer et al. 2009).

2.2.2 Tree frog (*Hyla arborea*)

The distribution range of *Hyla arborea* is decreasing in Switzerland, due to increased anthropogenic modification of water bodies (Schmidt & Zumbach 2005). Therefore, the species is classified as endangered on the Swiss Red List (Schmidt & Zumbach 2005). The spawning season of *H. arborea* starts between March and April, peaks in May and can extend until July (Meyer et al. 2009). A female deposits 4-10 small clutches that contain on average 15-80 ova (Mermod et al. 2010). Depending on temperatures, the larvae hatch after one to two weeks and reach metamorphosis after two to three more months (Meyer et al. 2009).

2.3 Animal husbandry

For both species, clutches were collected in Laupenau. Fresh clutches with similar developmental stages of the eggs were chosen (Gosner stage 1-13 (Gosner 1960)). While for *R. temporaria* clutch collection took place on 21 March, *H. arborea*, clutches were collected on 21 and 22 May.

Clutches
Until hatching, I kept individual clutches of both species in water-filled boxes (32x20.5x9 cm). These clutch boxes were floating on the water within the experimental boxes into which the tadpoles will be released later on.

Tadpoles
After hatching, tadpoles remained in the clutch boxes until they were free swimming and feeding (Gosner stage 26 (Gosner 1960)) and most of them reached a minimal size of 12 mm. As soon as this was the case, I transferred them to the experimental boxes (60x40x32 cm), which were located within the waterbodies of the ponds. I released *R. temporaria* tadpoles around two weeks after collection, on 1 and 4 April, whereas I released *H. arborea*, tadpoles after four weeks, on 23 June.

Metamorphs

With the emergence of the front limbs (Gosner stage 42 (Gosner 1960)), metamorphosing tadpoles were transferred into separate boxes (18.5x12x15 cm), which were floating on the water within the experimental boxes. In these metamorph boxes, I placed two rolls of household paper on the sides and added two small cups of water, allowing the metamorph to crawl from the water on land. Additionally, the boxes were sealed with mesh. All metamorphs stayed in these boxes until complete resorption of the tails (Gosner stage 46 (Gosner 1960)). After, the juveniles were released at the sites they were raised.

Experimental boxes

I used experimental boxes with a holey surface, which I had lined with a fine mesh (Figure 2). The mesh allowed water flow and nutrient exchange and protected the tadpoles against predation at the same time. Besides, the boxes were covered with a lid, which I had spanned with a fine mesh as well, such that sunlight got through. All boxes were put in the water ten days before the release of the tadpoles, allowing periphyton to grow thereon. In addition, four stones were laid on the ground where periphyton accumulated as well. These served as food for the tadpoles during the whole course of the experiment and made extra feeding redundant.

Figure 2: Examples of experimental boxes in which tadpoles were kept until they reached metamorphosis.

2.4 Experimental design

To assess if there are differences in development attributable to pesticide contamination, I collected and kept multiple clutches in Laupenau (source pond), but additionally transferred fractions of each clutch to the three other ponds (receiving ponds). The procedure of transfer was the same for both study organisms; however, shifted in time and differing in numbers (Figure 3, Table 1).

For *R. temporaria,* I placed six experimental boxes in each pond. Next, I collected a total of six clutches in the source pond. Per clutch, I randomly selected 4x12 tadpoles and assigned 1x12 to one box of each of the four ponds. Consequently, I raised in each of the six boxes per pond 12 tadpoles, whereof all 12 originated from the same clutch. For the random selection of the tadpoles, I followed the protocol for mesocosm studies of Josh van Buskirk (www.ieu.uzh.ch /research/ecology/change/labprotocols/03_mesocosm_experiments.pdf), with the exception that I drew random variables in R to assign tadpoles to boxes and did not do it haphazardly.

For *H. arborea*, I reduced the set-up to four experimental boxes per pond. Further, I collected eleven clutches in the source pond. After hatching, I mixed the tadpoles of all clutches before randomly selecting 4x4x10 tadpoles. The reason why I mixed the clutches and reduced the set-up, as well as the numbers, was that individual clutches were too small to provide enough tadpoles for all four ponds. In addition, a fourth of all tadpoles went missing after a heavy thunderstorm. Thus, I raised in each of the four boxes per pond 10 tadpoles originating from different clutches.

For both species, surplus tadpoles from individual clutches were rereleased in the source pond.

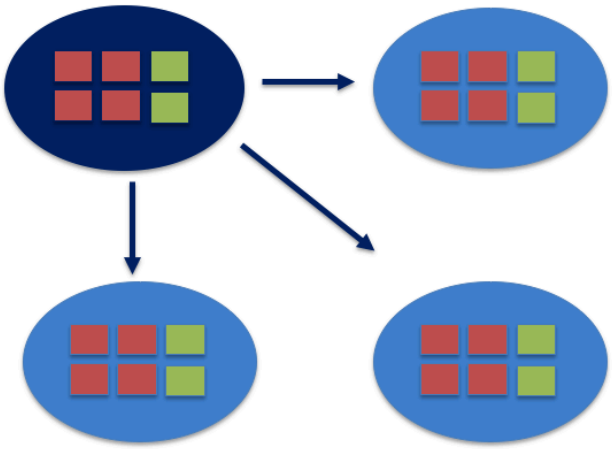

Figure 3: Scheme of transfer of the tadpoles: Light blue circles represent the three receiving ponds. The dark blue circle represents the source pond where all clutches were collected and from where tadpoles were distributed to the receiving ponds (dark blue arrows). Squares indicate the experimental boxes: Six boxes per pond in the case of *R. temporaria* (red + green) and four boxes in the case of *H. arborea* (red).

Table 1: Summary of the number of tadpoles which were raised per experimental box, pond and species (Rt refers to numbers of *R. temporaria* tadpoles and Ha refers to numbers of *H. arborea* tadpoles).

Box		Pond 1		Pond 2		Pond 3		Pond 4		Sum	
Rt	Ha	Rt	Ha	Rt	Ha	Rt	Ha	Rt	Ha	Rt	Ha
1	1	12	10	12	10	12	10	12	10	48	40
2	2	12	10	12	10	12	10	12	10	48	40
3	3	12	10	12	10	12	10	12	10	48	40
4	4	12	10	12	10	12	10	12	10	48	40
5		12		12		12		12		48	
6		12		12		12		12		48	
										288	160

2.5 Data collection

I started the field experiment on 21 March and stopped it on 18 August after five months. During this time, I measured a set of environmental variables, but also multiple life history traits. However, due to very high temperatures during the end of June in combination with a period of sporadic precipitation events, Fofere dried out completely on 3 July. Hence, I could not continue collecting data there. As a consequence, I could only incorporate Laupenau, Muttli, and Viadukt in the analysis of *H. arborea* data.

2.5.1 Environmental variables

Pesticide concentrations
On 20 May I took one 250 mL water sample per pond. These samples were sent to Bachema AG to perform a water residue analysis for pesticides. Using LC-MS/MS, they screened for the presence and concentration of 60 commonly used pesticides with a detection limit of 20 ng/L. With the results of their analysis I was able to calculate the total pesticide concentration of each pond, as well as the number of pesticides above the critical value of 100 ng/L.

Temperature
The temperature was continuously measured with HOBO Pendant® temperature data loggers. Three loggers were placed in three different experimental boxes of each pond. I started the measurement on 25 March and stopped it on 18 August. During this period, the loggers measured the temperature every two hours. These measurements allowed me to calculate the mean, the variance, maxima and minima of water temperature for each of the two larval periods.

Resource availability
I assessed the resource availability per pond by measuring the accumulated algal biomass on experimental glass slides. I inserted three glass slides per pond and covered them with a small pet bottle to prevent tadpole grazing. I started the measurement on 6 May and terminated it on 3 July. After removal, I airdried the glass slides for one week at room temperature. Then, I scraped the biomass from both sides onto pre-weighed boats and weighed them to the nearest microgram. The mean weight of algal biomass of the three slides per pond gave me a proxy for the resource availability per pond.

2.5.2 Life history traits

After releasing the tadpoles into the experimental boxes, I visited each pond at least two times per week. On the first weekly visit, I counted all tadpoles, looked if any deformities were exhibited and assessed their growth. On the second weekly visit, I checked the water level and the placement of the boxes in the ponds.

Counts
To count the number of tadpoles in each box, I caught tadpoles individually with a cup and transferred them to a small box filled with water. By doing so, I counted how many tadpoles were still present and alive. Death was recorded when a tadpole was immobile, or only remains of the skeleton were observable. If none of this was the case, the tadpole was recorded as missing. Using these counts per date, I was able to calculate mortality and survival rates per box, pond, and species. Survival was calculated by dividing the number of tadpoles surviving through metamorphosis, or still present in the boxes by the end of the experiment, by the total number of tadpoles released into the box. Further, I made a distinction between early mortality and late mortality since missing tadpoles occurred almost exclusively during the first two weeks after transfer. Early mortality was defined as disappearance within the first 14 days and late mortality as death starting from the 15^{th} day after the transfer.

Deformities
To assess deformities, a presence-absence based approach was used (Böll et al. 2013). Tadpoles exhibiting a deformation would have been excluded from the experiment. During the whole experiment, however, I did not detect any deformity, such that I did not include this parameter in my further analysis.

Growth
To assess growth, all tadpoles which were alive and in good shape were transferred to a small aquarium filled up to 1 cm with water. On the bottom of the aquarium, I placed a graph paper (scale 1 mm). As soon as the tadpoles were resting and in a straight position, I took a photo. From this picture, I extracted the length and width of each tadpole with the software Piximètre (Version 5.9) (Figure 4a). These weekly length and width measures allowed me to compare growth curves between species and ponds.

The procedure of two weekly visits continued until the first tadpoles developed forelimbs. Starting from then, I visited each pond every second day.

Metamorphosis
As soon as the tail resorption was completed (Gosner stage 46 (Gosner 1960)), I caught the juveniles with a small container and weighed them on a KERN® CM pocket scale to the nearest centigram. Next, I put them in a small transparent box and photographed them over graph paper (scale 1 mm). From there, their snout-vent length (SVL) was extracted with the software Piximètre (Figure 4b). Additionally, I recorded how many days had passed since their release to the big boxes (day zero). These measurements of body mass and total length (size) at metamorphosis, as well as the length of the larval period, could then be compared between ponds and species.

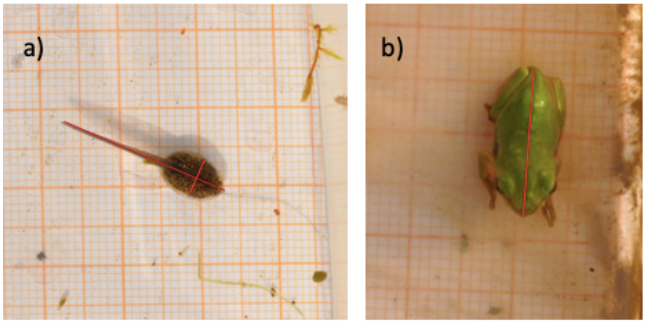

Figure 4: Measurement of sizes in tadpoles and juveniles: a) Length of tadpoles was measured from the snout to the end of the tail and width was measured behind the point where front limbs will emerge, as indicated by the red lines. b) SVL of juveniles was measured as indicated by the red line.

2.6 Population projection (*R. temporaria*)

To assess how tadpole mortality (early and late mortality) observed in the experiment may affect population dynamics, I used a population model. I worked with the population matrix model presented by Biek et al. (2002) (Table 2) and parametrized it with the mortalities that I had observed in my experiment. To do so, I multiplied the pre-juvenile to juvenile survival rate of the paper with one of the four survival rates I had seen in the different ponds. Since the survival rate suggested by Biek et al. (2002) is high, I assumed that it represented the rate under optimal

growth conditions for tadpoles and the rates that I had observed represented decreases due to environmental stressors like pesticide exposure, scarce resources or high temperatures. Next, I calculated with the altered matrix the population growth rate (λ_{SSD}) to investigate to what extent λ_{SSD} was affected.

In the following step, I examined what impact simultaneous decreases of multiple vital rates would yield on the population growth rate since also the λ_{SSD} reported by Biek et al. (2002) is high, with a value of 1.33. Therefore, I decreased adult survival from 0.43 to 0.3 and clutch size from 650 to 500, in addition to the decreases in pre-juvenile to juvenile survival, and calculated λ_{SSD} again.

Table 2: Lefkovitch matrix, according to Biek et al. (2002), consisting of the three stages; prejuvenile (embryo, larva, overwintering metamorph), juvenile and reproductive adult. Rates were estimated as follows: Embryo survival = 0.92, larval survival = 0.06, metamorph survival = 0.34, juvenile survival = 0.33, juvenile to juvenile = 0.25, juvenile to adult = 0.08, adult survival = 0.43, probability of laying = 1, clutch size = 650.

0	Probability of juvenile becoming adult x probability of laying x clutch size	Adult survival x probability of laying x clutch size
Embryo survival x larval survival x metamorph survival	Probability of remaining a juvenile	0
0	Probability of juvenile becoming adult	Adult survival

2.7 Comparison between years

The datasets used to compare parameters between years included the one of Pittet & Zumbach (2018), who performed a very similar study to mine on a smaller scale, and the one of Stoffel (2016), who repeatedly assessed water quality in multiple ponds in the Berner Seeland. I included the data of the second assessment of Stoffel (2016) since its date (23 May) corresponded approximately with the date, I took my samples (20 May). The two datasets allowed me to compare pesticide concentrations over three years (2016, 2018, 2019), except for Viadukt and Fofere where only two measurements were available (2016, 2019). Further, I could compare life history traits of *H. arborea* over two years (2018, 2019) between the ponds Laupenau and Muttli.

2.8 Data analysis

I used the software R (R Core Team 2015) and general linear models with binomial family distribution to analyse the count data (survival and mortality). As a fixed effect I entered one environmental variable (pesticide concentration, resource availability or temperature) per model. I did not include random effects since there were not enough data points for such an analysis. Residual plots did not show any obvious violations of the assumptions. Thus, among all models, the most parsimonious one was selected using the AIC.

I did not analyse the relationships between growth and environmental variables since the associated patterns recurred in the relationships between life history traits associated with metamorphosis (size/mass at metamorphosis and length of larval period) and environmental

variables (pesticide concentration, resource availability or temperature). To analyse these relationships, I performed a linear mixed effect analysis using the R package nmle (Pinheiro et al. 2017). As fixed effects, I entered one environmental variable per model. I did not include multiple variables or interactions since this would have overparameterized the model. As random effects, I nested box number in ponds, to account for their non-independence. I did not incorporate clutch ID as a random effect in the analysis of *R. temporaria* data since boxplots did not reveal apparent differences among clutches (see Figures 30-32, Appendix). For the cases where visual inspection of the residual plots revealed deviations from homoscedasticity, I used the variance structure varIdent, to allow different variances for different levels of the environmental variables. AIC values were used to find the best predictor and p-values were obtained by likelihood ratio tests of the full model with the effect in question against an intercept only model without the effect in question.

For the comparison between the years 2018 and 2019, I used the package nmle again to perform a linear mixed effect analysis. I incorporated year and pesticide concentration as fixed effects, pond as a random effect and either size or length of the larval period as a dependent variable. For the cases, where visual inspection of the residual plots revealed deviations from homoscedasticity, I used the variance structure varIdent. P-values were obtained using likelihood ratio tests.

3 Results

3.1 Environmental variables

Water residue analysis for pesticides revealed that the summed concentration of all formulations detected (total pesticide concentration) was highest in Muttli, followed by Laupenau. Both Fofere and Viadukt contained relatively low total pesticide concentrations (Figure 5a, Table 3).

Further, the analysis revealed that one single formulation exceeded the critical value of 100 ng/L in Fofere, Laupenau and Muttli, whereas there was no formulation above the critical value detected in Viadukt (Figure 5b).

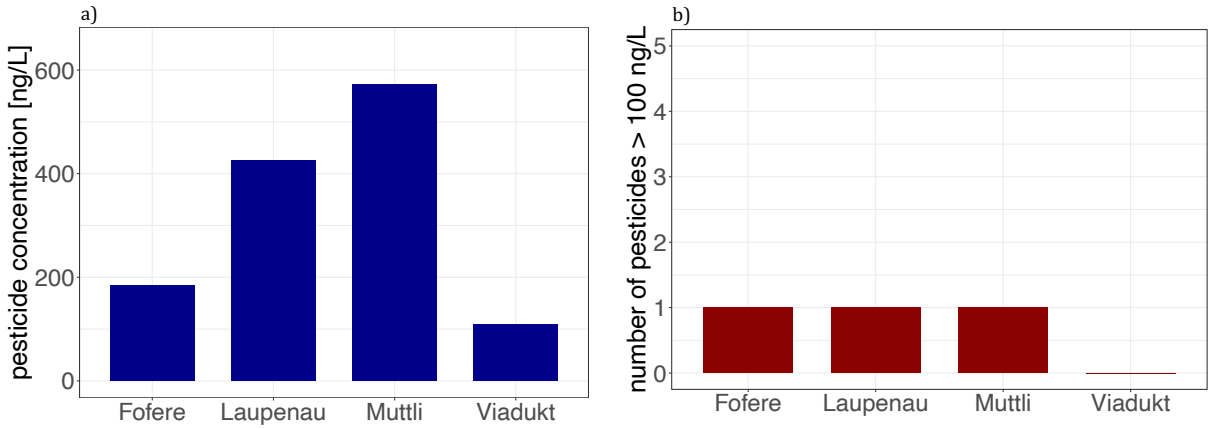

Figure 5: a) Blue bars show the total pesticide concentration in the water [ng/L] of the four ponds (Fofere, Laupenau, Muttli and Viadukt). Samples were taken on 20 May. b) Red bars show the number of pesticide formulations exceeding the critical value of 100 ng/L in the four ponds (Fofere, Laupenau, Muttli and Viadukt). Samples were taken on 20 May.

The highest level of dried algal biomass (resource availability) accumulated in Viadukt, followed by Muttli. Laupenau and Fofere both had relatively low resource availability (Figure 6, Table 3).

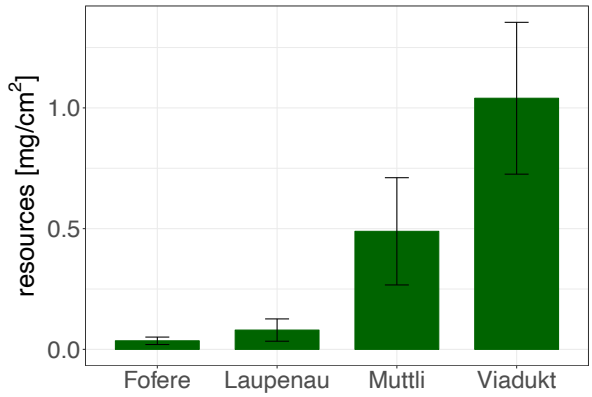

Figure 6: Green bars show the means of three measurements of resource availability [mg/cm^2] per pond (Fofere, Laupenau, Muttli, Viadukt). Black error bars represent the standard deviation. Resource measurement started on 6 May and ended on 3 July.

Water temperatures fluctuated substantially throughout the larval period of both species. During larval development of *R. temporaria*, water temperatures of all ponds were initially in a similar range, but starting from mid-June, Laupenau got considerably warmer. This trend continued, such that during the whole larval phase of *H. arborea*, water temperatures in Laupenau remained higher than in the other three ponds (Figure 7, Table 3).

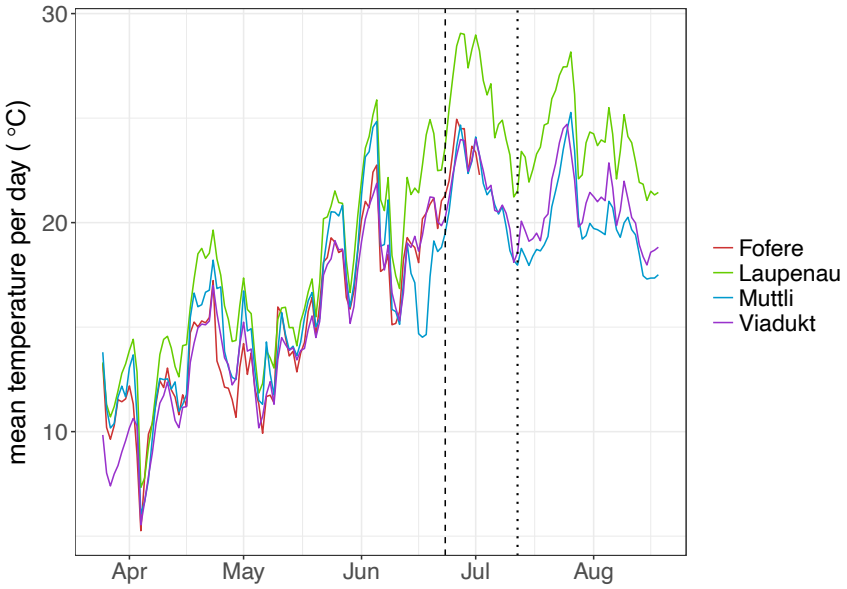

Figure 7: Lines show the temperature trajectory during larval development; the mean of 12 temperature measurements per day (°C) is plotted against the corresponding date. Measurement started on 25 March and stopped on 18 August. The dashed line indicates the start of the larval period of *H. arborea* (23 June). The dotted line indicates the end of the larval period of *R. temporaria* (12 July). Different colours represent the four different ponds (Fofere, Laupenau, Muttli and Viadukt).

Table 3: Summary of the environmental conditions per pond and period. Rt indicates parameters of the *R. temporaria* period and Ha indicates parameters of the *H. arborea* period.

Pond	Pesticide concentration [ng/L]	Resource availability [g]	Mean water temperature (°C)		Variance water temperature (°C)		Minimal water temperature (°C)		Maximal water temperature (°C)	
			Rt	Ha	Rt	Ha	Rt	Ha	Rt	Ha
Fofere	185	0.001	16.02	-	28.19	-	4.52	-	30.86	-
Laupenau	425	0.003	18.57	24.65	32.82	10.54	5.55	18.33	33.01	33.01
Muttli	580	0.018	16.38	20.40	23.01	4.97	5.45	16.52	30.05	28.55
Viadukt	110	0.039	15.80	21.06	23.14	6.28	4.62	15.76	25.13	32.09

3.2 Life history traits

3.2.1 Counts

The number of *R. temporaria* tadpoles in Laupenau was initially stable, but as metamorphosis approached, late mortality increased heavily (Figure 8, Table 4). The inverse was observed in Fofere, where numbers dropped drastically within the first weeks, but late mortality was low. Thus, survival was similar in these two ponds and on an intermediate level compared to the other two ponds. Muttli showed the highest survival rate, with low early but also low late mortality. Viadukt, on the other hand, showed both high early and high late mortality and, therefore, the lowest survival rate. Lowest AIC values for all response variables tested were associated with the intercept only model (Table 5). Consequently, there was neither a clear relationship between pesticide concentration and survival (p-value: 0.458, value: 2.259, std. error: 3.042) (Figure 9) nor between other environmental conditions and vital rates.

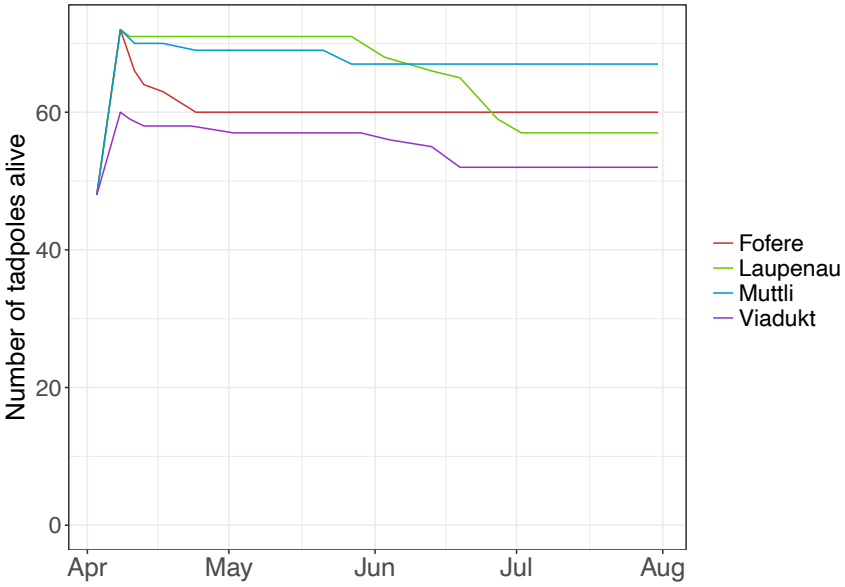

Figure 8: Lines show the trajectory of numbers of *R. temporaria* tadpoles that are either still present in the experimental boxes or successfully completed metamorphosis, plotted against the date of assessment. The reason for the initial increase in numbers is that I had to split the transfers of the 72 tadpoles per pond between two days (1 and 5 April). Different colours indicate the four different ponds (Fofere, Laupenau, Muttli and Viadukt).

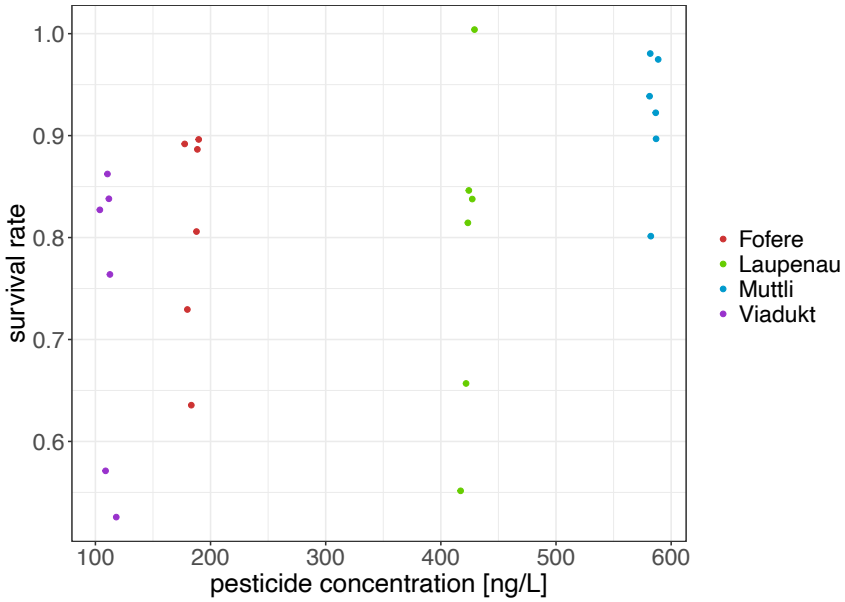

Figure 9: Points show survival rates of *R. temporaria* tadpoles per experimental box, plotted against total pesticide concentration in the water [ng/L] on 20 May. Different colours represent the four different ponds (Fofere, Laupenau, Muttli and Viadukt).

Early mortality for *H. arborea* tadpoles was low in Laupenau, but late mortality increased as metamorphosis approached. Still, survival was highest in Laupenau since in both Muttli and Viadukt early mortality was high. While late mortality in Viadukt was low, it stayed high in Muttli. Therefore, survival was lowest in Muttli (Figure 10, Table 4). AIC-based model selection revealed that the intercept only models were preferred for response variables describing survival (Table 5). Accordingly, there was neither a clear relationship between survival rate and total pesticide concentration (p-value: 0.974, value: -0.108, std. error: 3.345) (Figure 11) nor between any other environmental variable and vital rate.

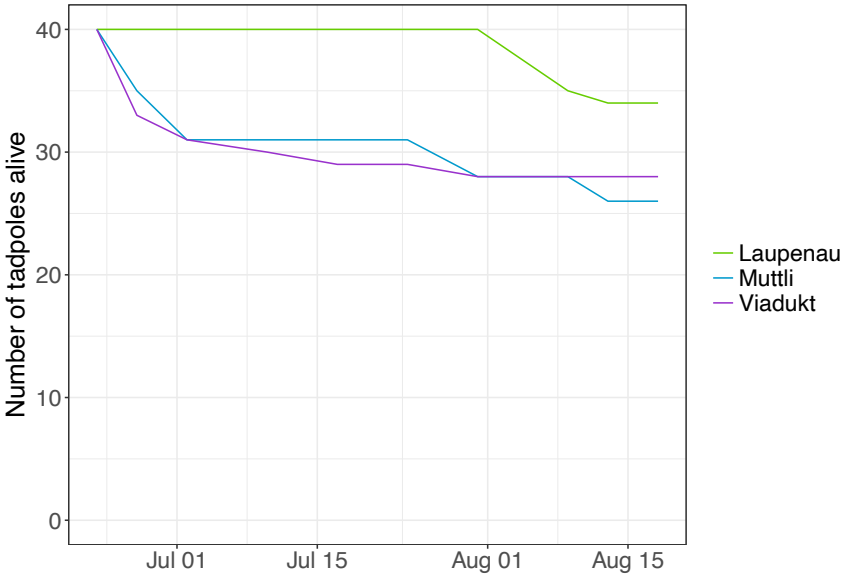

Figure 10: Lines show the trajectory of numbers of *H. arborea* tadpoles that are either still present in the experimental boxes or successfully completed metamorphosis, plotted against the date of assessment. Each pond started with a total of 40 tadpoles on 23 June. Different colours represent the three different ponds (Laupenau, Muttli and Viadukt).

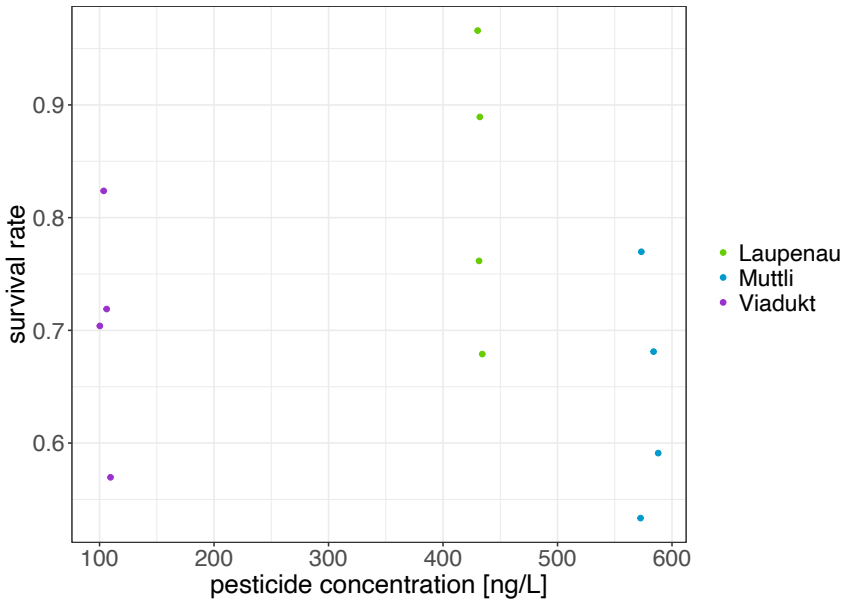

Figure 11: Points show survival rates of *H. arborea* tadpoles per experimental box, plotted against total pesticide concentration in the water [ng/L] on 20 May. Different colours indicate the three different ponds (Laupenau, Muttli and Viadukt).

Table 4: Summary of mean early and late mortality rates, as well as mean survival rates, per species and pond (± standard deviation).

Species	Pond	Early mortality	Late mortality	Survival
R. temporaria	Fofere	0.125 (± 0.069)	0.042 (± 0.069)	0.833 (± 0.105)
	Laupenau	0.014 (± 0.034)	0.194 (± 0.136)	0.792 (± 0.147)
	Muttli	0.028 (± 0.043)	0.042 (± 0.046)	0.931 (± 0.063)
	Viadukt	0.194 (± 0.155)	0.083 (± 0.053)	0.722 (± 0.146)
H. arborea	Laupenau	0.000 (± 0.000)	0.150 (± 0.129)	0.850 (± 0.129)
	Muttli	0.225 (± 0.126)	0.125 (± 0.050)	0.650 (± 0.129)
	Viadukt	0.250 (± 0.129)	0.050 (± 0.058)	0.700 (± 0.082)

Table 5: Summary of AIC values obtained through general linear model analysis of late mortality rate, early mortality rate or survival rate against the explanatory variables for both study organisms (*R. temporaria* and *H. arborea*). Lowest AIC values **(highlighted)** are for each response variable associated with the intercept only model.

Response variable	Explanatory variable	AIC (*R. temporaria*)	AIC (*H. arborea*)
Late mortality rate	~ 1 (intercept only)	**6.542**	**4.752**
	~ pond	12.660	8.779
	~ box number	8.635	6.752
	~ family	8.542	-
	~ pesticide concentration	8.543	6.771
	~ resources	8.548	6.779
	~ mean temperature	8.639	6.761
	~ variation temperature	8.609	6.759
	~ minimal temperature	8.573	6.773
	~ maximal temperature	8.562	6.752
Early mortality rate	~ 1 (intercept only)	**6.542**	**6.137**
	~ pond	12.703	10.341
	~ box number	8.547	8.267
	~ family	8.551	-
	~ pesticide concentration	8.685	8.154
	~ resources	8.603	8.312
	~ mean temperature	8.637	8.347
	~ variation temperature	8.576	8.343
	~ minimal temperature	8.676	8.363
	~ maximal temperature	8.661	8.214
Survival rate	~ 1 (intercept only)	**14.583**	**11.467**
	~ pond	19.671	14.838
	~ box number	16.492	12.975
	~ family	16.678	-
	~ pesticide concentration	15.852	13.423
	~ resources	16.028	13.645
	~ mean temperature	16.607	12.887
	~ variation temperature	16.752	12.809
	~ minimal temperature	16.241	13.401
	~ maximal temperature	15.926	12.674

3.2.2 Growth

Throughout the whole larval phase, *R. temporaria* tadpoles clearly grew fastest in Muttli and slowest in Fofere. In Viadukt and Laupenau, however, growth of the tadpoles was intermediate and very similar (Figure 12).

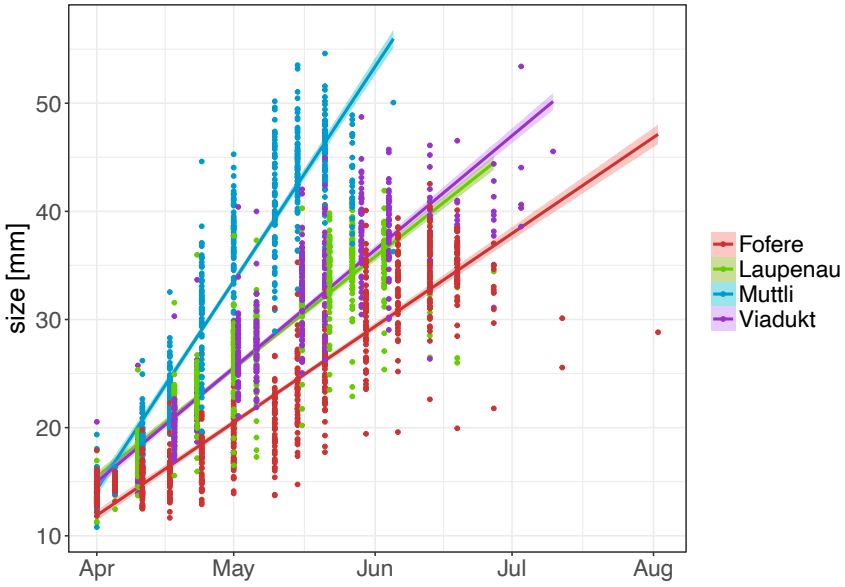

Figure 12: Points show total length (size) [mm] of individual tadpoles of *R. temporaria*, plotted against the date of measurement to assess growth throughout the whole larval phase. Different colours indicate the four different ponds (Fofere, Laupenau, Muttli and Viadukt) and pale bands represent the 95% confidence interval.

The differences in the growth of *H. arborea* tadpoles are not pronounced between the ponds. Tadpoles growing in Muttli seemed to have the fastest growth, followed by tadpoles raised in Viadukt and Laupenau (Figure 13).

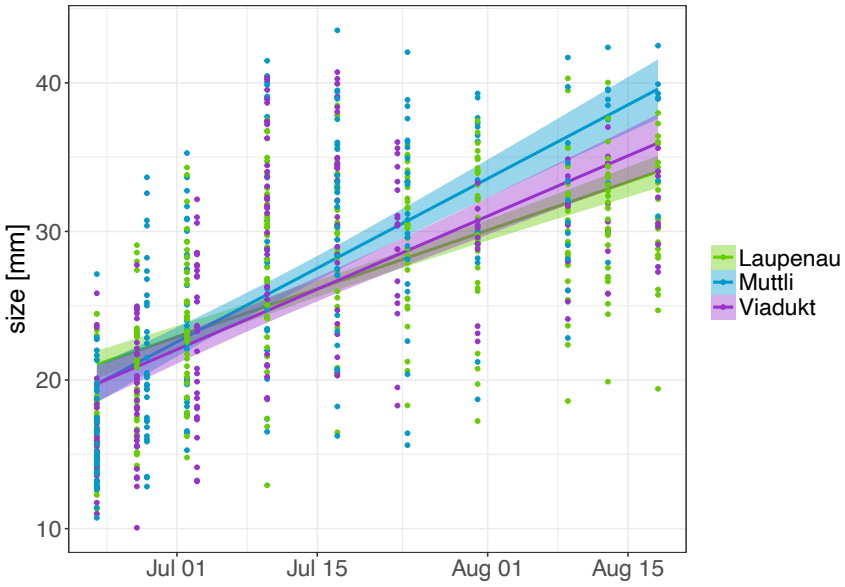

Figure 13: Points show total lenght (size) [mm] of individual tadpoles of *H. arborea*, plotted against the date of measurement to assess growth throughout the whole larval phase. Different colours indicate the three different ponds (Laupenau, Muttli and Viadukt) and pale bands represent the 95% confidence interval.

3.2.3 Metamorphosis

Juveniles

There were pronounced differences in the number of tadpoles that completed metamorphosis (juveniles) between the two species (Table 6). Not completing metamorphosis differs from mortality since multiple *H. arborea* tadpoles were not able to reach metamorphosis before the

termination of the experiment on 18 August. This was also one reason for the differences in numbers between the species. Another reason was that the experimental set-up for *H. arborea* included only 55.5% of the total number of tadpoles used for *R. temporaria* and lastly, there were differences in survival rates between the two species.

Table 6: Summary of total numbers of tadpoles completing metamorphosis per species and pond.

	Fofere	Laupenau	Muttli	Viadukt
R. temporaria	59	57	68	51
H. arborea	-	9	19	19

Size

R. temporaria juveniles from Muttli clearly reached the largest sizes at metamorphosis. Juveniles from the other three ponds finished their metamorphosis with similar sizes (Table 7, see Figure 24, Appendix). The AIC values of all models tested were in a narrow range (Table 8). Neither pesticide concentration (p-value: 0.136, value: 4.534, std. error: 2.636) (Figure 14) nor any other environmental variable showed a clear relationship with size.

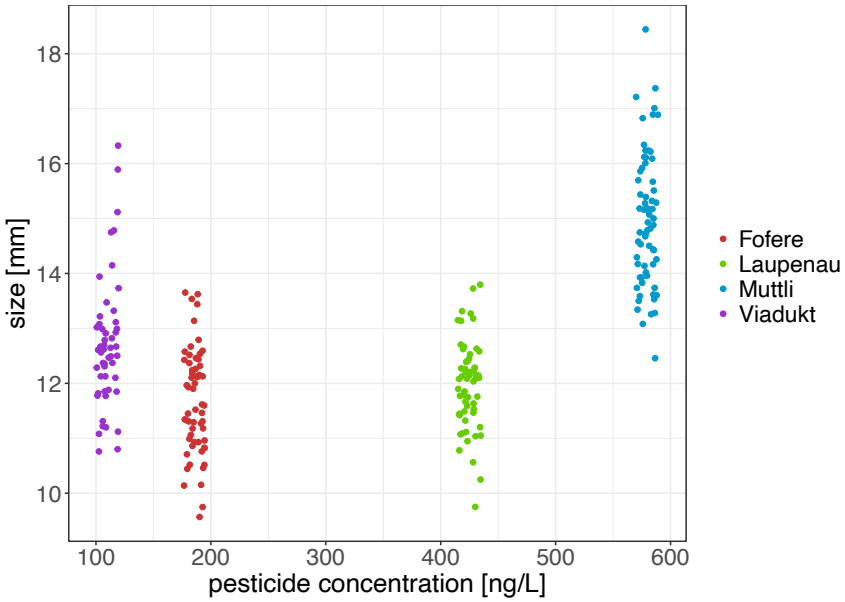

Figure 14: Points show size [mm] of individual *R. temporaria* juveniles at metamorphosis, plotted against total pesticide concentration in the water [ng/L] on 20 May. Different colours indicate the four different ponds (Fofere, Laupenau, Muttli and Viadukt).

H. arborea juveniles did not show pronounced differences in size at metamorphosis (Table 7, see Figure 25, Appendix). Juveniles from Muttli were slightly larger than those from Laupenau and Viadukt. Displaying the lowest AIC value of all models tested (Table 8), total pesticide concentration in the water showed a positive relationship with size at metamorphosis (p-value: 0.009, value: 3.003, std. error: 0.815) (Figure 15). The other environmental variables, however, did not correlate clearly with size.

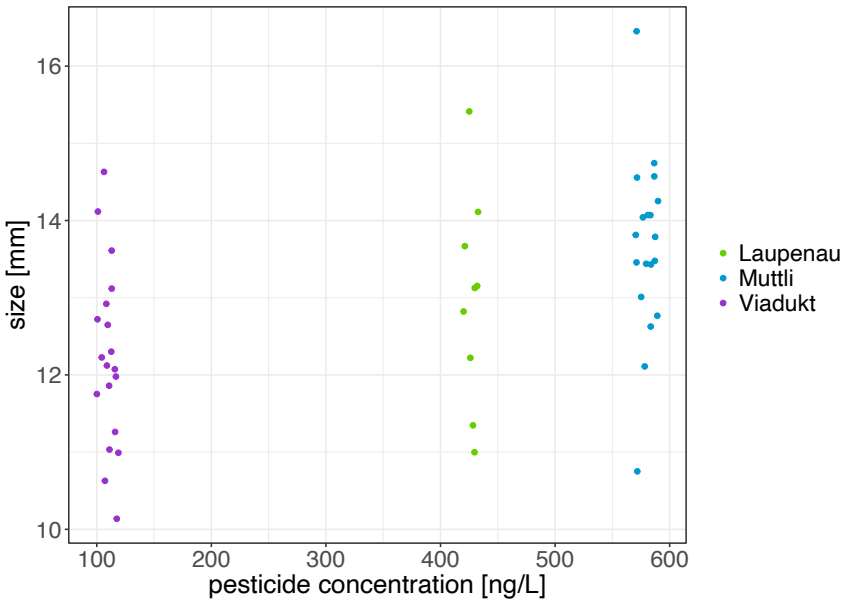

Figure 15: Points show size [mm] of individual *H. arborea* juveniles at metamorphosis, plotted against total pesticide concentration in the water [ng/L] on 20 May. Different colours represent the three different ponds (Laupenau, Muttli and Viadukt).

Body mass

R. temporaria juveniles from Muttli were clearly the heaviest, whereas the juveniles of the three other ponds were all in a similar range (Table 7, see Figure 26, Appendix). The relationships between body mass and environmental variables were following the ones between size and environmental variables (Table 8, see Figure 35, Appendix).

The body mass of *H. arborea* juveniles in Muttli aligned approximately with the body mass of the juveniles in Laupenau (Table 7, see Figure 27, Appendix). Juveniles that grew up in Viadukt displayed the lowest mean body mass. The observed relationships between body mass and environmental variables corresponded with the ones between size and environmental variables (Table 8, see Figure 36, Appendix) with one exception; a negative correlation between resource availability and body mass of juveniles was detected (p-value: 0.026, value: -2.129, std. error: 0.664) (Figure 16).

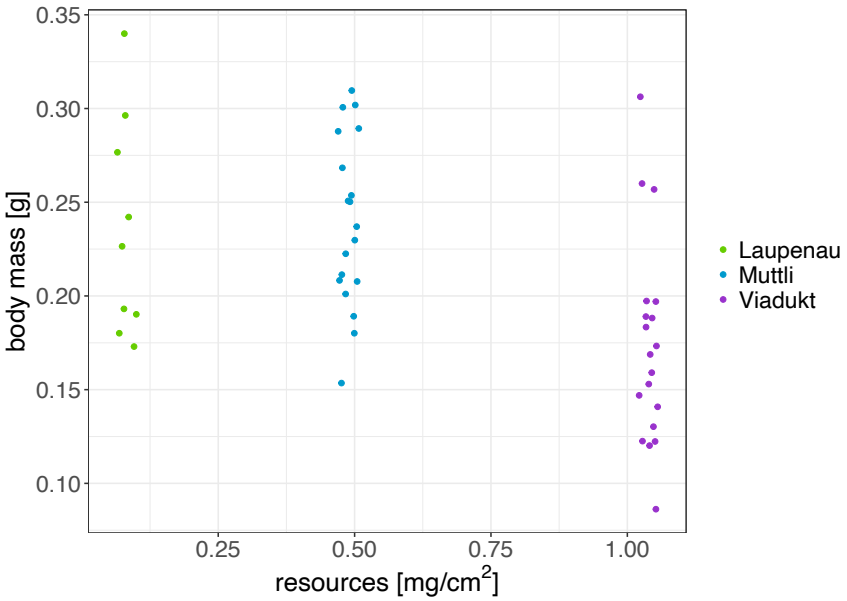

Figure 16: Points show body mass [g] of individual *H. arborea* juveniles at metamorphosis, plotted against the resources [mg/cm^2] that accumulated between the 6 May and 3 July. Different colours indicate the three different ponds (Laupenau, Muttli and Viadukt).

Length of larval period

R. temporaria juveniles that grew up in Muttli had the shortest larval period, followed by juveniles from Laupenau (Table 7, see Figure 28, Appendix). Juveniles from Viadukt took an intermediate number of days. However, compared to the other three ponds, the variance in Viadukt was much higher. The longest larval period was found in Fofere. AIC values differed considerably between the models tested (Table 8). Total pesticide concentration and length of the larval period showed a clear, negative correlation (p-value: 0.003, value: -48.024, std. error: 8.312) (Figure 17). Additionally, there was a negative relationship between minimal temperature during the larval period and length of the larval period (p-value: 0.019, value: -17.439, std. error: 5.081) (Figure 18). The effects of other temperature variables and resource availability on the length of the larval period were not strong.

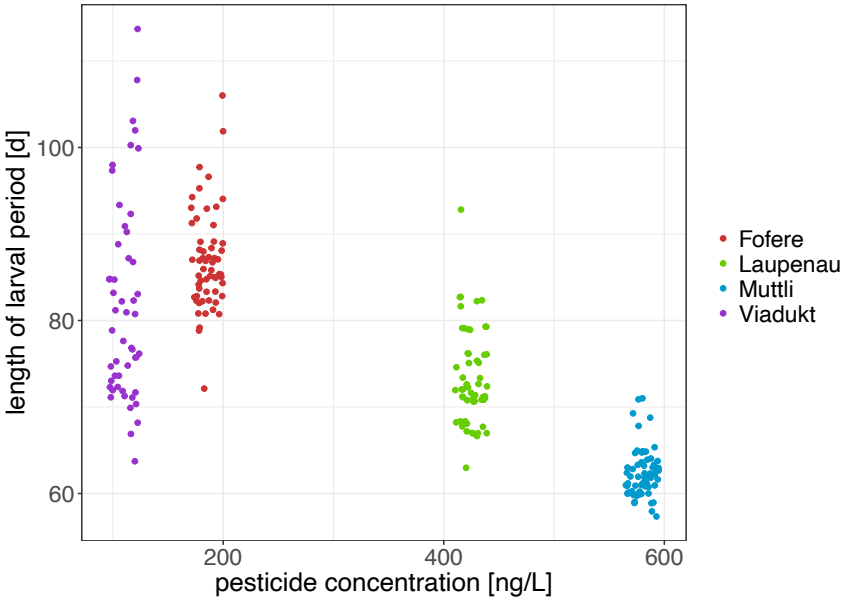

Figure 17: Points show the length of the larval period [d] of individual *R. temporaria* juveniles, plotted against total pesticide concentration in the water [ng/L] on 20 May. Different colours represent the four different ponds (Fofere, Laupenau, Muttli and Viadukt).

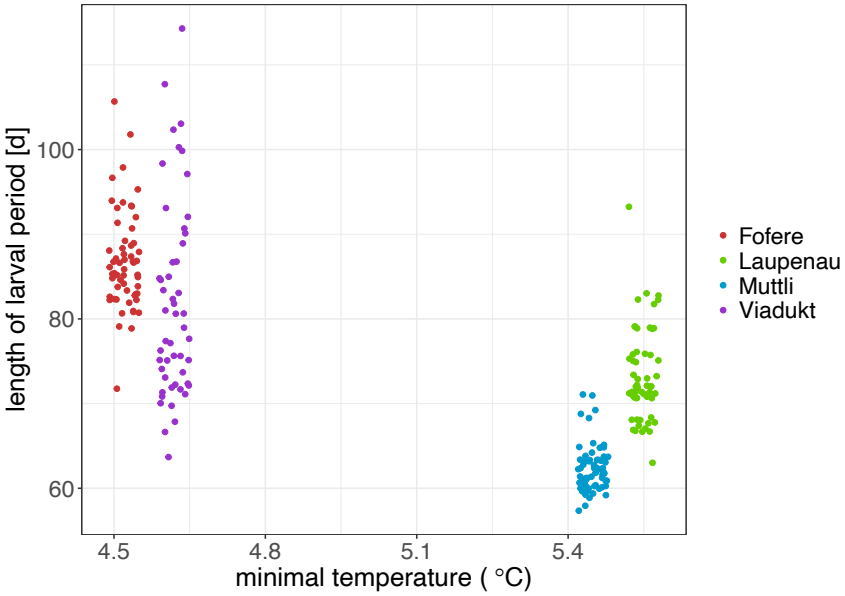

Figure 18: Points show the length of the larval period [d] of individual *R. temporaria* juveniles, plotted against minimal temperature (°C) during the larval period. Different colours represent the four different ponds (Fofere, Laupenau, Muttli and Viadukt).

For *H. arborea*, the length of the larval period was similar in all ponds (Table 7, see Figure 29, Appendix). Additionally, the overall variance was much higher due to a lower number of data points. Model selection revealed that the AICs of all models tested were close to the intercept only model (Table 8). Accordingly, there was no clear relationship between total pesticide concentration and length of the larval period (p-value: 0.302, value: 7.42, std. error: 7.29) (Figure 19). Furthermore, neither temperature variables nor resource availability affected the length of the larval period clearly.

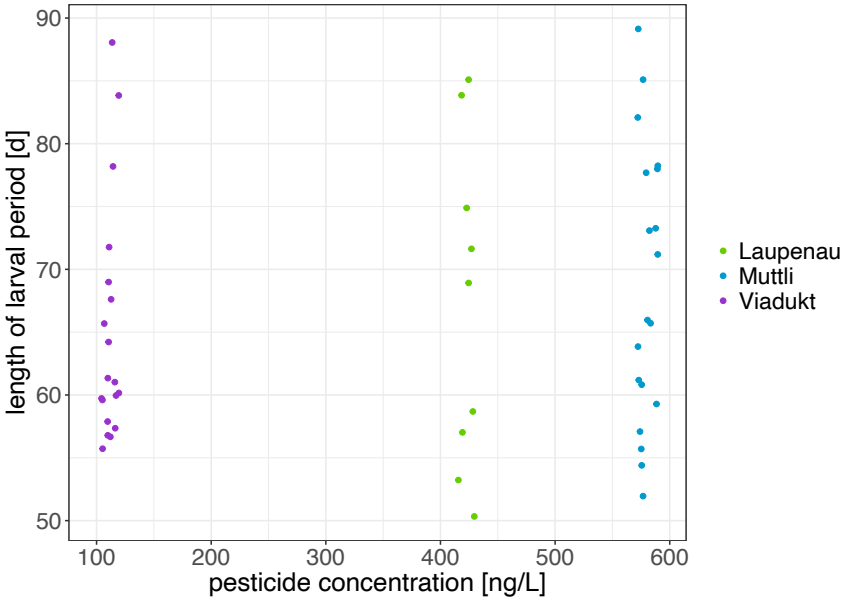

Figure 19: Points show length of larval period [d] of individual *H. arborea* juveniles, plotted against total pesticide concentration in the water [ng/L] on 20 May. Different colours represent the three different ponds (Laupenau, Muttli and Viadukt).

Table 7: Summary of mean body mass and mean size at metamorphosis, as well as mean length of the larval period per species and per pond (± standard deviation). Rt stands for parameters of *R. temporaria* and Ha for parameters of *H. arborea*.

Pond	Size [mm]		Body mass [g]		Length of larval period [d]	
	Rt	Ha	Rt	Ha	Rt	Ha
Fofere	11.67 (± 0.96)	-	0.149 (± 0.032)	-	86.81(± 5.74)	-
Laupenau	11.97 (± 0.81)	12.98 (± 1.36)	0.146 (± 0.040)	12.98 (± 1.36)	73.18 (± 5.44)	67.11 (± 13.01)
Muttli	14.98 (± 1.20)	13.65 (± 1.17)	0.317 (± 0.077)	13.65 (± 1.17)	62.25 (± 2.78)	68.58 (± 11.05)
Viadukt	12.70 (± 1.18)	12.22 (± 1.16)	0.174 (± 0.049)	12.22 (± 1.16)	81.88 (±11.49)	65.05 (± 9.39)

Table 8: Summary of AIC values obtained through linear mixed model analysis of body mass, size and length of larval period against the explanatory variables for both study organisms (*R. temporaria* und *H. arborea*). AIC values indicating relationships between explanatory and response variables are **highlighted**.

Response variable	Explanatory variable	AIC (*R. temporaria*)	AIC (*H. arborea*)
Size	~ 1 (intercept only)	681.769	162.903
	~ pesticide concentration	681.541	**158.019**
	~ resources	683.031	163.393
	~ mean temperature	683.497	164.863
	~ variation temperature	681.051	164.781
	~ minimal temperature	682.906	164.550
	~ maximal temperature	683.577	162.282
Body mass	~ 1 (intercept only)	-757.567	-130.851
	~ pesticide concentration	-758.089	**-135.845**
	~ resources	-755.921	**-133.822**
	~ mean temperature	-755.891	-129.169
	~ variation temperature	-757.977	-129.022
	~ minimal temperature	-756.347	-130.924
	~ maximal temperature	-755.630	-129.383
Length of larval period	~ 1 (intercept only)	1568.183	363.125
	~ pesticide concentration	**1475.904**	364.058
	~ resources	1484.539	364.558
	~ mean temperature	1484.102	365.111
	~ variation temperature	1484.392	365.073
	~ minimal temperature	**1479.013**	364.923
	~ maximal temperature	1484.264	364.458

3.3 Population projection (*R. temporaria*)

Different pond conditions led to different survival rates from the pre-juvenile to the juvenile stage. However, even in Viadukt, where survival was the lowest, the population was still projected to grow ($\lambda_{SSD} > 1$) (Figure 20). Nevertheless, the population growth rate showed a substantial decrease of 11% from highest to lowest λ_{SSD}.

If, in addition to decreases in pre-juvenile to juvenile survival, adult survival was reduced from 0.43 to 0.3 and clutch size was decreased from 650 to 500 ova, the population growth rate (λ_{SSD}) declined to rates between 1.01 and 1.11, approaching stable population sizes (Figure 20).

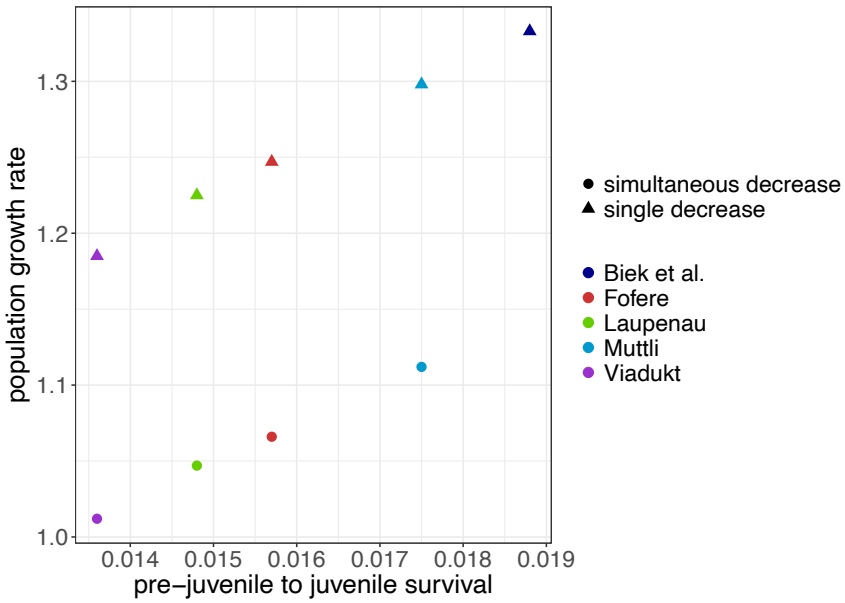

Figure 20: Triangles show the population growth rate (λ_{SSD}) associated with the different pre-juvenile to juvenile survival rates per pond. Red, green, light blue and violet represent the four different ponds (Fofere, Laupenau, Muttli, Viadukt) and dark blue the baseline of Biek et al. (2002). Points show the population growth rate (λ_{SSD}), if in addition to the decrease in pre-juvenile to juvenile survival rate, adult survival rate and clutch size is decreased.

3.4 Comparison between years

Pesticide contamination

Muttli displayed a pronounced decrease in total pesticide concentration by 88.8% within the three years of measurement. The other three ponds did not show such a clear pattern and fluctuated over the years within a much smaller range (Figure 21a).

When looking at the number of pesticides over the critical value of 100 ng/L, Muttli again showed a substantial decrease from nine formulations to only one formulation within the three years of measurement. The other three ponds did not show such a rapid decrease. Fofere even showed a minor increase and Laupenau fluctuated between 2 and 1. Viadukt was in both measurements the cleanest with no pesticide exceeding the critical value in both years of measurement (Figure 21b).

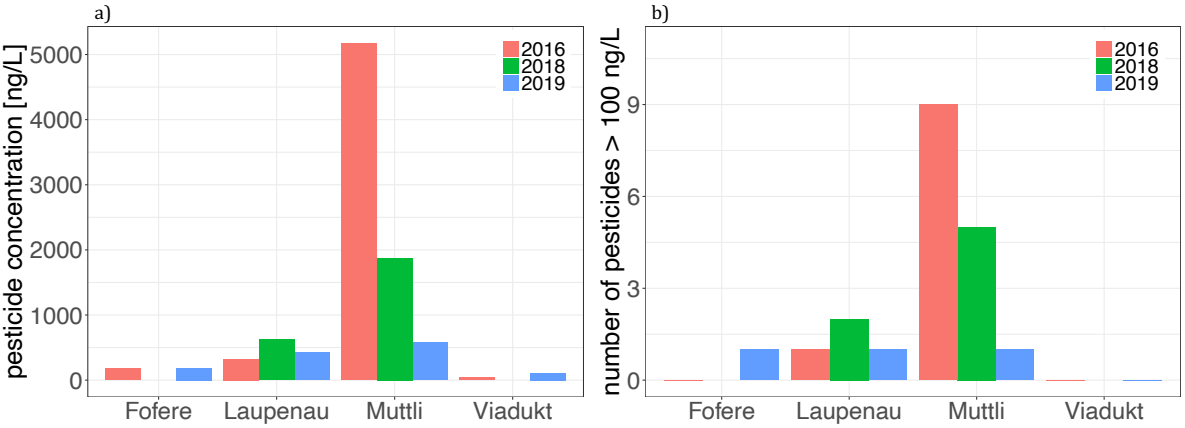

Figure 21: a) Bars show total pesticide concentration in the water [ng/L] of the four ponds (Fofere, Laupenau, Muttli, Viadukt) with different colours representing different years. While for Laupenau and Muttli three years of data is available (2016, 2018, 2019), for Fofere and Viadukt two years of data is available (2018, 2019). b) Bars show the total number of pesticides which exceeded the critical value of 100 ng/L per pond (Fofere, Laupenau, Muttli, Viadukt) with different colours representing different years. For Laupenau and Muttli three years of data is available (2016, 2018, 2019), whereas for Fofere and Viadukt two years of data is available (2018, 2019).

H. arborea *life history traits*

A comparison between the datasets of 2018 and 2019 revealed that survival rates of tadpoles were lower for both Laupenau and Muttli in 2019, but especially for Muttli (Table 9).

When looking at the size at metamorphosis, one can see pronounced differences in the study of 2018 between the two ponds. Juveniles raised in Muttli were substantially larger than their conspecifics raised in Laupenau (Table 9). However, in 2019, differences vanished almost completely (Figure 22). There was a clear, positive relationship between total pesticide concentration and size at metamorphosis (p-value: < 0.0001, value: 0.0032, std. error: 0.0002). Year, however, did not correlate clearly with size (p-value: 0.152, value: 0.422, std. error: 0.298).

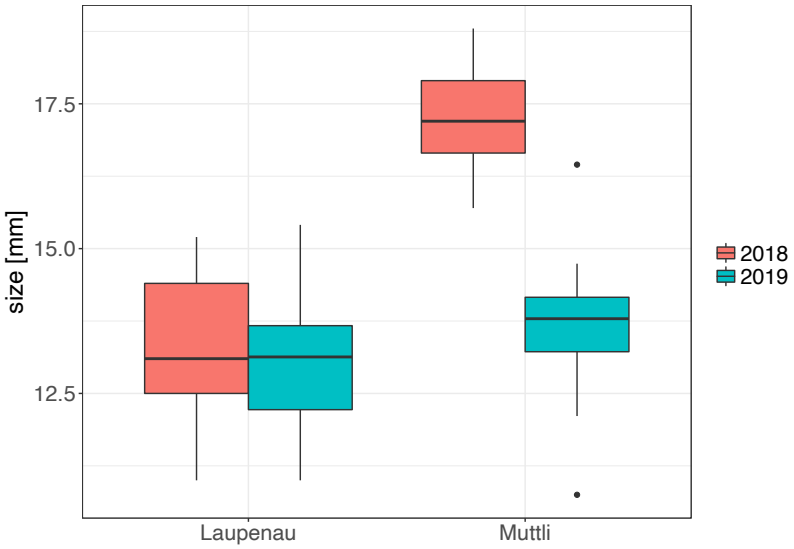

Figure 22: Boxplots show sizes of *H. arborea* juveniles at metamorphosis [mm] per pond (Laupenau, Muttli) and year. Black bars represent the median, whiskers the 95% confidence interval, boxes the quantiles and different colours the two years of measurement.

When looking at the length of the larval period, one can see that in 2018, juveniles raised in Muttli finished metamorphosis earlier than juveniles raised in Laupenau (Table 9). One year later, however, the differences vanished to a large extent (Figure 23). There was a clear, negative correlation between total pesticide concentration and length of the larval period (p-value: < 0.0001, value: -0.0276, std. error: 0.0016). Additionally, year displayed a negative relationship with length of the larval period (p-value: 0.003, value: -9.209, std. error: 3.000).

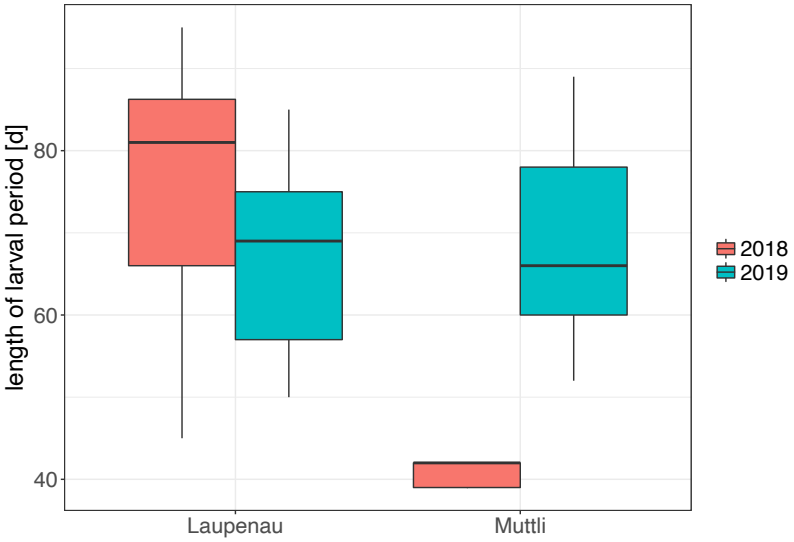

Figure 23: Boxplots show length of larval period [d] of *H. arborea* juveniles per pond (Laupenau, Muttli) and year. Black bars represent the median, whiskers the 95% confidence interval, boxes the quartiles and different colours the two years of measurement.

Table 9: Summary of mean survival rates, mean length of larval period and mean size at metamorphosis of *H. arborea* per year and pond (± standard deviation).

	2018 (Pittet & Zumbach 2018)		2019	
	Laupenau	Muttli	Laupenau	Muttli
Survival rate	0.95 (± 0.07)	0.93 (± 0.05)	0.85 (± 0.13)	0.65 (± 0.13)
Length of larval period [d]	75.10 (± 13.6)	40.60 (± 1.5)	67.10 (± 13.0)	68.60 (± 11.1)
Size [mm]	13.33 (± 1.09)	17.40 (± 0.86)	12.98 (± 1.36)	13.65 (± 1.17)

4 Discussion

Based on the results of numerous laboratory studies, I expected to find negative effects of elevated pesticide concentrations (> 500 ng/L) and pesticide mixtures on amphibian growth, development, and survival under natural conditions. However, most of the hypotheses were not supported by my experimental results, suggesting that the situation under natural conditions is not as unambiguous as under laboratory conditions.

Environmental variables known to affect the performance of larval amphibians differed considerably among the four ponds. While Viadukt displayed the highest resource availability, Laupenau reached the highest water temperatures and Muttli contained the highest total pesticide concentration. Such variability in conditions generates noise, which would not be allowed in classical laboratory studies assessing the risk posed by pesticides. However, the reason to carry out a field experiment was exactly to allow such variability and to investigate how pesticides affect amphibians under actual exposure conditions. That actual exposure conditions can differ substantially from laboratory conditions was, for instance, reported by Allran & Karasov (2001). At the end of their study, they concluded that pesticide concentrations inducing deleterious effects on larvae in the laboratory might be considerably higher than those typically found in surface water. This highlights that ultimately; the findings relevant for conservation measures are those observed under natural conditions.

I hypothesized that tadpoles would exhibit a lower survival rate when exposed to elevated pesticide concentrations. My experimental results supported this hypothesis only partially. While survival of *H. arborea* tadpoles was indeed lowest in Muttli, where total pesticide concentration was highest, the exact opposite was found for *R. temporaria* tadpoles whose survival was highest in Muttli. One explanation could be that *H. arborea* reacts more sensitively to pesticide exposure than *R. temporaria*. Such variable sensitivity was also found by Bridges & Semlitsch (2000), who demonstrated that multiple ranid species responded differently to sublethal concentrations of carbaryl. They concluded, however, that comparing the sensitivity of species is often difficult because of the variation at the individual, family and population level. In my data, reasonable variability existed at the individual level, but variability among clutches of *R. temporaria* was small, substantiating the fact that different sensitivities within the population under observation could be possible. Alternatively, the observed difference in survival could also have been confounded by the fact that the experiment had to be terminated before all *H. arborea* tadpoles were able to complete metamorphosis. Especially in Laupenau, where I observed the highest survival rate for *H. arborea*, late mortality increased substantially for *R. temporaria* as metamorphosis approached and might have done the same for *H. arborea*. This would indicate that pesticide exposure did not affect larval survival of either species noticeably under the conditions observed.

A possible explanation for the substantial increase in late mortality in Laupenau might be that larvae become increasingly sensitive to stressors as they are approaching metamorphosis. Metamorphosis is a developmentally and physiologically challenging process (Howe et al. 1998), where growth and differentiation occur rapidly (Smith-Gill & Berven 1979). Laboratory studies have shown that growth is often highest when resource level and temperature are suitable (Alvarez & Nicieza 2002, Schmidt et al. 1998). Therefore, the combination of limited resource availability, high water temperatures and possibly a consequent high pH might have led to

metabolic stress in Laupenau. High metabolic stress during a sensitive stage could be responsible for the observed mass loss followed by death in several tadpoles, suggesting that not all developmental stages are equally affected by suboptimal growth conditions. Accordingly, Howe et al. (1998) observed that *Bufo americanus* and *Rana pipiens* larvae close to metamorphosis reacted more sensitively to herbicide exposure than early stage larvae.

Next, I hypothesized that tadpoles exhibit slower growth, are therefore smaller and have lower mass at metamorphosis when raised in a pond with elevated pesticide concentrations. However, I found that the tadpoles of both species grew fastest in Muttli, where pesticide concentration was highest. Furthermore, total pesticide concentration correlated positively with size and body mass of *H. arborea* juveniles at metamorphosis, whereas it correlated negatively with the length of the larval period of *R. temporaria* juveniles.

Multiple laboratory and mesocosm studies have shown that pesticides can inhibit the growth of tadpoles, lowering body mass (Brunelli et al. 2009) and size at metamorphosis (Mazanti et al. 2003, Bridges 2000), as well as prolonging the larval phase (Hayes et al. 2006). Such inhibition can be detrimental, as small and thin juveniles are more prone to starvation and desiccation and less likely to escape predators (Smith 1987, Berven & Gill 1983). Long larval periods can be disadvantageous as well, especially when not associated with larger sizes. For temporary pond breeders, long larval periods are particularly unfavourable since pond drying is an important mortality factor (Cooke 1985). Even though I did find positive correlations between certain life history traits and total pesticide concentration, the possibility of growth inhibition cannot be excluded since I do not know whether growth would have been higher without pesticides in the water. However, my results suggest that patterns observed in the field cannot be directly predicted based on insights from laboratory studies. It seems like some environmental factors might have a stronger influence on growth and development than environmentally relevant concentrations of pesticides.

Resource availability, for example, was shown to influence size and mass at metamorphosis, as well as the length of the larval period under laboratory conditions (Leips & Travis 1994, Rohr et al. 2004). Besides, it was demonstrated that amphibians also exhibit a strong dependence of growth and differentiation processes upon temperature (Sanuy et al. 2008, Brattstrom, 1963). Yet, my results showed only few clear relationships between life history traits and these environmental variables. There was a negative relationship between the length of the larval period and minimal temperature in *R. temporaria*. Contrary to my expectation, body mass of *H. arborea* correlated negatively with resource availability. Possibly, taking only one resource measurement during the whole experiment prevented me from detecting differences in resource availability during critical stages of development. Still, my experimental results suggest that also patterns associated with temperature and resource availability might differ under natural conditions from what is expected based on laboratory studies.

Likely, interactions between different environmental conditions contributed to the observed patterns. My results show evidence that certain life history traits were simultaneously affected by multiple environmental conditions. A comprehensive review of Holmstrup et al. (2010) revealed that while high temperatures were found to increase toxicity in a wide range of studies with aquatic organisms, the interaction of resource availability and pesticide toxicity is less clear. Either high resource availability provided the energy needed for detoxification processes or lead

to increased chemical uptake through bioconcentration. Therefore, interactions between nutritional conditions and toxicants may be very complex, either masking or enhancing the potential effects of pesticides on tadpoles. With the presence of interactions, or even indirect effects, the significance of classical ecotoxicological risk assessment (ERA) decreases. The laboratory-based single-product single-species approach might ignore crucial modulating factors with the consequence that results gained in the laboratory could not be directly extrapolated to actual exposure conditions.

Further, Agostini et al. (2020), who conducted a similar study to mine, but with much higher concentrations, found severe lethal and sublethal effects in four anuran species. This suggests that besides environmental conditions, the dose might be key in determining the effect. While high concentrations could be acutely toxic, low to moderate concentrations might not be deleterious under natural conditions. Therefore, a threshold response toward pesticide concentrations is likely. Johansson et al. (2006), for example, observed strong negative effects on growth and survival when acutely exposing *R. temporaria* tadpoles to high concentrations of six pesticides in a laboratory study. Though, when exposing them to low concentrations, negative effects on both parameters vanished, even in chronic exposure tests. Accordingly, Davidson (2004) observed that when upwind pesticide use was above a certain threshold value, the five anuran species under investigation were overwhelmingly absent from field sites. This could point out that concentrations that impact tadpoles negatively might be higher than those present in the ponds I conducted my study in. The several free-living amphibian species, which could be observed in all four ponds, substantiate this explanation. Alternatively, it could indicate that up to certain concentrations, environmental conditions with stronger effects can compensate for or mask impacts of pesticides, but above a certain threshold, effects of pesticides could outweigh other influences.

Further, I hypothesized that decreases in the pre-juvenile to juvenile survival rate of *R. temporaria* might not translate into effects at the population level. Indeed, my results suggest that the population was still projected to grow, despite the observed decreases. Yet, the vital rates presented by Biek et al. (2002) result in a high λ_{SSD}, indicating that they are likely too optimistically estimated. Nevertheless, the implementation of the observed decreases lowered the population growth considerably. An elasticity analysis by Biek et al. (2002) unveiled that elasticities of individual vital rates did not differ greatly, but were highest for juvenile survival. However, in amphibians, the survival of juveniles often depends on the size at metamorphosis (Smith 1987), highlighting the importance of growth conditions during the larval stage. Further, Biek et al. (2002) conducted a life stage simulation analysis (LSA) to take also the observed variation of each vital rate into account. The LSA revealed that larval survival can have a strong effect on the population growth, because of its high variability among years. If the lower end of the variability could be eliminated, the positive effect on the population growth rate should be strong (Biek et al. 2002). Hence, improving the growth conditions of larvae, thereby increasing size at metamorphosis and larval survival, provides an effective way to increase overall population growth.

Differences in the population growth rate are exacerbated, if vital rates that are likely to be simultaneously affected are combined to cumulative elasticities (Biek et al. 2002). Simultaneous decreases in pre-juvenile and adult survival are realistic since adults are likely to experience pesticide exposure as well when migrating from and to spawning waters in agricultural

landscapes (Brühl et al. 2013). The combination of decreases in pre-juvenile to juvenile survival and adult survival, as well as in clutch size, reinforced the effect on the population growth rate, decreasing λ_{SSD} close to one. This implies that risk assessment, which considers only vital rates associated with tadpoles, might underestimate the threat posed by pesticides (Ockleford et al. 2017).

Lastly, I compared the patterns observed in the studies of 2016 and 2018 with those of 2019. While total pesticide concentration, as well as the number of pesticide formulations exceeding the critical value, decreased heavily in Muttli, they only changed moderately in the other three ponds. This emphasizes that pesticide load can vary substantially between ponds and years. Yet, the continuous decrease in pesticide load in Muttli aligns with the incorporation of the swiss federal 'Aktionsplan Pflanzenschutzmittel' in 2017. In this plan of action, a reduction of the risk originating from pesticides is intended and alternatives to pesticides are promoted. To verify if the decrease will be permanent, or if fluctuations will continue, further measurements in the future are needed. However, my results demonstrate that taking only one pesticide measurement in a single year is not enough. Pesticide levels have to be assessed over multiple years to evaluate actual exposure conditions.

A similar trend as in pesticide load was observed when comparing life history traits among the years. While in 2018, differences in life history traits of *H. arborea* among ponds were substantial, they vanished to a large extent in 2019, showing that fluctuations between ponds and years can be considerable. Total pesticide concentration correlated positively with size at metamorphosis and negatively with length of the larval period. This indicates that the assessment of life history traits in one year allows the detection of decisive patterns. However, the comparison between the two years of data revealed that to clearly evaluate the effects of pesticides, multiple years of assessment of life history traits are required.

Certainly, there is need for further research and actions in multiple areas. Firstly, it is crucial to obtain more monitoring data of pesticide levels in small, standing waters. With continuous measurement and surveillance, the prediction of exposure can be improved. Further, the payoff of measures like the 'Aktionsplan Pflanzenschutzmittel' can be quantified and, if necessary, adapted. Such monitor programs already exist for rivers and streamlets in Switzerland, where monthly water samples are collected and analysed (Balsiger 2007) and could serve as an example. Secondly, tadpoles, juveniles and adults should be monitored to quantify individual and population level effects (Böll et al. 2013). Monitoring could help to answer questions like whether pesticide exposure as a larva leads to fitness consequences in later life stages under natural conditions. Lastly, repeating this study with a higher number of ponds and more pronounced differences in pesticide load among the ponds would allow investigating interactions between pesticides and different environmental conditions. Insights from monitoring, as well as insights in interactive effects, could then be implemented in ERA to make results more meaningful.

Conclusion

This study shows that the impacts of pesticide concentrations on amphibian growth, development and survival under natural conditions are not as clear-cut as in the laboratory. Considerable variation in the investigated variables existed among ponds, years and especially among individuals; however, certain life history traits were clearly affected by some environmental conditions. Even though inhibiting effects of pesticides on growth cannot be

precluded and responses might be dose-dependent, my results suggest that some environmental conditions with a strong effect on growth and development, or interactions thereof, can override potential effects of pesticides. Therefore, knowledge acquired in the laboratory cannot be extrapolated directly to the field, highlighting the importance of conducting more studies under natural conditions.

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6 Statement of Authorship

I declare that I have used no other sources and aids other than those indicated. All passages quoted from publications or paraphrased from these sources are indicated as such, i.e. cited and/or attributed. This thesis was not submitted in any form for another degree or diploma at any university or other institution of tertiary education.

Zurich, 30.04.2020

7 References

Agostini MG, Roesler I, Bonetto C, Ronco AE, Bilenca D (2020) Pesticides in the real world: The consequences of GMO-based intensive agriculture on native amphibians. Biol Conserv 241:108355

Aldrich A, Junghans M, Aeberli C, Brühl CA, Streissl F, Schmidt BR (2016) Amphibians and plant-protection products: what research and action is needed? Environ Sci Eur 28:17

Allran JW, Karasov WH (2001) Effects of atrazine on embryos, larvae, and adults of anuran amphibians. Environ Toxicol Chem 20:769–775

Alvarez D, Nicieza AG (2002) Effects of temperature and food quality on anuran larval growth and metamorphosis. Funct Ecol 16:640–648

Balsiger C (2007) Gewässerbelastung durch Pestizide. Zürich

Bernabò I, Sperone E, Tripepi S, Brunelli E (2011) Toxicity of Chlorpyrifos to Larval Rana dalmatina: Acute and Chronic Effects on Survival, Development, Growth and Gill Apparatus. Arch Environ Contam Toxicol 61:704–718

Berrill M, Bertram S, Wilson A, Louis S, Brigham D, Stromberg C (1993) LETHAL AND SUBLETHAL IMPACTS OF PYRETHROID INSECTICIDES ON AMPHIBIAN EMBRYOS AND TADPOLES. Environ Toxicol Chem 12:525–539

Berven KA, Gill DE (1983) Interpreting Geographic Variation in Life-History Traits. Am Zool 23:85–97

Biek R, Funk WC, Maxell BA, Mills LS (2002) What is missing in amphibian decline research: Insights from ecological sensitivity analysis. Conserv Biol 16:728–734

Blaustein AR, Romansic JM, Kiesecker JM (2003) Ultraviolet Radiation, Toxic Chemicals and Amphibian Population Declines. Divers Distrib 9:123–140

Böll S, Schmidt BR, Veith M, Wagner N, Rödder D, Weimann C, Kirschey T, Lötters S (2013) Amphibians as indicators of changes in aquatic and terrestrial ecosystems following GM crop cultivation: a monitoring guideline. BioRisk 8:39–51

Bridges CM (2000) Long-Term Effects of Pesticide Exposure at Various Life Stages of the Southern Leopard Frog (Rana sphenocephala). Arch Environ Contam Toxicol 39:91–96

Bridges CM, Semlitsch RD (2000) Variation in Pesticide Tolerance of Tadpoles among and within Species of Ranidae and Patterns of Amphibian Decline. Conserv Biol 14:1490–1499

Brogan WR, Relyea RA (2013) Mitigating with macrophytes: Submersed plants reduce the toxicity of pesticide-contaminated water to zooplankton. Environ Toxicol Chem 32:699–706

Brühl CA, Schmidt T, Pieper S, Alscher A (2013) Terrestrial pesticide exposure of amphibians: An underestimated cause of global decline? Sci Rep 3:1135

Brunelli E, Bernabò I, Berg C, Lundstedt-Enkel K, Bonacci A, Tripepi S (2009) Environmentally relevant concentrations of endosulfan impair development, metamorphosis and behaviour in Bufo bufo tadpoles. Aquat Toxicol 91:135–142

Damalas CA (2009) Understanding benefits and risks of pesticide use. Sci Res Essay 4:945–949

Davidson C (2004) DECLINING DOWNWIND: AMPHIBIAN POPULATION DECLINES IN CALIFORNIA AND HISTORICAL PESTICIDE USE. Ecol Appl 14:1892–1902

Davidson C, Shaffer HB, Jennings MR (2002) Spatial Tests of the Pesticide Drift, Habitat Destruction, UV-B, and Climate-Change Hypotheses for California Amphibian Declines. Conserv Biol 16:1588–1601

Dirzo R, Young HS, Galetti M, Ceballos G, Isaac NJB, Collen B (2014) Defaunation in the Anthropocene. Science (80-) 345:401–406

Foley JA, Defries R, Asner GP, Barford C, Bonan G, Carpenter SR, Chapin FS, Coe MT, Daily GC, Gibbs HK, Helkowski JH, Holloway T, Howard EA, Kucharik CJ, Monfreda C, Patz JA, Prentice IC, Ramankutty N, Snyder PK (2005) Global Consequences of Land Use. Science (80-) 309:570–574

Fryday S, Thompson H (2012) Toxicity of pesticides to aquatic and terrestrial life stages of amphibians and occurrence, habitat use and exposure of amphibian species in agricultural environments. Sand Hutton

Gibbons MM, McCarthy TK (1986) The reproductive output of frogs Rana temporaria (L.) with particular reference to body size and age. J Zool 209:579–593

Gosner K (1960) A simplified table for staging anuran embryos and larvae with notes on identification. Herpetologica 16:183–190

Grube A, Donaldson D, Kiely T, Wu L (2011) Pesticide Industry Sales and Usage Report: 2006 and 2007 Market Estimates. Washington DC

Hayes TB, Case P, Chui S, Chung D, Haeffele C, Haston K, Lee M, Mai VP, Marjuoa Y, Parker J, Tsui M (2006) Pesticide mixtures, endocrine disruption, and amphibian declines: are we underestimating the impact? Environ Health Perspect 114:40–50

Holmstrup M, Bindesbøl AM, Oostingh GJ, Duschl A, Scheil V, Köhler HR, Loureiro S, Soares AMVM, Ferreira ALG, Kienle C, Gerhardt A, Laskowski R, Kramarz PE, Bayley M, Svendsen C, Spurgeon DJ (2010) Interactions between effects of environmental chemicals and natural stressors: A review. Sci Total Environ 408:3746–3762

Houlahan JE, Findlay CS, Schmidt BR, Meyer AH, Kuzmin SL (2000) Quantitative evidence for global amphibian population declines. Nature 404:752–755

Howe GE, Gillis R, Mowbray RC (1998) Effect of chemical synergy and larval stage on the toxicity of atrazine and alachlor to amphibian larvae. Environ Toxicol Chem 17:519–525

Johansson M, Piha H, Kylin H, Merilä J (2006) TOXICITY OF SIX PESTICIDES TO COMMON FROG (RANA TEMPORARIA) TADPOLES. Environ Toxicol Chem 25:3164–3170

Lambin EF, Meyfroidt P (2011) Global land use change, economic globalization, and the looming land scarcity. PNAS 108:3465–3472

Leips J, Travis J (1994) Metamorphic Responses to Changing Food Levels in Two Species of Hylid Frogs. Ecology 75:1345–1356

Mann RM, Hyne R V, Choung CB, Wilson SP (2009) Amphibians and agricultural chemicals:

Review of the risks in a complex environment. Environ Pollut 157:2903–2927

Mazanti L, Sparling DW, Rice C, Bialek K, Stevenson C, Teels B (2003) Synergistic Effects of a Combined Exposure to Herbicides and an Insecticide in Hyla versicolor. ASTM Int 1443:111–129

Mermod M, Zumbach S, Lippuner M, Pellet J, Schmidt B (2010) Praxismerkblatt Artenschutz Laubfrosch. Neuchâtel

Meyer A, Zumbach S, Schmidt BR, Monney J-C (2009) Auf Schlangenspuren und Krötenpfaden, 1st edn. Haupt, Bern

Ockleford C, Adriaanse P, Berny P, Brock T, Duquesne S, Grilli S, Hernandez-Jerez AF, Hougaard Bennekou S, Klein M, Kuhl T, Laskowski R, Machera K, Pelkonen O, Pieper S, Stemmer M, Sundh I, Teodorovic I, Tiktak A, Topping CJ, Wolterink G, Aldrich A, Berg C, Ortiz-Santaliestra M, Weir S, Streissl F, Smith RH (2017) Scientific Opinion on the state of the science on pesticide risk assessment for amphibians and reptiles. EFSA J 16:301

Ortiz-Santaliestra ME, Maia JP, Egea-Serrano A, Lopes I (2018) Validity of fish, birds and mammals as surrogates for amphibians and reptiles in pesticide toxicity assessment. Ecotoxicology 27:819–833

Pimentel D, Levitan L (1986) Pesticides: Amounts applied and amounts reaching pests. Bioscience 36:86–91

Pittet M, Zumbach S (2018) Impact de la qualité de l ' eau et des pesticides sur le développement de têtards d ' Hyla arborea. Neuchâtel

R Core Team (2015). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL https://www.R-project.org/.

Relyea RA (2005a) THE IMPACT OF INSECTICIDES AND HERBICIDES ON THE BIODIVERSITY AND PRODUCTIVITY OF AQUATIC COMMUNITIES. Ecol Appl 15:618–627

Relyea RA (2005b) THE LETHAL IMPACT OF ROUNDUP ON AQUATIC AND TERRESTRIAL AMPHIBIANS. Ecol Appl 15:1118–1124

Rohr JR, Elskus AA, Shepherd BS, Crowley PH, Mccarthy TM, Niedzwiecki JH, Sager T, Sih A, Palmer BD (2004) Multiple Stressors and Salamanders: Effects of an Herbicide, Food Limitation, and Hydroperiod. Ecol Appl 14:1028–1040

Sanuy D, Oromi N, Galofré A (2008) Effects of temperature on embryonic and larval development and growth in the natterjack toad (Bufo calamita) in a semi-arid zone. Anim Biodivers Conserv 31:41–46

Schmidt BR, Hotz H, Anholt BR, Guex G-D, Semlitsch RD (1998) Factors contributing to the maintenance of the genetic polymorphism at the locus LDH-B in the pool frog, Rana lessonae. Can Jounral Zool 76:795–804

Schmidt BR, Zumbach S (2005) Rote Liste der gefährdeten Amphibien der Schweiz. Bern

Schmidt BR, Zumbach S, Tobler U, Lippuner M (2015) Amphibien brauchen temporäre Gewässer. Zeitschrift für Feldherpetologie 22:137–150

Smith-Gill SJ, Berven KA (1979) Predicting Amphibian Metamorphosis. Am Nat 113:563–585

Smith DC (1987) ADULT RECRUITMENT IN CHORUS FROGS: EFFECTS OF SIZE AND DATE AT METAMORPHOSIS. Ecology 68:344–350

Stoffel D (2016) The detection of aquatic contamination with plant protection products in amphibian reproduction sites. University of Zurich

Stuart SN, Chanson JS, Cox NA, Young BE, Rodrigues ASL, Fischman DL, Waller RW (2004) Status and trends of amphibian declines and extinctions worldwide. Science (80-) 306:1783–1786

Tilman D (1999) Global environmental impacts of agricultural expansion: The need for sustainable and efficient practices. PNAS 96:5995–6000

Tilman D, Fargione J, Wolff B, D'Antonio C, Dobson A, Howarth R, Schindler D, Schlesinger WH, Simberloff D, Swackhamer D (2001) Forecasting agriculturally driven global environmental change. Science (80-) 292:281–284

Vitousek PM, Mooney HA, Lubchenco J, Melillo JM (1997) Human Domination of Earth's Ecosystems. Science (80-) 277:494–499

Zaga A, Little EE, Rabeni CF, Ellersieck MR (1998) Photoenhanced toxicity of a carbamate insecticide to early life stage anuran amphibians. Environ Toxicol Chem 17:2543–2553

8 Appendix

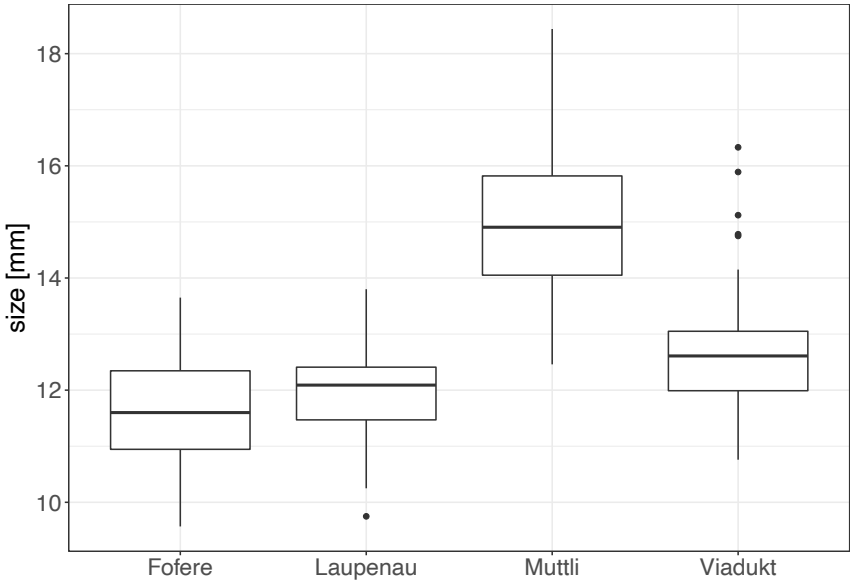

Figure 24: Boxplots show size [mm] of *R. temporaria* juveniles per pond. Black bars represent the median, whiskers represent the 95% confidence interval and boxes the quartiles.

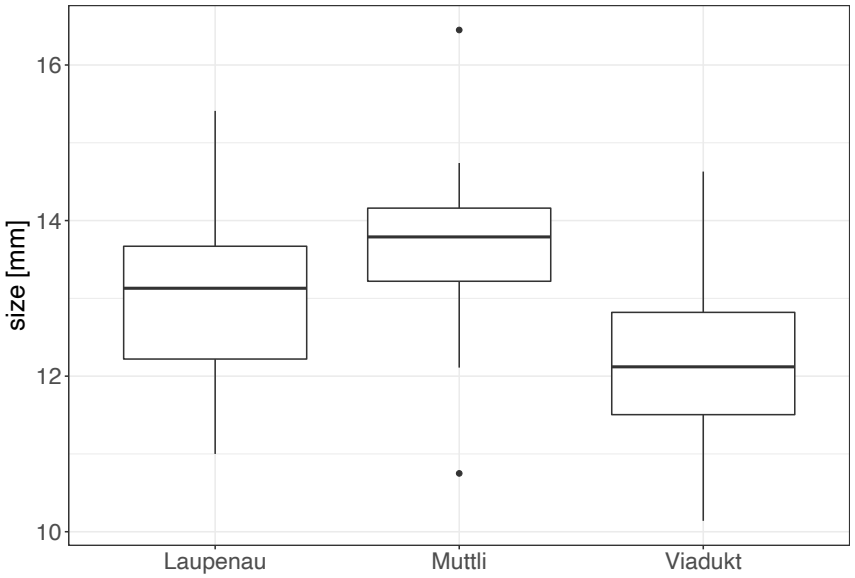

Figure 25: Boxplots show size [mm] of *H. arborea* juveniles per pond. Black bars represent the median, whiskers represent the 95% confidence interval and boxes the quartiles.

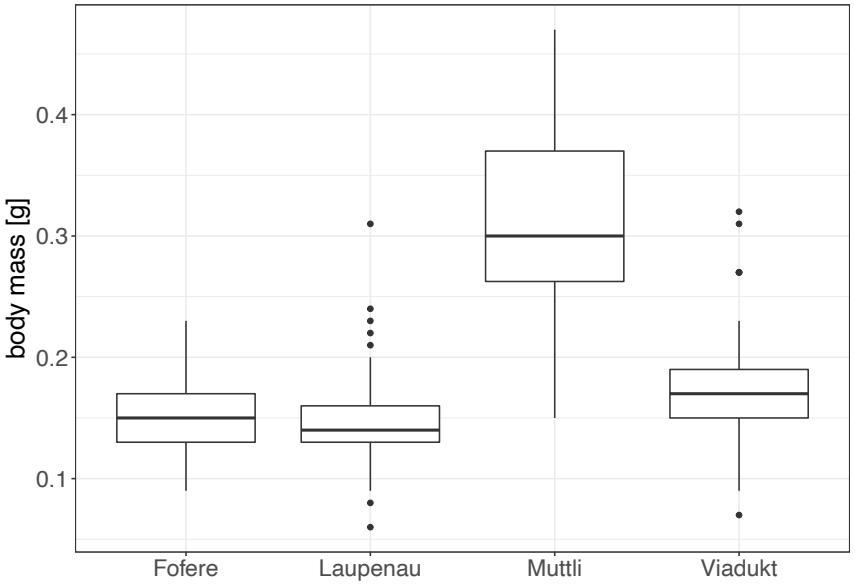

Figure 26: Boxplots show body mass [g] of *R. temporaria* juveniles per pond. Black bars represent the median, whiskers represent the 95% confidence interval and boxes the quartiles.

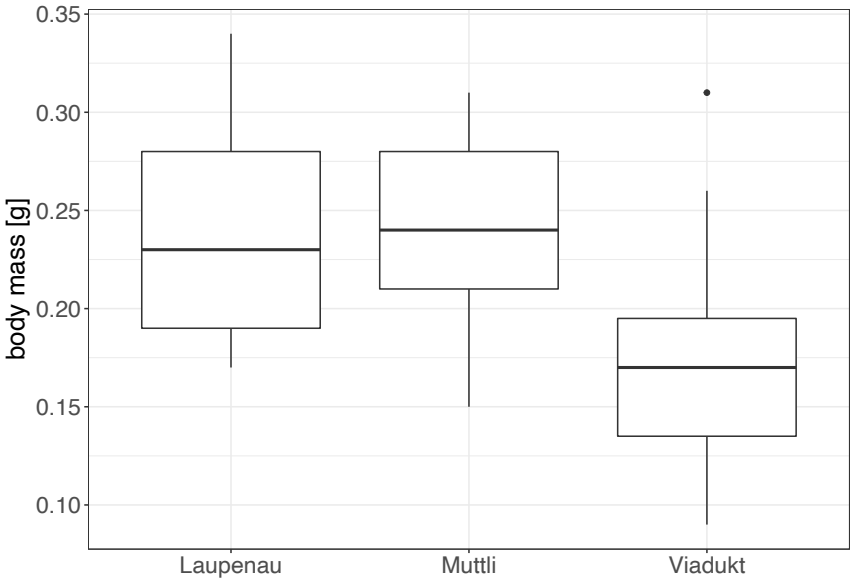

Figure 27: Boxplots show body mass [g] of *H. arborea* juveniles per pond. Black bars represent the median, whiskers represent the 95% confidence interval and boxes the quartiles.

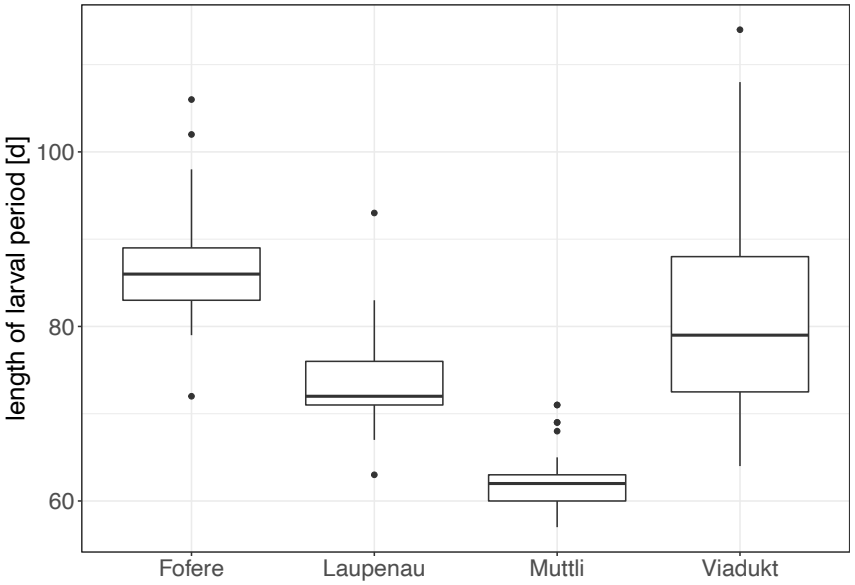

Figure 28: Boxplots show length of larval period [d] of *R. temporaria* juveniles per pond. Black bars represent the median, whiskers represent the 95% confidence interval and boxes the quartiles.

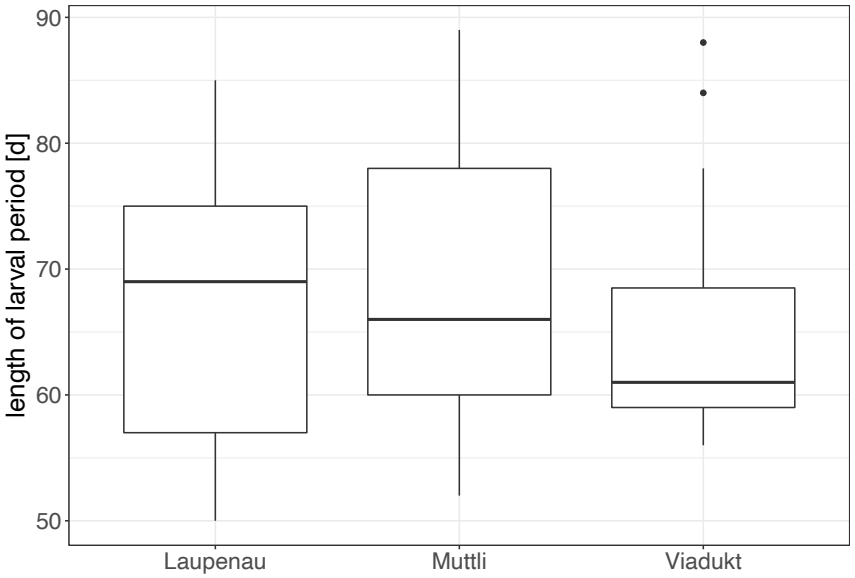

Figure 29: Boxplots show length of larval period [d] of *H. arborea* juveniles per pond. Black bars represent the median, whiskers represent the 95% confidence interval and boxes the quartiles.

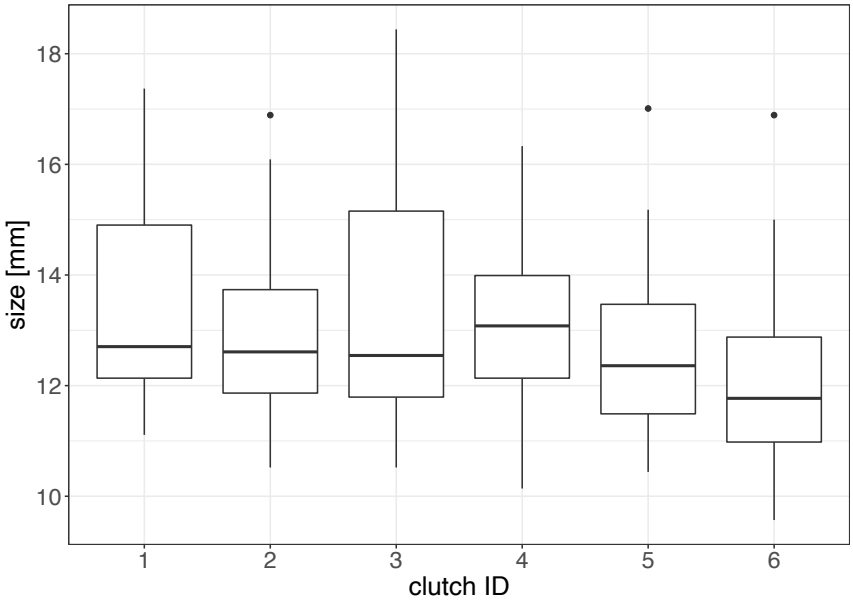

Figure 30: Boxplots show size [mm] of *R. temporaria* juveniles per clutch. Black bars represent the median, whiskers represent the 95% confidence interval and boxes the quartiles.

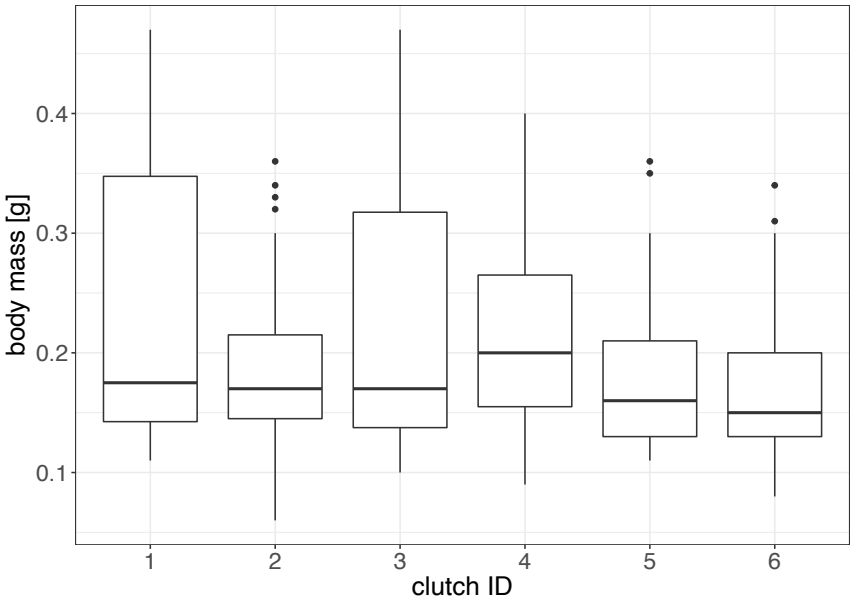

Figure 31: Boxplots show body mass [g] of *R. temporaria* juveniles per clutch. Black bars represent the median, whiskers represent the 95% confidence interval and boxes the quartiles.

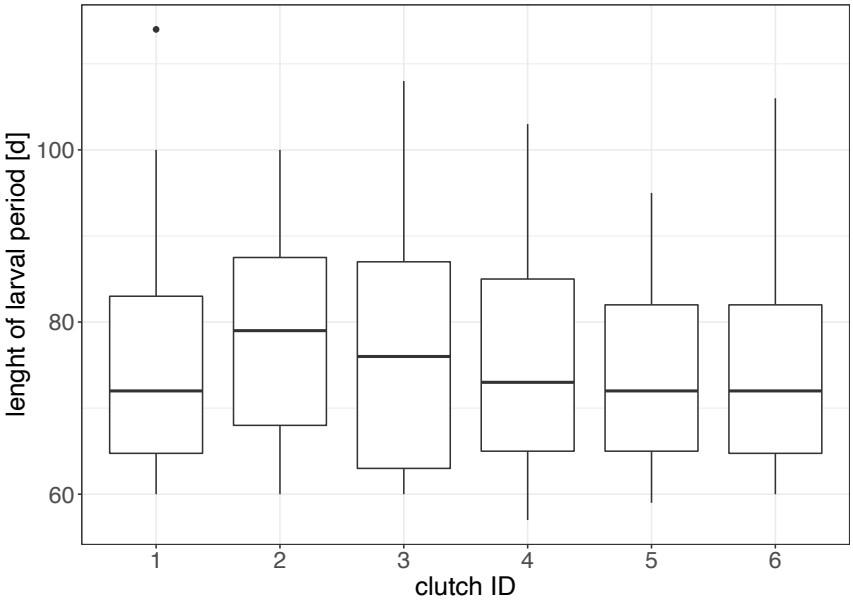

Figure 32: Boxplots show length of larval period [d] of *R. temporaria* juveniles per clutch. Black bars represent the median, whiskers represent the 95% confidence interval and boxes the quartiles.

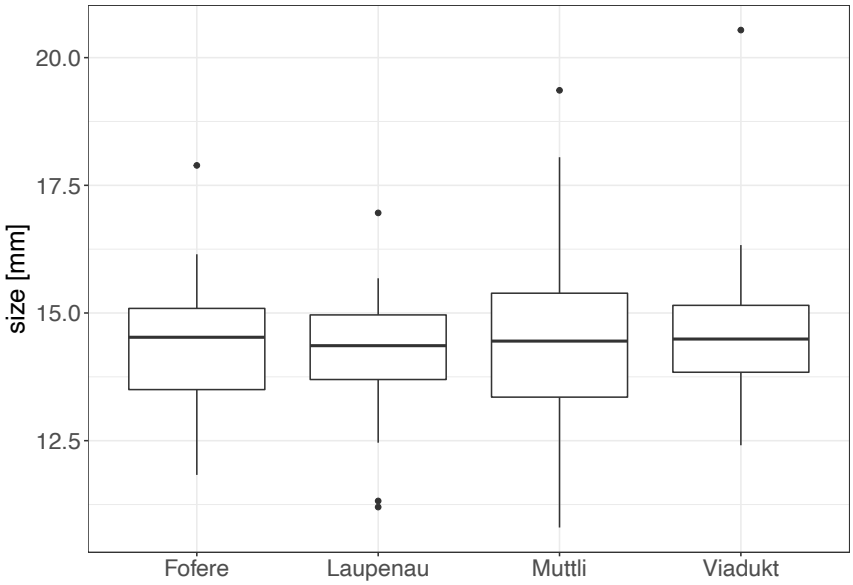

Figure 33: Boxplots show size [mm] of *R. temporaria* tadpoles on the day they were released into the experimental boxes per pond. Black bars represent the median, whiskers represent the 95% confidence interval and boxes the quartiles.

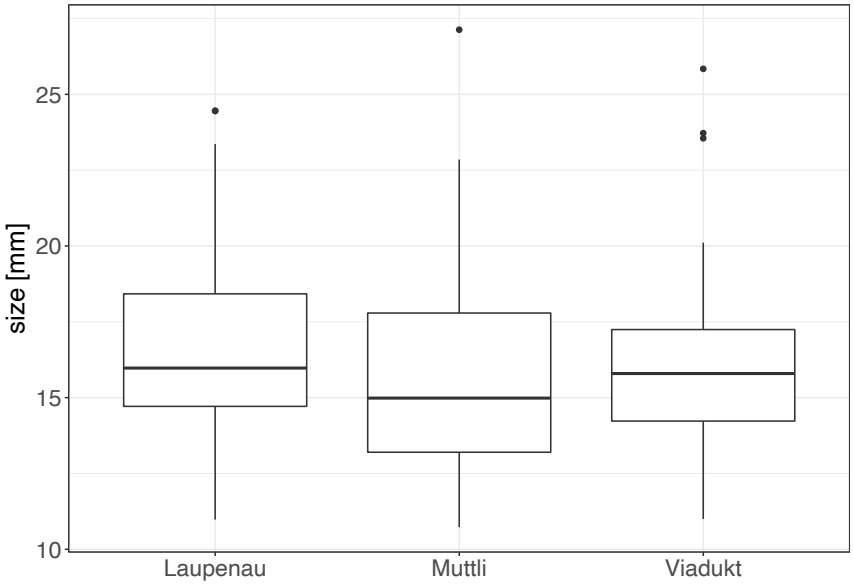

Figure 34: Boxplots show size [mm] of *H. arborea* tadpoles on the day they were released into the experimental boxes per pond. Black bars represent the median, whiskers represent the 95% confidence interval and boxes the quartiles.

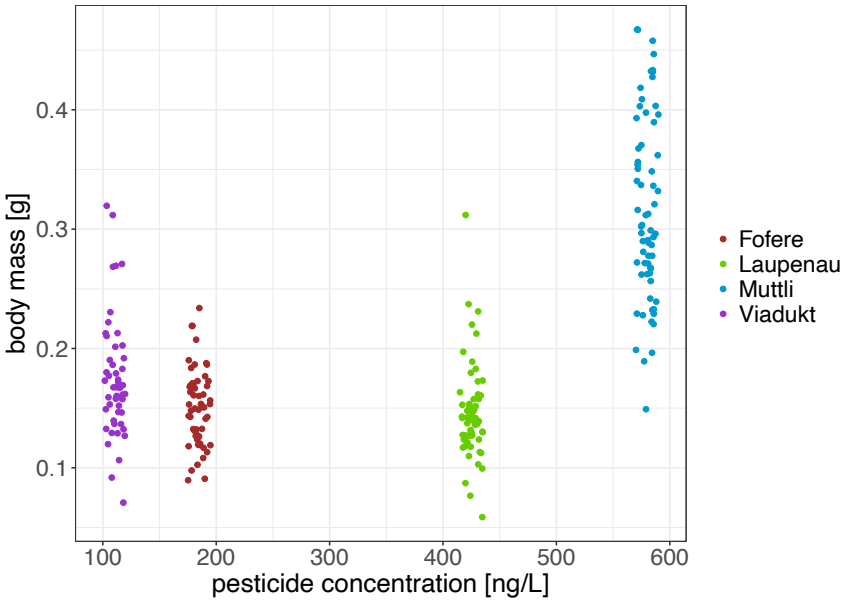

Figure 35: Points show the body mass [g] of *R. temporaria* juveniles, plotted against total pesticide concentration in the water [ng/L] on 20 May. Different colours represent the four different ponds (Fofere, Laupenau, Muttli and Viadukt).

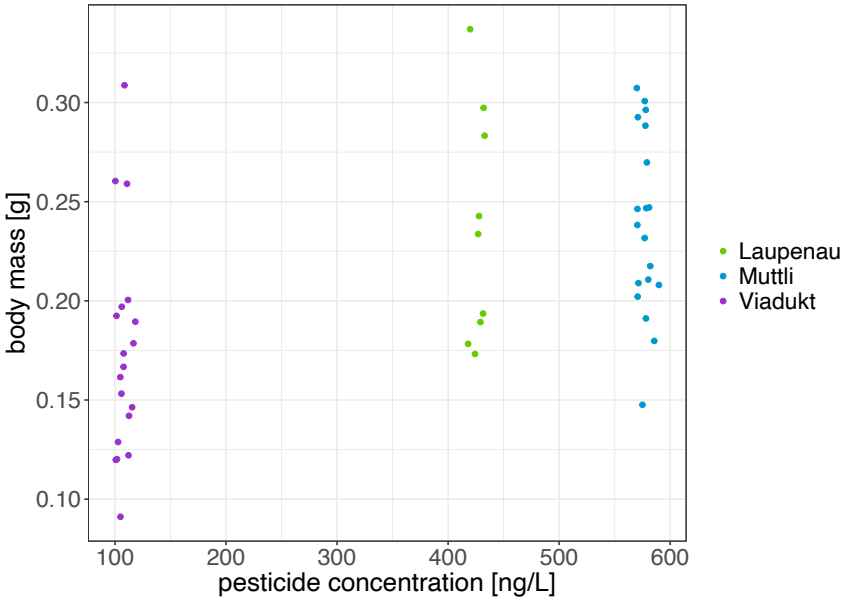

Figure 36: Points show the body mass [g] of of *H. arborea* juveniles, plotted against total pesticide concentration in the water [ng/L] on 20 May. Different colours represent the three different ponds (Laupenau, Muttli and Viadukt).