Low Temperatures Lead to Higher Toxicity of the Fungicide Folpet to Larval Stages of *Rana temporaria* and *Bufotes viridis*

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Abstract

Pesticides are one of the main drivers of the worldwide amphibian decline. Their actual toxicity depends on a number of factors, like the species in focus or the developmental stage of exposed individuals. As ectothermic species, the metabolism of amphibians is influenced by ambient temperature. Therefore, temperature also affects metabolic rates and thus processes that might enhance or reduce toxic effects. Studies about the interactive effect of temperature and toxicity on amphibians are rare and deliver contrasting results. To investigate the temperature-dependent pesticide sensitivity of larvae of two European species we conducted acute toxicity tests for the viticultural fungicide Folpan® 500 SC with the active ingredient folpet at different temperatures (6°C, 11°C, 16°C, 21°C, 26°C). Sensitivity of *Rana temporaria* and *Bufotes viridis* was highly affected by temperature: early larvae (Gosner stage 20) were about twice more sensitive to Folpan® 500 SC at 6°C compared to 21°C. Next to temperature, species and developmental stage of larvae had an effect on sensitivity. The most sensitive individuals (early stages of *R. temporaria* at 6°C) were 14.5 times more sensitive than the least sensitive ones (early stages of *B. viridis* at 26°C). Our results raise concerns about typical ecotoxicological studies with amphibians that are often conducted at temperatures between 15°C and 20°C. We suggest that future test designs should be performed at temperatures that reflect the temperature range amphibians are exposed to in their natural habitats. Variations in the sensitivity due to temperature should also be considered as an uncertainty factor in upcoming environmental risk assessments for amphibians.

1 Introduction

To improve crop yields about 360 million kg of pesticide formulations are used per year on agricultural fields in the European Union (data from 2017; Eurostat, 2020). Only a small part of these pesticides reaches their target organism [2], and due to spray drift and run-off they can get into water bodies

within or near agricultural fields [3,4]. Such agricultural ponds can be important breeding habitats for amphibians [5–7], which are therefore exposed to pesticides during their aquatic life stages. Pesticides were shown to have adverse effects on amphibians in several studies (e.g. [8–12], and are consequently identified as one of the main drivers in the global amphibian decline [13,14]. The actual toxicity of pesticides for amphibians depends on a number of factors, including the active ingredients [8], formulation additives [9,10,15], the species in focus [12], a previous exposure to pesticides [10,11] and the developmental stage [10,12,16] of the tested individuals.

Also water temperature during pesticide exposure of larvae has an impact on the toxicity. Amphibians are ectothermic species and behavior and physiology are fundamentally influenced by environmental temperature [17]. Therefore, metabolic rates and thus processes that might enhance or reduce toxic effects, like the uptake of substances, the metabolic oxygen demand, and detoxification processes are temperature-dependent [18]. However, studies on the combined effects of temperature and pesticides on amphibians reveal contrasting results. Some observed that higher temperatures increased toxicity [19–21], while others showed a reduced toxic effect of pesticides on exposed amphibians [22–24]. For *Oligosoma polychroma*, a skink (reptile) and thus also an ectothermic vertebrate species, even a heat-seeking behavior was observed, that can be interpreted as response to increase the metabolism to better deal with stress after exposure to a glyphosate formulation [25].

Detailed knowledge of the relationship of pesticide sensitivity and temperature is central for two reasons. First, we are facing a global warming caused by climate change with more frequent temperature extremes [26]. Understanding the combined effect of this temperature increase and pesticides will help to better estimate the impact of climate change on amphibian populations, to identify potential threats on species and to set mitigation measures. Second, laboratory toxicity tests for pesticides with amphibian larvae are typically performed at temperatures between 15°C and 20°C (e.g. Mann et al., 2003; Johansson et al., 2006; Brühl et al., 2013; Wagner et al., 2017). These standard

temperatures might not reflect the natural range of temperatures at which a species is exposed to pesticides in its habitat. For example, larvae of *Rana temporaria* can be found in European ponds with water temperatures only a few degrees above the freezing point [27]. In this study, the average water temperature during the aquatic development was 9.7°C and the maximum temperature 23°C [27]. However, in small water bodies the maximum water temperatures might be above 30°C, as even in high-altitudes breeding ponds with temperatures of up to 26.5°C can be found [28]. Therefore, standard laboratory toxicity tests might lead to the underestimation of possible sublethal or even lethal effects that occur at lower or higher temperatures. Thus, knowing the temperature at which amphibians are most sensitive will allow a more reliable assessment of the actual risk of pesticides.

In the present study, we conducted aquatic acute toxicity tests at temperatures between 6°C and 26°C to investigate the effect of the temperature on the sensitivity of amphibian larvae to the fungicide Folpan® 500 SC with the active ingredient folpet. With up to eight applications per growing season, folpet is, next to sulfur, the most common fungicide in German vineyards and is preventively used to protect plants primarily from mildew [29]. In general, fungicides are underrepresented in ecotoxicological studies compared to other pesticide classes [30]. To identify potential species and developmental stage specific differences in pesticide sensitivity, we tested early and late larval stages of the common frog (*Rana temporaria* Linnaeus, 1758) and the green toad (*Bufotes viridis* Laurenti, 1768), two temperate species that can be found in breeding ponds in German vineyards [6]. Both species are listed as “least concern” by the IUCN [31,32] and are widespread in Europe. *R. temporaria* is discussed as a model organism for European amphibian species in toxicological studies [33]. This species uses a variety of different water bodies for mating, which usually takes place in March, but can start as early as the end of January [34] when water temperatures are above 5°C for some days [34,35]. However, even at temperatures only a few degrees above freezing point spawning can be observed [36] and early larvae can be found [27]. Preferred temperatures of early *R. temporaria* larvae from Germany

are between 14.8°C and 19.6°C, and between 16.5°C and 26.0°C of late larvae stages [37]. In contrast to *R. temporaria*, *B. viridis* is considered to be a thermophile species with preferred spawning temperatures between 16°C and 20°C. The optimum thermal tolerance limits for early larvae are between 12°C and 25°C [38].

The aim of the study was to get a better understanding about the temperature-dependent pesticide sensitivity of two European amphibian species. We hypothesized 1) that the sensitivity of larvae to Folpan® 500 SC is highly affected by water temperature, 2) that early larvae are more sensitive than late larval stages (see Adams and Brühl, 2020), and 3) that pesticide sensitivity differs between species.

2. Material and methods

2.1 Sampling and animal husbandry

Up to 300 eggs of eight and seven different clutches of *R. temporaria* and *B. viridis*, respectively, were collected in March and May 2018. The spawning pond of *R. temporaria* is located in the Palatinate Forest (Rhineland-Palatinate, Germany; 49.262433 N, 8.061896 E (WSG84), 242 m asl), distant from any pesticide use. The pond of the *B. viridis* population is located in a vineyard dominated area (Rhineland-Palatinate, Germany; 49.317490 N, 8.129091 E (WSG84), 194 m asl). Thus, the pond can be expected to be contaminated with various pesticides. Eggs were transferred to glass aquaria (30 x 20 x 20 cm) filled with tap water and kept in a climate chamber at 16°C with a 16:8 day-night-rhythm. For logistical reasons, not all acute toxicity tests for the same developmental stage were conducted at the same time. Therefore, parts of each clutch were kept at 10°C and daylight to slow down the development of the eggs. After hatching, larvae were kept in groups of 50 individuals in aerated glass aquaria filled with tap water at 21°C. As the larvae grew, we reduced their density to 20 larvae per aquaria. Cleaning of the aquaria and water renewal took place every second day. Larvae were fed daily *ad libitum* with commercial fish food, cooked salad, and cucumber.

2.2 Test substance

The fungicide Folpan® 500 SC (ADAMA Deutschland GmbH, Germany; purchased from a local distributor) with the active ingredient folpet (38-42% of weight; CAS number 133-07-03) was used for all tests. Folpet is an organochlorine phthalimide with a molecular weight of 296.6 g/mol and is used as a protective, broad-spectrum fungicide against leaf spot diseases in grapevines. Data on environmental contaminations are rare, but maximum measured concentrations of 50 ng/L in rivers [39] and 4.53 µg/L in ponds [40] have been reported. To assess the environmental realistic toxicity effect, the formulation was tested instead of the pure active ingredient. Other formulation ingredients are "alkylnaphthalensulfonic acid, polymer with formaldehyde, sodium salt" (3.5-5%), fumaric acid (1-1.5%), methenamine (0.5-1%) and 1,2-Benzisothiazoline-3-one (<0.1%). The acute aquatic toxicity of the formulation leads to a 96-h LC_{50} of 0.256 mg Folpan/L for the rainbow trout (*Oncorhynchus mykiss*) [41].

2.3 Experimental design

Acute toxicity of Folpan® 500 SC was determined in a full-factorial design with different temperature conditions and two developmental stages of both species. Early larval stages (Gosner stage 20; GS20; first hatchling stage with external gill circulation; see Gosner (1960) for classification) were tested at five different temperatures (6°C, 11°C, 16°C, 21°C, 26°C). Late larval stages (Gosner stage 36-41; GS40; larvae with at least hindlimbs) were tested at three different temperatures (6°C, 16°C, 26°C). For each combination of temperature, species and developmental stage (= 16 combinations in total), a 48 h static acute toxicity test was performed with six different pesticide concentrations, ranging between 0 (control) and 4.2 mg Folpan/L (see Supplementary Table 1). Fungicide concentrations were chosen based on range-finding tests and previous studies with folpet [16,43] to cover the concentration range at which ideally 0-100% mortality of the test organisms should be observed. Range finding tests

were performed as 48 h tests with three Folpan concentrations and a control group with three replicates of one individual for each species/developmental stage and different temperatures. For each pesticide concentration of the final acute toxicity test 25 (GS20) or 15 (GS40) individuals were used, resulting in 150 and 90 individuals per test, respectively. Tests were conducted in 1.7 L glass jars containing 1L FETAX medium [44] and the respective amount of Folpan® 500 SC. Before adding the folpet formulation, the jars with the FETAX medium were cooled or heated to the test temperature in climate chambers (WK 19'/+15-35, Weiss Technik GmbH, Reiskirchen, Germany; MLR-351H SANYO Versatile Environmental Test Chamber, SANYO Electric Co. Ltd., Moriguchi, Japan). To reduce the influence of thermal shock on the physiology of the animals, preselected larvae from different clutches of about the same size and developmental stage (GS20 or GS40) showing normal behavior were placed in plastic boxes and acclimated at least for one hour to the test temperature. Afterwards five (GS20) or three (GS40) larvae were randomly placed in a test jar, resulting in five replicates/jars per pesticide concentration. For each jar, the mortality of larvae was determined after 48 h of exposure, whereby dead larvae were removed after 2 h and 24 h from the test jars. In accordance with the test guideline for acute toxicity testing in fish (OECD test guideline No. 203, [45]), larvae were not fed during the experimental period. Tests were performed in climate chambers set to the according test temperature with a 16:8 day-night-rhythm.

2.4 Statistical analysis

For each test the median lethal concentration causing 50% mortality of test organisms (LC_{50} value) was determined using different concentration-response models (log-normal functions - LN.2, LN.3, LN.4; log-logistic functions - LL.2, LL.3u, LL.4, LL.5; and Weibull-functions - W1.2, W1.3, W1.4, W2.2, W2.3, W2.4) calculated with the R package “drc” [46]. To get the most accurate LC_{50} value, the model that best describes the observed mortality of larvae was selected based on the lowest Akaike’s Information Criterion for each test. LC_{50} values between different test temperatures for the same

158 species and development stage were compared by a confidence interval overlap test [47] with the
159 function "comped" implemented in "drc". As we hypothesised a correlation between temperature and
160 toxicity, we tested the LC_{50} of a species/developemental stage at a temperature only against the LC_{50}
161 of the next higher temperature to reduce the probability of an alpha error accumulation. In case the
162 difference was not significant, we also tested against the two steps higher temperature. Confidence
163 interval overlap tests were also used to compare LC50 values between species and developement stages
164 at the same test temperature. For all comparisons, p-values were calculated following the method
165 described by Altman & Bland [48]. When testing the same species and developmental stage at different
166 temperatures, or the same species or developemental stage at different temperatures, p-values were
167 adjusted with a Bonferroni correction. All statistical analyses were carried out in R (version 3.4.3; R
168 Core Team, 2019).

169 **2.5 Animal welfare**

170 The study was approved by the Landesuntersuchungsamt in Koblenz (Germany; approval number
171 G18-20-009), and the collection of clutches and the husbandry of larvae were permitted by the
172 "Struktur- und Genehmigungsdirektion Süd Referat 42 - Obere Naturschutzbehörde" (Neustadt an der
173 Weinstraße, Germany; approval number: 42/553-254/455-18). After the experiments all test organisms
174 were euthanized with a buffered 0.1% MS-222 solution.

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176

177 **3 Results**

178 The calculated LC_{50} values of Folpan® 500 SC ranged between 0.30 and 2.90 mg Folpan/L for *R.*
179 *temporaria* and 0.64 and 4.35 mg Folpan/L for *B. viridis* (Table 1). Toxicity decreased (i.e. increasing

180 LC_{50} values) with increasing temperature for both tested species and developmental stages (see Fig. 1).
181 In particular, the LC_{50} of GS20 at 21°C, the temperature at which toxicity tests are often conducted,
182 was 2 (*R. temporaria*) and 2.3 (*B. viridis*) times higher than the lowest observed LC_{50} value. A
183 temperature increase from 6°C to 16°C resulted in 1.7 to 2.0 and an increase from 16°C to 26°C in 1.2
184 to 3.8 times higher LC_{50} values. A temperature increase of 5°C (GS20) or 10°C (GS40) resulted always
185 in a significantly higher LC_{50} value (all $p \leq 0.038$, see Table 1), except for the comparison of 6°C and
186 11°C in GS20 in both species and 11°C and 16°C in GS20 *R. temporaria*. In general, the most sensitive
187 individuals (*R. temporaria* GS20 at 6°C) were 14.5 times more sensitive than the least sensitive ones
188 (*B. viridis* at GS20 26°C). Our analysis revealed that early larvae were more sensitive than late larvae,
189 with the expection of *B. viridis* at 26°C (Table 2). Comparing LC_{50} values between species showed
190 that *R. temporaria* is more sensitive in early and less sensitive in late developmental stages than *B.*
191 *viridis* (Table 3), suggesting an interaction between developmental stage and species. However, the
192 difference was not significant when comparing late developmental stages at 16°C and 26°C after a
193 Bonferroni correction. Across all temperature treatments in both developmental stages and species the
194 control and lowest concentration of 0.1 mg Folpan/L did not lead to any mortality in tested larvae.

195

196 **Figure 1**: Calculated LC_{50} values (± 95% CI) of early (GS20) and late (GS40) developmental stages
197 of *R. temporaria* and *B. viridis* at different temperatures. For detailed values and differences between
198 temperatures see Table 1.

199

200

Table 1: Calculated LC_{50} values for two developmental stages of *R. temporaria* and *B. viridis* at different temperatures with 95% confidence intervals and the used dose-response models. P-values show results from confidence interval overlap tests when testing against the next higher temperature. In case the difference was not significant, it was also tested against the two steps higher temperature. Significant differences after Bonferroni-correction are presented in bold.

	Developmental stage	T (°C)	Model	LC_{50} (mg Folpan/L)	95% CI (mg Folpan/L)	p (to next temperature)
Rana temporaria	GS20	6	W2.2	0.30	0.28 - 0.31	0.172 (11°C) / < **0.001** (16°C)
		11	W2.2	0.39	0.29 - 0.49	0.120 (16°C) / < **0.001** (21°C)
		16	W2.2	0.52	0.44 - 0.59	**0.001**
		21	W2.2	0.68	0.66 - 0.70	< **0.001**
		26	W1.2	1.12	1.10 - 1.15	-
	GS40	6	W2.2	1.29	1.22 - 1.36	< **0.001**
		16	W2.2	2.37	2.20 - 2.53	**0.004**
		26	W2.2	2.90	2.79 - 3.00	-
Bufotes viridis	GS20	6	W2.2	0.64	0.57 - 0.71	0.528 (11°C) / < **0.001** (16°C)
		11	W2.2	0.69	0.61 - 0.76	< **0.001**

	16	LN.2	1.15	1.09 - 1.21	**0.038**
	21	W2.2	1.28	1.20 - 1.36	< **0.001**
	26	W2.2	4.35	4.19 - 4.50	-
GS40	6	LN.2	1.04	0.95 - 1.14	< **0.001**
	16	W2.2	2.06	1.87 - 2.26	< **0.001**
	26	W1.2	2.56	2.32 - 2.80	-

206

207 **Table 2**: Comparison of LC_{50} values between developmental stages. Significant differences after

208 Bonferroni-correction are presented in bold.

Species	**T (°C)**	**GS20 vs. GS40**
R. temporaria	6	< **0.001**
	16	< **0.001**
	26	< **0.001**
B. viridis	6	< **0.001**
	16	< **0.001**
	26	< **0.001**

209

Table 3: Comparison of LC_{50} values between species. Significant differences after Bonferroni-correction are presented in bold.

Developmental stage	T (°C)	*R. temporaria* vs. *B. viridis*
GS20	6	< **0.001**
	11	< **0.001**
	16	< **0.001**
	21	< **0.001**
	26	< **0.001**
GS40	6	**0.002**
	16	0.088
	26	0.063

4 Discussion

In the present study, we demonstrated that the pesticide sensitivity of two European amphibian species is highly affected by temperature, with individuals of both tested developmental stages and species being more sensitive at lower temperatures. As we did not observe mortality at any temperature in controls, the tested temperatures are within a range that allows survival. Therefore, observed mortalities

219 are caused by Folpan® 500 SC, where the lethal concentration depends on the temperature.
220 Explanations for the relationship between temperature and sensitivity are diverse and depending on the
221 pesticide and organism in focus, but exact mechanisms often remain unknown. In our study, higher
222 temperatures might be nearer to the optimal temperature of the tested individuals, allowing effective
223 metabolism and detoxification. Likewise, low temperatures might be below the optimal temperature
224 range and result in additional stress, limiting the ability to cope with Folpan® 500 SC. Observed results
225 might also be caused by the characteristics of folpet, the active ingredient of the tested formulation
226 Folpan® 500 SC. In general, folpet degrades rapidly in aquatic environments and shows a half-life
227 (DT50) of 0.7 h at 25°C and 0.178 h at 40°C (both pH 7; EFSA, 2009). Further, the degradation depends
228 on the pH of the medium (DT50 pH 4, 25°C = 6.5 h; DT50 pH 4, 40°C = 1.06 h; DT50 ph 9, 25°C and
229 40°C = too rapid to measure; EFSA, 2009). Thus, the alkaline FETAX medium (ranging between pH
230 7.7 and 8.29 in our study) even accelerates the degradation. Although information about the
231 degradation below 25°C is lacking, a temperature-dependent degradation that could have caused the
232 observed effects can be expected. Because of the overall fast degradation, no analysis of the actual
233 folpet concentration at the start and the end of a test was possible. It remains also unknown if the
234 degradation of the formulation Folpan® 500 SC is similar to its active ingredient folpet, as additives
235 could increase the stability of the formulation. Additives might also influence the toxicity of the
236 formulation [9,10,15,51]. Regardless whether the lower sensitivity at higher temperatures is caused by
237 a more effective metabolism and detoxification, and thus reduced bioaccumulation, or by an increased
238 degeneration of folpet, Folpan® 500 SC is more toxic for the two tested amphibian species at lower
239 temperatures.

240 Thus, increasing environmental temperatures might seem to have a positive effect on amphibians in
241 terms of a reduced folpet toxicity. However, climate warming will also cause a shift in the breeding
242 season to an earlier time of the year in temperate species [52]. Lötters et al. [53] showed that a shift of

one month could decrease the glyphosate exposure risk during their migration to the breeding pond to about 50% for *R. temporaria*. Thus, also the exposure risk of larvae might be reduced. However, increased temperatures will also result in an earlier vegetation period of crops [54,55] and pesticides might be applied earlier. Consequently, the general exposure risk, but also the temperature at which amphibians will be exposed to pesticides in their aquatic habitats, will probably not change fundamentally. However, also more frequent temperature extremes can be expected [26], resulting in regional and temporary temperature drops so that also later larvae might be exposed to low temperatures. Climate change will also cause more frequent pesticide applications [56,57], resulting in higher overall pesticide loads in water bodies. Already today, many different pesticides can be found in ponds within agriculture [58,59]. Although higher temperatures might result in a lower sensitivity to folpet, contrary effects are possible for other pesticides and pesticide mixes. In vineyards, folpet is usually applied first in late May [60], when *R. temporaria* larvae occur in late development stages. At this time, *B. viridis* is still spawning and thus early larvae can be found. Only few data on actual environmental contamination with folpet are available and data show that maximum measured concentrations (50 ng/L [39]; 4.53 µg/L, [40]) are by a factor of at least 66 below the lowest LC_{50} values obtained in our study. We can therefore conclude that this pesticide will most likely not lethally affect the two tested amphibian species at the larval stage, but sublethal effects cannot be excluded. Thus, future studies should also focus on the effect of the temperature on sublethal endpoints like development or behavior.

Our results are in contrast to most studies that investigated the effect of temperature on pesticide toxicity for amphibian larvae in acute toxicity studies. In Materna et al. [20] leopard frog larvae (*Lithobates sp.*; former *R. pipiens* complex) showed higher mortalities in 96-h acute toxicity tests for the pyrethroid insecticide esfenvalerate at 22°C than at 18°C. Boone and Bridges [19] found the same relationship for *L. clamitans* (former *R. clamitans*) as the 96h-LC_{50} at 27°C was two times higher than

267 at 17°C. Lau et al. [21] calculated 96h-LC_{50} values for the pesticide methomyl for three Asian
268 amphibian species (*Duttaphrynus melanostictus*, *Polypedates megacephalus*, *Microhyla pulchra*) at
269 temperatures between 15°C and 35°C, and observed lower 96h-LC_{50} values at higher temperatures.
270 However, Chiari et al. [24] showed that increased temperature can also reduce the toxicity of a pesticide
271 in 96-h acute toxicity tests by comparing published LC_{50} values for copper sulfate of various amphibian
272 species. In contrast to most 96-h tests, reduced toxic effects of pesticides at higher temperatures can
273 also be found in studies with tests running over several weeks or until metamorphosis. Baier et al. [23]
274 found that the effects of the glyphosate formulation Roundup® PowerFlex on mortality, growth and
275 tail deformation of the common toad (*Bufo bufo*) were more pronounced at 15°C than at 20°C. In a
276 study on the glyphosate formulation Roundup® LB Plus, Baier et al. [22] also found increased effects
277 on the development of common toad larvae at lower temperatures (15°C compared to 20°C) when
278 exposure occurred already as egg. Rohr et al. [61] reported that an increased temperature reduced the
279 time to the metamorphosis of larval *Ambystoma barbouri* exposed to the herbicide atrazine. Hence,
280 also the total exposure to atrazine was reduced in this study, which ameliorated increased adverse
281 effects of the pesticide [61].

282 With the exception of *B. viridis* at 26°C, early larval stages were 1.6 to 4.5 times more sensitive than
283 late stages in both tested species. This is in line with the results from Adams and Brühl [16], where
284 early larvae of *R. temporaria* (Gosner stage 20) were two times more sensitive than late larvae (Gosner
285 stage 36) to the fungicide Folpan® 80 WDG with the same active ingredient folpet. Also Wagner et
286 al. [10] found late larval stages of *R. temporaria* to be less sensitive in acute tests with two herbicides.
287 Interestingly, in our study early larvae of *B. viridis* at 26°C were least sensitive. *Bufotes viridis* is a
288 thermophilic species, and the highest tested temperature is at the upper limit of its optimal thermal
289 range for development of early larvae (12°C - 25°C; Derakhshan and Nokhbatolfoghahai, 2015).
290 Hence, 26°C might allow optimal detoxification without causing temperature stress for early stages. In

late larval stages additional stress caused by processes linked to metamorphosis could countervail the advantages of high temperatures, resulting in late larvae of *B. viridis* being more sensitive than early larvae. Further, the optimal temperature of late *B. viridis* larvae could be even higher than 26°C. This might also explain why late larvae of *R. temporaria* (with assumed lower optimal temperature) were less sensitive than *B. viridis*, although *R. temporaria* is more sensitive in early stages. In general, species [12] and even population [10,11] specific differences in pesticide sensitivity are known. For example, Adams et al. [43] showed that out of eight central European amphibian species, the most sensitive species was five-times more sensitive than the least sensitive species towards the pesticide folpet. Therefore, differences in the sensitivity in our study species are not surprising. However, the original breeding pond where *B. viridis* eggs were obtained was situated within viticulture. Thus, it cannot be ruled out that differences in the sensitivity are the result of an adaption of the population to pesticides and not a species effect.

Folpet is, next to sulfur, the most common fungicide in German vineyards and thus understanding its toxicity on non-target organisms is of high relevance. However, the fast and temperature dependent degradation of folpet limits the conclusions drawn from our study. Thus, we recommend that future studies on the relationship of temperature and sensitivity of amphibians should focus on pesticides with a longer degradation time, not influenced as much by temperature. It might also be worth to consider pesticide mixtures, as often several formulations are applied at the same time [60] and a mixture of pesticides can be found in agricultural ponds [40]. It has recently been shown that the developmental temperature prior to ecotoxicological tests can have an influence on the organisms´ sensitivity to a test substance (Silva et al 2020) and should consequently also be considered in future amphibian tests.

To date, no standard test guideline for acute toxicity tests of European amphibian species exists and amphibians are also not explicitely considered in the environmntal risk assessment of pesticides. The results of our study raise concerns about typical ecotoxicological studies with amphibians that are often

conducted at temperatures between 15°C and 20°C, because early larvae at 6°C were about two times more sensitive to Folpan® 500 SC as at 21°C. Therefore, adverse effects in temperate amphibian species might only be observed at lower or, depending on the tested pesticide, higher temperatures. Based on the results we obtained in our study we conclude that an additional temperature related factor needs to be incorporated in an uncertainty factor of an upcoming environmental risk assessments for amphibians in the EU that reflects variations in pesticide sensitivity due to temperature. Additionally, we agree with recommendations of previous studies [19,21–23] that future test protocols should consider temperature as an important factor. Tests should be performed at temperatures that are reflecting the temperature range amphibians are exposed to in their natural habitats, possibly also including natural daily temperature fluctuations.

5 Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

6 Author contributions

CL, CB and KT conceived and designed the study. CL and LS performed the experiment. CL and LS analyzed the data and drafted the manuscript. KT acquired the funding of the project and supervised the work together with CB. All authors contributed to the writing process and approved the final manuscript.

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9 Data availability statement

The original contributions presented in the study are included in the article/supplementary files, further inquiries can be directed to the corresponding author/s.

10 Literature

1. Eurostat. Pesticide sales in the EU 2017 [Internet]. 2020 [cited 9 Feb 2020]. Available: https://appsso.eurostat.ec.europa.eu/nui/show.do?query=BOOKMARK_DS-382683_QID_1F97D137_UID_-3F171EB0&layout=PESTICID,L,X,0;TIME,C,X,1;GEO,L,Y,0;UNIT,L,Z,0;INDICATORS,C,Z,1;&zSelection=DS-382683UNIT,KG;DS-382683INDICATORS,OBS_FLAG;&rankName1=UNIT_1_2_-1_2&

2. Pimentel D. Amounts of pesticides reaching target pests: Environmental impacts and ethics. J Agric Environ Ethics. 1995;8: 17–29. doi:10.1007/BF02286399

3. Zhang X, Luo Y, Goh KS. Modeling spray drift and runoff-related inputs of pesticides to receiving water. Environ Pollut. Elsevier Ltd; 2018;234: 48–58. doi:10.1016/j.envpol.2017.11.032

4. Reichenberger S, Bach M, Skitschak A, Frede H. Mitigation strategies to reduce pesticide inputs into ground- and surface water and their effectiveness; A review. Sci Total Environ.

2007;384: 1–35. doi:10.1016/j.scitotenv.2007.04.046

5. Knutson MG, Richardson WB, Reineke DM, Gray BR, Parmelee JR, Weick SE. Agricultural ponds support amphibian populations. Ecol Appl. 2004;14: 669–684. doi:10.1890/02-5305

6. Lenhardt PP, Schäfer RB, Theissinger K, Brühl CA. An expert-based landscape permeability model for assessing the impact of agricultural management on amphibian migration. Basic Appl Ecol. Elsevier GmbH; 2013;14: 442–451. doi:10.1016/j.baae.2013.05.004

7. Berger G, Pfeffer H, Kalettka T. Amphibienschutz in kleingewässerreichen Ackerbaugebieten. Rangsdorf: Natur & Text; 2011.

8. Johansson M, Piha H, Kylin H, Merilä J. Toxicity of six pesticides to common frog (*Rana temporaria*) tadpoles. Environ Toxicol Chem. 2006;25: 3164. doi:10.1897/05-685R1.1

9. Mann R, Tyler M, Bidwell J. Toxicity of herbicide formulations to frogs and the implications for product registration: A case study from Western Australia. Appl Herpetol. 2003;1: 13–22. doi:10.1163/157075403766451199

10. Wagner N, Veith M, Lötters S, Viertel B. Population and life-stage-specific effects of two herbicide formulations on the aquatic development of European common frogs (Rana temporaria). Environ Toxicol Chem. 2017;36: 190–200. doi:10.1002/etc.3525

11. Hua J, Morehouse NI, Relyea R. Pesticide tolerance in amphibians: Induced tolerance in susceptible populations, constitutive tolerance in tolerant populations. Evol Appl. 2013;6: 1028–1040. doi:10.1111/eva.12083

12. Biga LM, Blaustein AR. Variations in lethal and sublethal effects of cypermethrin among aquatic stages and species of anuran amphibians. Environ Toxicol Chem. 2013;32: 2855–

2860. doi:10.1002/etc.2379

13. Collins JP, Storfer A. Global amphibian declines: Sorting the hypotheses. Divers Distrib. 2003;9: 89–98. doi:10.1046/j.1472-4642.2003.00012.x

14. Hayes TB, Falso P, Gallipeau S, Stice M. The cause of global amphibian declines: a developmental endocrinologist's perspective. J Exp Biol. 2010;213: 921–933. doi:10.1242/jeb.040865

15. Brühl CA, Schmidt T, Pieper S, Alscher A. Terrestrial pesticide exposure of amphibians: An underestimated cause of global decline? Sci Rep. 2013;3: 1135. doi:10.1038/srep01135

16. Adams E, Brühl CA. Fungicide Exposure Induces Sensitivity Differences in Aquatic Life Stages of European Common Frogs (*Rana temporaria*). J Herpetol. 2020;54: 331–336. doi:10.1670/19-004

17. Wells KD. The ecology and behavior of amphibians. Chicago: The University of Chicago Press; 2007.

18. Honkanen JO, Kukkonen JVK. Environmental temperature changes uptake rate and bioconcentration factors of bisphenol A in tadpoles of Rana temporaria. Environ Toxicol Chem. 2006; doi:10.1897/05-586R1.1

19. Boone MD, Bridges CM. The effect of temperature on the potency of carbaryl for survival of tadpoles of the green frog (*Rana clamitans*). Environ Toxicol Chem. 1999;18: 1482–1484. doi:10.1897/1551-5028(1999)018<1482:TEOTOT>2.3.CO;2

20. Materna EJ, Rabeni CF, Lapoint TW. Effects of the synthetic pyrethroid insecticide, esfenvalerate, on larval leopard frogs (*Rana* spp.). Environ Toxicol Chem. 1995;14: 613–622.

399 doi:10.1002/etc.5620140409

400 21. Lau ETC, Karraker NE, Leung KMY. Temperature-dependent acute toxicity of methomyl
401 pesticide on larvae of 3 Asian amphibian species. Environ Toxicol Chem. 2015;34: 2322–
402 2327. doi:10.1002/etc.3061

403 22. Baier F, Jedinger M, Gruber E, Zaller JG. Temperature-Dependence of Glyphosate-Based
404 Herbicide's Effects on Egg and Tadpole Growth of Common Toads. Front Environ Sci.
405 2016;4: 1–10. doi:10.3389/fenvs.2016.00051

406 23. Baier F, Gruber E, Hein T, Bondar-Kunze E, Ivanković M, Mentler A, et al. Non-target effects
407 of a glyphosate-based herbicide on Common toad larvae (Bufo bufo, Amphibia) and
408 associated algae are altered by temperature. PeerJ. 2016;4: e2641. doi:10.7717/peerj.2641

409 24. Chiari Y, Glaberman S, Serén N, Carretero MA, Capellini I. Phylogenetic signal in amphibian
410 sensitivity to copper sulfate relative to experimental temperature. Ecol Appl. 2015;25: 596–
411 602. doi:10.1890/14-0439.1

412 25. Carpenter JK, Monks JM, Nelson N. The effect of two glyphosate formulations on a small,
413 diurnal lizard (Oligosoma polychroma). Ecotoxicology. Springer US; 2016;25: 548–554.
414 doi:10.1007/s10646-016-1613-2

415 26. IPCC. Climate Change 2013: The Physical Science Basis. Contribution of Working Group I to
416 the Fifth Assessment Report of the Intergovernmental Panel on Climate Change. Stocker T.,
417 Qin D, Plattner G-K, Tignor M, Allen SK, Boschung J, et al., editors. Cambridge, United
418 Kingdom and New York, NY, USA: Cambridge University Press; 2013.
419 doi:10.1017/CBO9781107415324.Summary

420 27. Baumgartner C, Bitschi N, Ellinger N, Gollmann B, Gollmann G, Köck M. Laichablage und

Embryonalentwicklung von Springfrosch (Rana dalmatina BONAPARTE , 1840) und Grasfrosch (Rana temporaria LINNAEUS , 1758) in einem syntopen Vorkommen. Herpetozoa. 1996;9: 133–150.

28. Sztatecsny M, Gallauner A, Klotz L, Baierl A, Schabetsberger R. The presence of common frogs (Rana temporaria) increases the body condition of syntopic Alpine newts (Ichthyosaura alpestris) in oligotrophic high-altitude ponds : benefits of high-energy prey in a low-productivity habitat. 2013;2450: 209–215.

29. Roßberg D, Ipach R. Erhebungen zur Anwendung von Pflanzenschutzmitteln im Weinbau. J für Kult. 2015;67: 410–416. doi:10.5073/JFK.2015.12.03

30. Zubrod JP, Bundschuh M, Arts G, Brühl CA, Imfeld G, Knäbel A, et al. Fungicides: An Overlooked Pesticide Class? Environ Sci Technol. 2019;53: 3347–3365. doi:10.1021/acs.est.8b04392

31. Kuzmin S, Ishchenko V, Tuniyev B, Beebee T, Andreone F, Nyström P, et al. *Rana temporaria* (European Common Frog). IUCN Red List Threat Species. 2009; e.T58734A86470817. doi:http://dx.doi.org/10.2305/IUCN.UK.2009.RLTS.T58734A11834246.en

32. Aghasyan A, Avci A, Tuniyev B, Crnobrnja-Isailovic J, Lymberakis P, Andrén C, et al. *Bufotes viridis*. IUCN Red List Threat Species. 2015; e.T155333A86444583. doi:http://dx.doi.org/10.2305/IUCN.UK.2015- 1.RLTS.T155333A74514442.en

33. Wagner N, Viertel B. Chapter 16. The Use of Aquatic Life-stages of European Amphibians in Toxicological Studies. In: Larramendy ML, editor. Ecotoxicology and Genotoxicology: Non-traditional Aquatic Models. Royal Society of Chemistry; 2017. pp. 371–406.

443 doi:10.1039/9781782629887-00371

444 34. Günther R. Die Amphibien und Reptilien Deutschlands. Heidelberg: Spektrum Akademischer
445 Verlag; 2009.

446 35. Muir AP, Biek R, Mable BK. Behavioural and physiological adaptations to low-temperature
447 environments in the common frog, Rana temporaria. BMC Evol Biol. 2014;14: 1–11.
448 doi:10.1186/1471-2148-14-110

449 36. Loman J. Breeding phenology in Rana temporaria. Local variation is due to pond temperature
450 and population size. Ecol Evol. 2016;6: 6202–6209. doi:10.1002/ece3.2356

451 37. Drakulić S, Feldhaar H, Lisičić D, Mioč M, Cizelj I, Seiler M, et al. Local differences of
452 thermal preferences in European common frog (Rana temporaria Linnaeus, 1758) tadpoles.
453 Zool Anz. 2017;268: 47–54. doi:10.1016/j.jcz.2017.04.005

454 38. Derakhshan Z, Nokhbatolfoghahai M. Thermal tolerance limits and effects of temperature on
455 the growth and development of the green toad, *Bufotes viridis*. Salamandra. 2015;51: 129–136.

456 39. Konstantinou IK, Hela DG, Albanis TA. The status of pesticide pollution in surface waters
457 (rivers and lakes) of Greece. Part I. Review on occurrence and levels. Environ Pollut.
458 2006;141: 555–570. doi:10.1016/j.envpol.2005.07.024

459 40. Adams E, Leeb C, Brühl CA. Pesticide exposure affects reproductive capacity of common
460 toads (Bufo bufo) in a viticultural landscape. Ecotoxicology. 2021;30: 213–223.
461 doi:10.1007/s10646-020-02335-9

462 41. Adama. Safety data sheet according to regulation (EC) No. 1907/2006 (REACH) - Product
463 Number FNG56820-A (Folpan 500 SC). 2015;

42. Gosner KL. A Simplified Table for Staging Anuran Embryos Larvae with Notes on Identification. Herpetologica. 1960;16: 183–190. doi:10.2307/3890061

43. Adams E, Leeb C, Roodt AP, Brühl CA. Interspecific sensitivity of European amphibians towards two pesticides and comparison to standard test species. Environ Sci Eur. Springer Berlin Heidelberg; 2021;33: 1–7. doi:10.1186/s12302-021-00491-1

44. Dawson DA, Bantle JA. Development of a reconstituted water medium and preliminary validation of the Frog Embryo Teratogenesis Assay—Xenopus (FETAX). J Appl Toxicol. 1987;7: 237–244. doi:10.1002/jat.2550070403

45. OECD. Test No. 203: Fish, Acute Toxicity Test. OECD Guidelines for the Testing of Chemicals, Section 2. Paris: OECD Publishing; 2019. doi:10.1787/9789264069961-en

46. Ritz C, Baty F, Streibig JC, Gerhard D. Dose-Response Analysis Using R. PLoS One. 2015;10: e0146021. doi:10.1371/journal.pone.0146021

47. Wheeler MW, Park RM, Bailer AJ. Comparing median lethal concentration values using confidence interval overlap or ratio tests. Environ Toxicol Chem. 2006;25: 1441–1444. doi:10.1897/05-320R.1

48. Altman DG, Bland MJ. How to obtain the P value from a confidence interval. BMJ. 2011; 343:d2304. doi:10.1136/bmj.d2304

49. R Development Core Team. R: A language and environment for statistical computing. R Foundation for Statistical Computing. Vienna, Austria; 2020. doi:10.1111/j.1365-2621.1979.tb03829.x

50. EFSA. Conclusion on the peer review of the pesticide risk assessment of the active substance

485 folpet. EFSA Sci Rep. 2009;297: 1–80.

486 51. Adams E, Gerstle V, Brühl CA. Dermal Fungicide Exposure at Realistic Field Rates Induces
487 Lethal and Sublethal Effects on Juvenile European Common Frogs (*Rana temporaria*).
488 Environ Toxicol Chem. 2021; etc.4972. doi:10.1002/etc.4972

489 52. Blaustein AR, Walls SC, Bancroft BA, Lawler JJ, Searle CL, Gervasi SS. Direct and indirect
490 effects of climate change on amphibian populations. Diversity. 2010;2: 281–313.
491 doi:10.3390/d2020281

492 53. Lötters S, Filz KJ, Wagner N, Schmidt BR, Emmerling C, Veith M. Hypothesizing if
493 responses to climate change affect herbicide exposure risk for amphibians. Environ Sci Eur.
494 2014;26: 1–5. doi:10.1186/s12302-014-0031-4

495 54. Chmielewski FM, Müller A, Bruns E. Climate changes and trends in phenology of fruit trees
496 and field crops in Germany, 1961-2000. Agric For Meteorol. 2004;121: 69–78.
497 doi:10.1016/S0168-1923(03)00161-8

498 55. Lavalle C, Micale F, Houston TD, Camia A, Hiederer R, Lazar C, et al. Climate change in
499 Europe. 3. Impact on agriculture and forestry. A review. Agron Sustain Dev. 2009;29: 433–
500 446. doi:10.1051/agro/2008068

501 56. Delcour I, Spanoghe P, Uyttendaele M. Literature review: Impact of climate change on
502 pesticide use. Food Res Int. Elsevier Ltd; 2015;68: 7–15. doi:10.1016/j.foodres.2014.09.030

503 57. Olesen JE, Bindi M. Consequences of climate change for European agricultural productivity,
504 land use and policy. Eur J Agron. 2002;16: 239–262. doi:10.1016/S1161-0301(02)00004-7

505 58. Bundschuh M, Elsaesser D, Stang C, Schulz R. Mitigation of fungicide pollution in detention

ponds and vegetated ditches within a vine-growing area in Germany. Ecol Eng. Elsevier B.V.; 2016;89: 121–130. doi:10.1016/j.ecoleng.2015.12.015

59. Allinson G, Zhang P, Bui AD, Allinson M, Rose G, Marshall S, et al. Pesticide and trace metal occurrence and aquatic benchmark exceedances in surface waters and sediments of urban wetlands and retention ponds in Melbourne, Australia. Environ Sci Pollut Res. 2015;22: 10214–10226. doi:10.1007/s11356-015-4206-3

60. Leeb C, Brühl C, Theissinger K. Potential pesticide exposure during the post-breeding migration of the common toad (*Bufo bufo*) in a vineyard dominated landscape. Sci Total Environ. 2020;706: 134430. doi:10.1016/j.scitotenv.2019.134430

61. Rohr JR, Sesterhenn TM, Stieha C. Will climate change reduce the effects of a pesticide on amphibians?: Partitioning effects on exposure and susceptibility to contaminants. Glob Chang Biol. 2011;17: 657–666. doi:10.1111/j.1365-2486.2010.02301.x

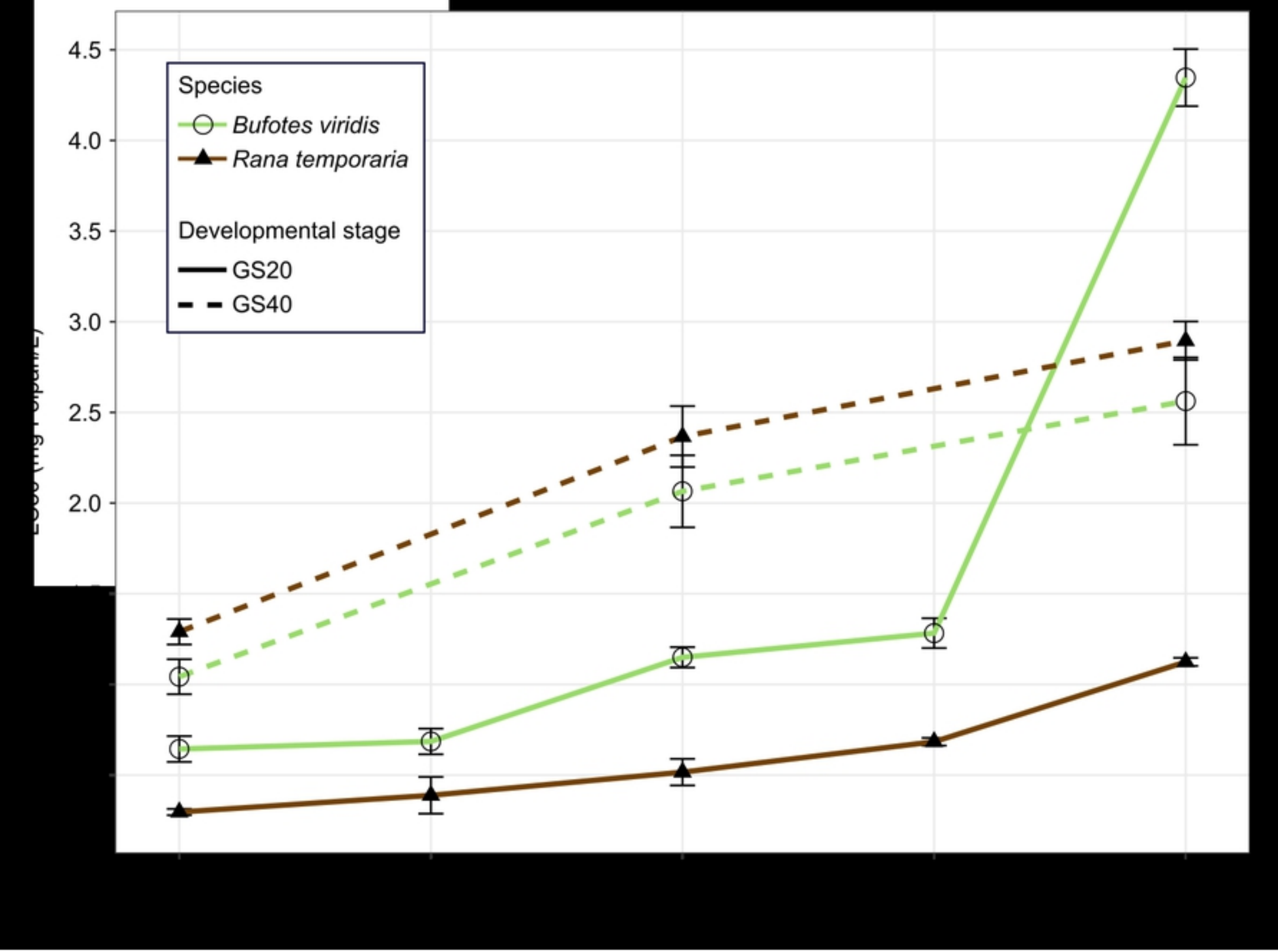
4.5
4.0
3.5
3.0
2.5
2.0
Species
Bufotes viridis
Rana temporaria
Developmental stage
GS20
GS40

Figure 1