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Environmental Toxicology and Chemistry, Vol. 16, No. 9, pp. 1940–1947, 1997

Printed in the USA
0730-7268/97 $6.00 + .00

EFFECTS OF TRIPHENYLTIN AND pH ON THE GROWTH AND DEVELOPMENT OF *RANA LESSONAE* AND *RANA ESCULENTA* TADPOLES

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(*Received* 8 *August* 1996; *Accepted* 26 *February* 1997)

Abstract—We tested the susceptibility of parental (*Rana lessonae*) and hemiclonal hybrid tadpoles of the *R. esculenta* complex to triphenyltin (TPT), a fungicide commonly used in agriculture, at renewed concentrations of 0.11, 0.81, and 1.87 μg/L over the entire larval period. Because habitats of *R. lessonae* are often characterized by a low pH and disturbed habitats such as gravel pits by high pH, we also tested whether pH 6.4 or pH 8.1 modifies their susceptibility to triphenyltin. We measured survival to metamorphosis, body mass at day 30, body mass at metamorphosis, and time to metamorphosis of individually reared tadpoles. Crosses of *R. lessonae* males with *R. lessonae* females and with two hemiclones of *R. esculenta* females produced tadpoles of *R. lessonae*, *R. esculenta* (GUT1) and *R. esculenta* (GUT2). At increasing triphenyltin concentrations, survival and growth rate decreased and time to metamorphosis increased. The GUT1 larvae of *R. esculenta* were the least and larvae of *R. lessonae* the most sensitive. The GUT2 larvae of *R. esculenta* were intermediate in sensitivity. The effect of pH also depended on the genotype. Tadpoles of *R. lessonae* had a higher growth rate and a shorter larval period at pH 6.4. The pH effect on tadpoles of *R. esculenta* was slight, but growth rate was lower and larval period longer at pH 6.4. Because TPT most strongly affected the larval period, exposure to TPT could decrease survival in natural ponds because of the increased risk of predation in permanent ponds or desiccation in drying ponds as well as the increased risk of additional chemical exposure. Our results indicate that the sexual parental species *R. lessonae* is more sensitive to environmental chemicals such as triphenyltin than is the hemiclonal hybrid *R. esculenta*.

Keywords—Amphibian Growth Metamorphosis pH Survival Triphenyltin

INTRODUCTION

Abiotic factors play an important role in the distribution and abundance of animal species. They not only limit the possible habitats where organisms are found, but they can also determine how ''viable'' a population is in the current environment. Factors like pH and pollution are important because of increasing habitat acidification and chemical contamination of natural habitats caused by human activities. Most experiments dealing with environmental pollution have determined short-term effects of acute toxicity on survival and have less often addressed sublethal effects of long-term exposure to chronic concentrations. Changes in life history traits related to fitness, such as growth rate and the timing of developmental events, can be just as critical as direct mortality. It is therefore particularly important to identify sublethal effects to fully assess responses to pollution, especially those affecting juvenile recruitment and population growth.

Triphenyltin (TPT) compounds are used worldwide as agricultural fungicides. The most important field of application is the control of blight in root crops such as sugar beets and potatoes, but they are also used on celery, carrots, onions, rice, pecan nuts, hops, coffee, and peanuts [1]. Its widespread agricultural use has resulted in the contamination of aquatic environments either through direct application (e.g., rice fields) or indirectly by runoff into ditches, temporary pools, and lakes during rainfall events. Inclusion of TPT in antifouling paints resulted in the contamination of harbors and rivers where concentrations up to 0.2 μg/L have been found [2–4]. After a single treatment of a rice field with 1.12 kg TPT/ha, an average water concentration of 146 μg/L was measured. All aquatic fauna and floating algae were killed [5]. Routine spraying on a potato field resulted in TPT concentrations of 1.5 to 2.7 μg/L in an adjacent ditch. Depending on how often these routine sprayings are taking place, and on the persistence of TPT in the aquatic environment, TPT can have a strong effect on fish and other aquatic organisms such as mosquito larvae, dragonfly larvae, and snails [1]. For sensitive fish species like *Pimephales promelas*, a mean lethal concentration (LC50) (96 h) is 7.1 μg/L [6]. *Oncorhynchus mykiss* showed the same effect at 15 μg/L of TPT hydroxide [7]. In *P. promelas*, sublethal effects could be detected at 0.23 μg/L TPT hydroxide after 30 d [6].

Little is known about the toxicity of TPT compounds to amphibians. It has recently been shown that TPT negatively affects feeding and swimming behavior of tadpoles after short-term exposure to 5 to 20 μg/L TPT [8]. In general, amphibians have been underrepresented in ecotoxicological studies of vertebrates, although amphibian biomass often constitutes an important component of many ecosystems [9]. In addition, their position in both aquatic and terrestrial food webs as primary and secondary consumers as well as prey makes them particularly sensitive to ecosystem deterioration and, consequently, useful as a bioindicator.

Rana esculenta and *R. lessonae* were chosen because of their sympatric and broad geographic distribution in Europe and ubiquitous presence in aquatic habitats, including agricultural landscapes [10]. *Rana esculenta* is a natural hybrid of *R. ridibunda* and *R. lessonae* that reproduces by hybridogenesis [11,12]. During gametogenesis, these hybrids eliminate the *R. lessonae* genome prior to meiosis and the *R. ridibunda* genome is transmitted clonally [reviewed in 12,13].

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In the *R. lessonae–esculenta* (L–E) system, hybridity in *R. esculenta* is restored each generation by sexual reproduction with its genetic host *R. lessonae*. This hemiclonal hybrid seems to be more tolerant of environmental conditions than *R. lessonae* [14]. The ecological success of *R. esculenta* is likely a result of its ability to metamorphose earlier than *R. lessonae* when resources are limited [15]. *Rana esculenta* is also more tolerant of anoxic conditions during hibernation than either parental species [16]. These findings together suggest that *R. esculenta* may possess a ''general-purpose'' genotype with broad tolerances to environmental extremes [17]. If *R. esculenta* does possess a general-purpose genotype, its phenotypic response (e.g., sensitivity to environmental factors such as TPT) should be lower across all levels of exposure than in the parental species *R. lessonae*.

In this study, we determined the sensitivity of tadpoles of the parental *R. lessonae* and two hemiclonal hybrid genotypes of *R. esculenta* to TPT by measuring survival and growth and development to metamorphosis at three different renewed concentrations (0.11, 0.81, and 1.87 μg/L). Because natural habitats of *R. lessonae* often are characterized by a low pH whereas *R. esculenta* predominate in areas that often are characterized by a high pH (e.g., gravel pits), we additionally tested whether pH 6.4 or pH 8.1 modified their susceptibility to the experimental TPT concentrations.

MATERIALS AND METHODS

Experimental design

Although other stages of growth and development are important, our focus in this study was on genotypic differences and fitness-related larval traits. Thus, tadpoles of three genotypes were reared at three TPT concentrations (plus two controls) and at two pH levels only from hatching to metamorphosis. This design yielded a total of 30 treatment combinations that were each replicated 10 times and resulted in a total of 300 tadpoles each reared in individual containers. Tadpoles from each genotype were selected and counted into cups (one tadpole per cup) and then randomly assigned to treatment and dishpan (one tadpole per dishpan). Single tadpoles were reared in 1 L of aged tap water in plastic dishpans (31 × 21 × 11 cm). Dishpans were arranged into five randomized-complete blocks (two replicates per block) according to shelf height to control for possible environmental gradients of temperature. The treatments were assigned randomly within blocks. The TPT concentrations used in this study were chosen according to a pilot study using *R. temporaria* tadpoles and were all below the 96-h LC50 [18].

Breeding design

We used artificial fertilizations to produce offspring of the parental species *R. lessonae* (LL) and of two different coexisting *R. esculenta* hemiclones (GUT1 and GUT2 [19]). Adult frogs of *R. lessonae* and *R. esculenta* used for the crosses were collected on May 12 and 15, 1993, from a pond near Gütighausen, Kanton Zürich, Switzerland. All frogs were held in outdoor enclosures until used. During the 1993 breeding season, the frog population at Gütighausen consisted of 57% of the host species *R. lessonae* and 43% of the hybridogenetic *R. esculenta*. The *R. esculenta* subpopulation consisted of four different hemiclones, distinguished by *R. ridibunda* alleles at the enzyme loci glucose phosphate isomerase (GPI), manose phosphate isomerase (MPI), and lactate dehydrogenase-B (LDH-B): hemiclone GUT1 (GPI *a*, MPI *c*, and LDH-B *c*) 68%, GUT2 (GPI*a*, MPI*a*, and LDH-B*c*) 23%, GUT3 (GPI*d*, MPI*c*, and LDH-B*c*) 7%, and GUT4 (GPI*a*, MPI*c*, and LDH-B*a*) 2% ([19] unpublished data). The taxon and hemiclone of each adult was determined before the crosses were made by protein electrophoresis using the discriminating enzymes GPI, MPI, and LDH-B [20]. Because of the limited availability of GUT3 and GUT4 hemiclones, only the two most common hemiclones, GUT1 and GUT2, were used in this study.

All females were injected with LH–RH hormone (luteinizing hormone–releasing hormone; H-7525; BACHEM, Bubendorf, Switzerland) to induce ovulation (approx. 24 h). Eggs from a female were stripped into a sperm suspension made by crushing both testes of a male in pond water. After 5 min, the sperm suspension was rinsed into a new petri dish (if used again) or discarded, and fresh pond water was added to cover the fertilized eggs. Eggs of the next female were then fertilized with the same sperm suspension or a newly prepared suspension. Replicate females and males of the parental species were crossed to create multiple full-sibling families. For *R. esculenta*, one female of each hemiclone was crossed using the sperm of the same *R. lessonae* male ($n = 3$ males total). Three replicate females of each hemiclone ($n = 6$ females total) were used to account for individual female maternal effects. The offspring of families resulting from this procedure were pooled into groups called *R. esculenta* (GUT1) and *R. esculenta* (GUT2). All crosses were made within 12 h on May 20 and tadpoles hatched on May 25 to 26, 1993. After hatching, all tadpoles were transferred to larger containers with 1.0 L of pond water.

Experimental treatments

Tadpoles were exposed to three TPT concentrations for the entire larval period with chemical renewal and water changes every 3 d. Water temperature during this time ranged between 23 and 25 °C. Tadpoles were fed a finely ground food ration (3:1 by mass of rabbit chow and TetraMin® fish flakes) every 3 d, starting at 5 mg/d [18]. Nominal TPT concentrations of 0.09, 0.91, and 1.82 μg/L (as TPT^+ ion) were achieved by dilution of a stock solution (FlukaAG, Buchs, Switzerland). Concentrations of TPT were relatively stable over the 3-d exposures and resulted in significant accumulation in tadpole body tissues [18]. The stock solution for all TPT treatments contained 50 mg of TPT chloride dissolved in 50 ml acetone, was stored at −20°C, and wrapped with aluminium foil to prevent photodegradation. Subsamples of 100 μl of each concentration were added to the respective experimental containers. Two additional treatments, pure aged tap water and aged tap water with 100 μl acetone, were used to control for the effect of the composition of the tap water and a standard TPT solvent. The acetone treatment controlled for the minimum dose of the solvent needed to deliver an accurate low-level dose of TPT. The tap water was aged 3 d in a 500-L Nalgene® container prior to use.

Experimental water samples (70 ml each) from 30 dishpans were taken and pooled across each genotype for a total of 2.0 L per treatment on June 17, 1993, for analysis of TPT and its transformation products. Dynamics of the TPT in the experimental containers during the 3-d periods between water changes and chemical additions were monitored extensively in pilot studies [18]. After the acidification of the water samples to pH 2.0 and the addition of 5 ml of 5% formaldehyde, they were stored in the dark at 4°C until analysis. The water

Table 1. An analysis of survival with an estimation of deviance for the model

Source of variation	*d.f.*	Deviance
1 Block + pH × TPT[a] × Genotype (Gen)	270	102.18
2 Block + pH + TPT + Gen + pH × TPT + pH × Gen + TPT × Gen	278	102.19
3 Block + pH + TPT + Gen + pH × TPT + pH × Gen	286	118.57
4 Block + pH + TPT + Gen + pH × TPT + TPT × Gen	280	123.82
5 Block + pH + TPT + Gen + pH × Gen + TPT × Gen	282	124.01

[a] TPT = triphenyltin.

was analysed in subsamples of 1 L for the controls and the lowest TPT dose, in subsamples of 500 ml for the medium dose, and in subsamples of 250 ml for the high dose. The analytical technique employed high-resolution capillary gas chromatography with flame photometric detection [21]. The normal and consistent pH in our aged tap water was 8.1. Concentrated H_2SO_4 was diluted with distilled H_2O in the ratio of 1:10 and then 0.65 ml of the diluted (1.81 N) H_2SO_4 were added to each container to average pH 6.4 over 72 h. Changes in pH were measured by a Metrohm pH-Meter 691 and recorded every hour initially and every 24 h on the second and third day. The low pH (below 6.2) was maintained for 24 h after the addition of H_2SO_4 but was then increased gradually to pH 7.2 by the third day [18]. The high pH was relatively stable during the 3-d renewal period [18]. Water was not buffered artificially because the buffer alone could have differentially affected larval growth and development. The pH values given in this study (pH 8.1 and 6.4) are the mean values over a 72-h period. These values reflect average pH exposure of the tadpoles and are not unlike the natural fluctuations in small ponds after heavy rain or during drying (unpublished data).

Data and statistical analyses

The experiment started on May 30, 1993, and ended after the last tadpole completed metamorphosis on August 12, 1993. Survival, body mass at day 30, body mass at metamorphosis, and time to metamorphosis were measured for each tadpole that survived. Each individual was weighed twice, once at day 30 and a second time after tail resorption was complete (3–4 d after forelimb emergence). Time to metamorphosis was defined as days from the start of the experiment to forelimb emergence at stage 42 [22]. Because all tadpoles were the same age, all treatments were started on the same day, and metamorphosis had a constant well-defined endpoint (stage 42), the number of days to metamorphosis was used as a measure of the developmental rate of tadpoles.

Survival was analyzed as a generalized-linear model using the GLM function in S-Plus, with binomial-distributed errors. This type of analysis allows for the estimation of whether or not the probability of survival is affected by treatment and accounts for the binary nature of the data. Body mass at day 30, body mass at metamorphosis, and time to metamorphosis were logarithmically transformed before the analysis to reduce skewness. Differences in treatment effects and their interactions were analyzed with a univariate fixed-effect analysis of variance (ANOVA) using the GLM procedure of SAS (SAS Institute, Raleigh, NC, USA). For the tests of significance, Type III sums of squares were used to account for the unequal replication resulting from mortality. Levels of significance were adjusted according to the Bonferroni method to account for multiple tests of the data. All pairwise comparisons of means within significant treatments were made using Scheffe's tests that controlled the Type I experimentwise error rate. All correlations were calculated with Pearson product–moment correlations.

Table 2. Comparisons of the change in deviance when a term is dropped from the model (Table 1). The change in deviance is an asymptotic chi-square distribution with the degrees of freedom equal to the number of parameters dropped from the model ($\alpha \leq 0.05$)

Source of variation	*d.f.*	Deviance	*p* Value
pH × TPT[a] × Genotype (1[b] vs 2)	8	0.01	NS
TPT × Genotype (2 vs 3)	8	16.4	0.036
pH × Genotype (3 vs 4)	2	21.7	0.0002
pH × TPT (4 vs 5)	4	21.8	0.0002

[a] TPT = triphenyltin.
[b] Numerical terms refer to the sources of variation from the model presented in Table 1.

RESULTS

Nominal TPT concentrations and effective TPT concentrations, as determined by chemical analyses of the water did not differ significantly [18]. Effective TPT concentrations were 0.11, 0.81, and 1.87 μg/L and any decreases from the expected concentrations were a result of the adsorption of TPT to food particles and to the direct uptake through the skin of the tadpoles [18].

Block (or shelf) had a significant effect on all response variables except survival (Tables 1 to 5). The effect was likely caused by a vertical temperature gradient that varied with shelf height (approximately 5°C warmer on the top shelf) and interacted with both TPT and pH. This effect is not unexpected, nor does it detract from the main effects of TPT or pH because both main effects were highly significant and not likely to be explained just by the two-way interaction terms (Tables 3 to 5). It does suggest, however, that temperature can mediate TPT and pH effects.

Survival of the tadpoles depended on TPT, pH, and genotype because each of the two-way interactions was significant (Table 2). In control containers with tap water and acetone, survival was 92 to 95%, but survival decreased gradually with increasing levels of TPT to 38% (Fig. 1a). Most mortality occurred during the 3- to 5-d period of tail resorption (stages 42–46 [21]), but no gross developmental abnormalities were observed during this time. The mechanism for death is as yet unknown, but the time during metamorphosis seemed to be especially critical for tadpole survival, particularly for tadpoles at highest TPT levels. The genotype of the tadpoles also affected survival. Overall, 86% of the *R. esculenta* tadpoles (average of RL1 and RL2) survived through metamorphosis, whereas only 55% of the *R. lessonae* tadpoles survived through metamorphosis. A large portion of the difference in the survival of genotypes was manifested at the two pH levels

Table 3. A summary of the univariate analysis of variance (ANOVA) of body mass at day 30. Levels of significance were adjusted according to the Bonferroni correction to account for multiple testing of the data ($\alpha = 0.0167$, * $p < 0.0167$, ** $p < 0.001$, and *** $p < 0.0001$)

Source of variation	*d.f.*	Mean squares	*F* Ratio	*p* Value
Triphenyltin (TPT)	4	4.1182	64.44	0.0001***
pH	1	0.0025	0.04	0.8402
Genotype	2	1.4564	23.50	0.0001***
Block	4	0.1488	2.40	0.0534
TPT × pH	4	0.1749	2.82	0.0278
TPT × Genotype	8	0.1979	3.19	0.0025*
TPT × Block	16	0.0458	0.74	0.7496
pH × Genotype	2	0.2926	4.72	0.0106*
pH × Block	4	0.2218	3.58	0.0085*
Genotype × Block	8	0.0439	0.71	0.6835
TPT × pH × Genotype	8	0.1129	1.82	0.0791
TPT × pH × Block	16	0.0658	1.06	0.3991
TPT × Genotype × Block	29	0.0595	0.96	0.5303
pH × Genotype × Block	8	0.1066	1.72	0.1000
Four-way interaction	27	0.0413	0.67	0.8891
Error	125	0.0620		

(Fig. 1b). Although survival of the three genotypes at pH 6.4 was not significantly different, at pH 8.1 the survival of *R. lessonae* tadpoles was signifcantly lower than that of either hemiclone of *R. esculenta* (Fig. 1b).

Body mass at day 30 was strongly affected by the concentration of TPT (Table 3, Fig. 2a). Concentrations of 0.81 and

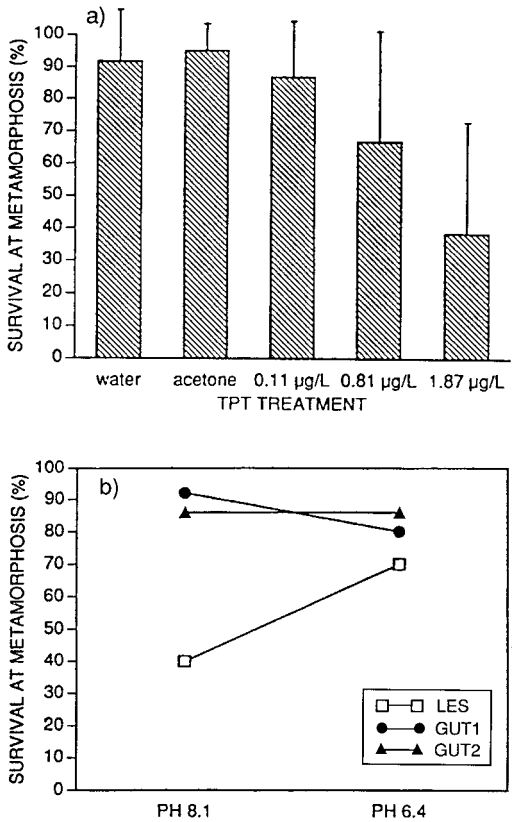

Fig. 1. (**a**) Mean percent survival of tadpoles in tap water, acetone controls, and at three different triphenyltin (TPT) concentrations and (**b**) the interaction of genotype and pH on survival of tadpoles to metamorphosis. Lines connect the least square means of all replicates. LES = *Rana lessonae* tadpoles, GUT1 = hemiclone 1 of *R. esculenta* tadpoles, and GUT2 = hemiclone 2 of *R. esculenta* tadpoles.

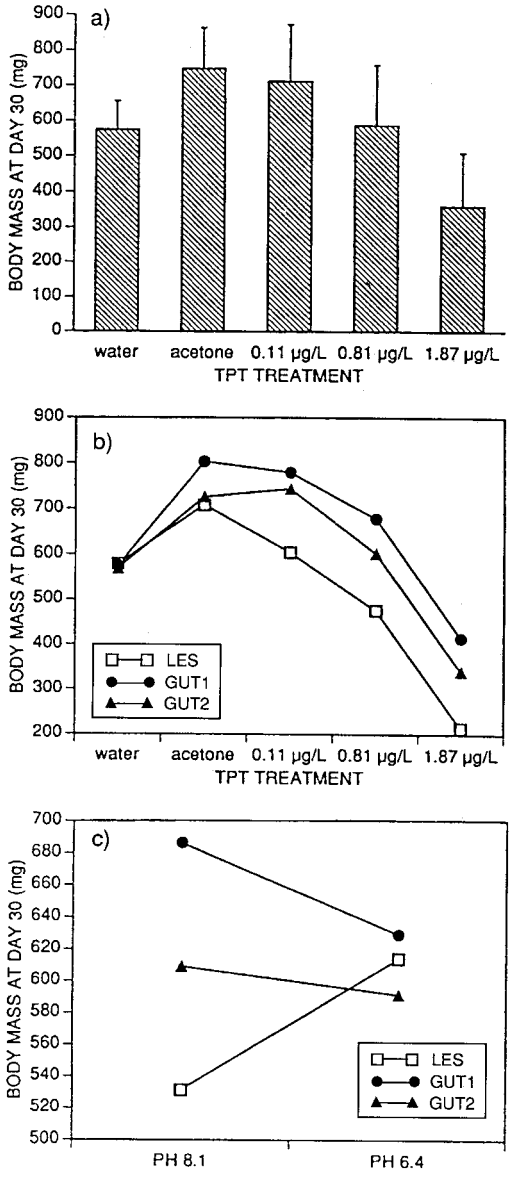

Fig. 2. (**a**) Mean body mass of tadpoles at day 30 (mg) in tap water, acetone controls, and at three different triphenyltin (TPT) concentrations. Bars represent mean values + one standard error. (**b**) Mean body mass of tadpoles at day 30 (mg) in tap water and acetone controls and at three different TPT concentrations plotted by genotype. Lines connect the least square means of all replicates. (**c**) Interaction of genotype and pH on mean body mass of tadpoles at day 30 (mg). Lines connect the least square means of all replicates. LES = *Rana lessonae* tadpoles, GUT1 = hemiclone 1 of *R. esculenta* tadpoles, and GUT2 = hemiclone 2 of *R. esculenta* tadpoles.

1.87 µg/L TPT significantly decreased the body mass at day 30, but there was no difference between the lowest TPT concentration of 0.11 µg/L and the acetone control (Fig. 2a). All genotypes performed equally well in the tap water control but diverged with the addition of acetone and TPT. Tadpoles of GUT1 were heaviest at day 30 ($\bar{X} \pm$ SE = 651 ± 24 mg), *R. lessonae* tadpoles were the lightest (501 ± 26 mg), and GUT2 tadpoles were intermediate (597 ± 23 mg) at all levels of TPT (Fig. 2b). Body mass at day 30 of the three genotypes also differed between the pH levels (Table 3). Body mass at pH 6.4 was similar among all genotypes but diverged significantly at pH 8.1, with body mass decreasing for *R. lessonae* tadpoles

Table 4. A summary of the univariate analysis of variance (ANOVA) of body mass at metamorphosis. Levels of significance were adjusted according to the Bonferroni correction to account for multiple testing of the data ($\alpha = 0.0167$, * $p < 0.0167$, ** $p < 0.001$, and *** $p < 0.0001$)

Source of variation	*d.f.*	Mean squares	*F* Ratio	*p* Value
Triphenyltin (TPT)	4	0.1849	15.70	0.0001***
pH	1	0.0036	0.31	0.5809
Genotype	2	0.0099	0.84	0.4352
Block	4	0.0761	6.46	0.0001**
TPT × pH	4	0.0121	1.03	0.3972
TPT × Genotype	7	0.0334	2.84	0.0090*
TPT × Block	16	0.0119	1.01	0.4493
pH × Genotype	2	0.0048	0.41	0.6672
pH × Block	4	0.0128	1.08	0.3673
Genotype × Block	8	0.0168	1.43	0.1905
TPT × pH × Genotype	6	0.0247	2.10	0.0588
TPT × pH × Block	14	0.0127	1.08	0.3854
TPT × Genotype × Block	27	0.0097	0.83	0.7098
pH × Genotype × Block	8	0.0069	0.58	0.7893
Four-way interaction	19	0.0271	2.31	0.0034
Error	118	0.0118		

Table 5. A summary of the univariate analysis of variance (ANOVA) of time to metamorphosis. Levels of significance were adjusted according to the Bonferroni correction to account for multiple testing of the data ($\alpha = 0.0167$, * $p < 0.0167$, ** $p < 0.001$, and *** $p < 0.0001$)

Source of variation	*d.f.*	Mean squares	*F* Ratio	*p* Value
Triphenyltin (TPT)	4	0.6049	162.19	0.0001***
pH	1	0.0084	2.25	0.1362
Genotype	2	0.1431	38.36	0.0001***
Block	4	0.0421	11.30	0.0001***
TPT × pH	4	0.0113	3.02	0.0207
TPT × Genotype	7	0.0100	2.67	0.0133*
TPT × Block	16	0.0094	2.52	0.0023*
pH × Genotype	2	0.0301	8.07	0.0005**
pH × Block	4	0.0133	3.58	0.0086*
Genotype × Block	8	0.0072	1.93	0.0622
TPT × pH × Genotype	6	0.0071	1.92	0.0838
TPT × pH × Block	14	0.0054	1.45	0.1402
TPT × Genotype × Block	27	0.0052	1.39	0.1165
pH × Genotype × Block	8	0.0055	1.47	0.1753
Four-way interaction	19	0.0043	1.14	.3173
Error	118	0.0037		

but increasing slightly for both GUT1 and GUT2 tadpoles of *R. esculenta* (Fig. 2c).

Body mass at metamorphosis was significantly affected by TPT concentration (Table 4, Fig. 3). Body mass was similar among the controls and the lowest two TPT concentrations but increased significantly at 1.87μg/L TPT (Fig. 3). There was also a significant interaction between genotype and TPT concentration with body mass (Table 4); that is, GUT1 tadpoles achieved a significantly larger body mass at 0.81μg/L TPT than did GUT2 tadpoles or *R. lessonae* tadpoles. In contrast to body mass at day 30, there was no effect of pH on body mass at metamorphosis.

The time to metamorphosis was significantly affected by TPT concentration (Table 5, Fig. 4a). The time to metamorphosis was significantly longer for the two highest TPT concentrations compared to the controls or the lowest TPT concentration (Fig. 4a). The time to metamorphosis for the two hemiclones were similar (GUT1 and GUT2; 45 ± 1 d) but were significantly shorter than for *R. lessonae* tadpoles (49 ± 1 d; Table 5). There was also a significant interaction between genotype and pH with time to metamorphosis (Table 5). For both hemiclones of *R. esculenta* and at the two pH levels, time to metamorphosis was short and was not significantly different (Fig. 4b), but the response of *R. lessonae* tadpoles was significantly longer compared to the hemiclones with time to metamorphosis increasing sharply at pH 8.1 (Fig. 4b).

Body mass at metamorphosis and time to metamorphosis

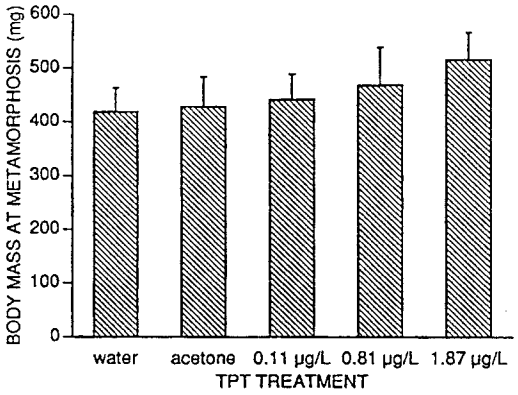

Fig. 3. Mean body mass at metamorphosis (mg) of tadpoles in tap water, acetone controls, and at three different triphenyltin (TPT) concentrations. Bars represent mean values + one standard error.

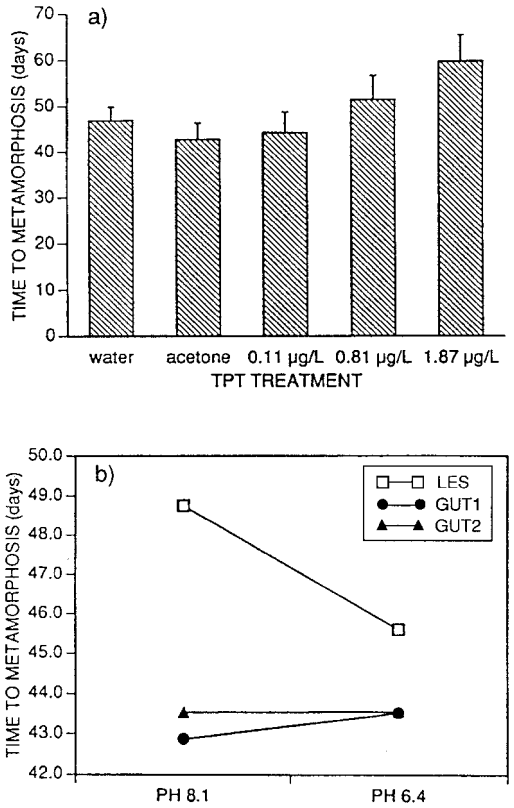

Fig. 4. (**a**) Mean time to metamorphosis (in days) of tadpoles in tap water, acetone controls, and at three different triphenyltin (TPT) concentrations. Bars represent mean values + one standard error. (**b**) Interaction of genotype and pH on mean time to metamorphosis (in days days) of tadpoles. Lines connect the least square means of all replicates. LES = *Rana lessonae* tadpoles, GUT1 = hemiclone 1 of *R. esculenta* tadpoles, and GUT2 = hemiclone 2 of *R. esculenta* tadpoles.

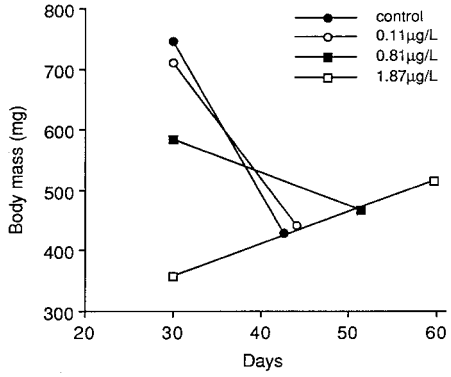

Fig. 5. Bivariate relationship between the time to metamorphosis and body mass at metamorphosis for the control (combined tap water and acetone) and three triphenyltin (TPT) concentrations. Lines connect the least square means of all replicates.

were significantly correlated and illustrate the interdependency of the larval traits. Overall, the correlation of body mass at metamorphosis with time to metamorphosis was positive (Pearson's Correlation; $r = 0.55$, $p < 0.0001$, $n = 190$). This positive relationship indicated that a large body mass at metamorphosis can only be attained by a long larval period and hence by a tradeoff in performance. However, the TPT concentrations used in our experiment altered the relationship between body mass at metamorphosis and days to metamorphosis from highly negative in the acetone control (no trade-off) to positive at the highest TPT concentration (indicating a trade-off, Fig. 5). The pattern of the trade-off was similar among the three genotypes [18].

DISCUSSION

In summary, our results show that all genotypes of tadpoles exhibited reduced survival and body mass at day 30 (growth rate) but exhibited increased body mass at metamorphosis and time to metamorphosis when exposed to the highest concentrations of TPT. Significant increases in body mass at day 30 of tadpoles in the acetone control and lowest TPT level suggest a hormetic effect of acetone that may have masked the effects of the lowest TPT levels. Survival to metamorphosis and body mass at metamorphosis were lower for *R. lessonae* tadpoles than for either hemiclone of *R. esculenta.* The hemiclones and parental tadpoles showed an asymmetrical response. A low pH enhanced the performance of *R. lessonae* tadpoles considerably. Larvae of *R. lessonae* increased their growth rate and metamorphosed faster at low pH, whereas the hybrids displayed a neutral or slightly negative response. Tadpoles of both hemiclones experienced reduced growth rates at low pH but exhibited no change in time to metamorphosis. Tadpoles of *R. lessonae* appeared to be more sensitive to TPT and pH variation than tadpoles of either hemiclone of *R. esculenta.*

It has recently been found that after a short-term exposure to TPT, tadpoles reduced their swimming and feeding activity significantly thereby limiting food availability [8]. Antecdotal observations of tadpoles in our containers also support a mechanism of reduced feeding activity. The time spent feeding is directly related to the level of food intake and weight increase in tadpoles [23]. Reduced food intake has dramatically negative effects on growth and development of amphibian larvae resulting directly in small body mass at metamorphosis and long larval periods [e.g., 15,24]. It can also indirectly affect individuals by reducing survival through the increasing risk of predation in permanent ponds or desiccation in ephemeral ponds and also by reducing adult reproductive fitness [e.g., 25–27]. Damage to the central nervous system similar to that seen in fish may account for changes in tadpole behavior [28], however the mode of action is unknown for tadpoles. It is also possible that TPT had a direct effect on the developmental rate of tadpoles, independent of food intake, because days to metamorphosis increased significantly. However, further simultaneous manipulation of food and TPT levels would be necessary to separate alternative mechanisms.

The interaction of TPT concentration and pH on survival found in our study supports results from previous investigations with carp and *Daphnia.* These studies demonstrated that the accumulation of TPT (and compounds similar to TBT) was pH dependent [29,30]. Both studies showed that animals accumulated higher amounts of organotins at high pH levels, and consequently, the same levels of TPT can be more toxic at high pH because of the higher TPT accumulation. Thus, one prediction was that tadpoles exposed to TPT should grow better at low pH if the pH alone had no adverse effects. However, a low pH (usually pH < 6.0) can cause the disruption of Na^+ and Cl^- regulation and greatly accelerate the Na^+ efflux [31,32]. Increasing the Na^+ influx to reduce the Na^+ loss is costly and can reduce growth and retard development [33]. Hence, it is difficult to separate effects caused by a higher TPT accumulation at high pH and effects caused by a higher hydrogen ion concentration at low pH. However, because the hydrogen ion concentration at a given pH is constant at all TPT levels, TPT and pH effects interacted significantly on survival (Table 1), thus supporting the hypothesis that the toxicity of TPT actually increases at high pH. It is also interesting to note that the three-way interaction (pH $\times$ TPT $\times$ genotype; Table 2) was not significant, indicating that the increased toxicity of TPT at high pH was a general effect on all tadpoles independent of genotype. At metamorphosis, this pH–TPT interaction was no longer significant for body mass but was nearly significant for time to metamorphosis. It is likely that TPT accumulation at metamorphosis had reached saturation and thus reduced the pH-specific TPT effect.

An important finding of our study was the shift in trade-offs between body mass at metamorphosis and time to metamorphosis at different TPT concentrations. The clear shift from a negative relationship between body mass and larval period under control conditions to a positive relationship at high TPT concentration (Fig. 5) indicates that tadpoles shift from no trade-off in fitness under favorable conditions (i.e., short larval period and large body size at metamorphosis) to a trade-off in fitness at high chemical exposure (i.e., either short larval period or large body size at metamorphosis). We suggest that the trade-off in fitness may be a general response of tadpoles to environmental stress that limits growth. Triphenyltin exposure probably limits the food intake and slows growth rate such that early metamorphosis is not possible. However, if tadpoles survive and continue to grow even slowly, they can achieve a relatively large size at metamorphosis [24,34]. Thus, the performance of tadpoles in ephemeral or time-limited habitats contaminated with environmental chemicals such as TPT will be especially poor, but performance may be near normal in more resource-rich habitats even if contaminated with sublethal levels of chemicals, assuming other factors such as predation or parasitism are equal between habitats.

Laboratory effects and application to the field

The susceptibility of the tadpoles to TPT could have been modified by the experimental conditions of the laboratory and may not be directly applicable to field conditions. In our experiment, *R. esculenta* tadpoles almost always performed better (i.e., had a larger body mass and a shorter time to metamorphosis) than *R. lessonae* tadpoles. However, under certain environmental conditions, notably in undisturbed ponds with low tadpole densities and unlimited food, *R. lessonae* tadpoles can have shorter larval periods and larger body sizes at metamorphosis than *R. esculenta* tadpoles [14,15]. Therefore, tadpoles of *R. lessonae* were expected to grow better than *R. esculenta* tadpoles at least in the control treatments, but they did not. We suggest that the artificial food supply, accumulation of wastes, or lack of ability to thermoregulate created a stressful environment for tadpoles even under the best laboratory conditions (i.e., controls). If all conditions in the experiment were selectively more stressful for *R. lessonae* tadpoles, then *R. esculenta* tadpoles could have been favored in all treatments simply because of its generally reduced susceptibility to environmental stress in the laboratory. A good indication of the presence of environmental stress during our experiment was the overall low body mass at metamorphosis, even in the control treatments, compared to tadpoles raised under semi-natural conditions in artificial ponds or in field cages [R.D. Semlitsch, unpublished data; 14,15]. However, our results indicate that *R. lessonae* tadpoles performed equally well as *R. esculenta* tadpoles in a number of benign treatments, and therefore we conclude that *R. esculenta* is relatively more tolerant to the chemical treatments in our experiment than *R. lessonae* and that our results can be extended to field conditions.

Species differences and distributions

If species respond differently to the same gradient of environmental variation, this variation can affect their coexistence by modifying their competitive relationships. This is especially true for this hybridogenetic system where *R. esculenta* must rely on the presence of its genetic host *R. lessonae* in the population for breeding. The differential response of *R. lessonae* and *R. esculenta* larvae to TPT and pH may, in part, explain their locally varying frequencies.

Our results, in addition to previous research, indicate that *R. lessonae* tadpoles are more sensitive to environmental stress or pollution than *R. esculenta* tadpoles [16,35]. This evidence correlates with the fact that *R. lessonae* prefers small lakes or ponds with a low nutrient content and rich vegetation in a pristine environment [12,36]. *Rana esculenta,* however, lives in a wide variety of habitats and is more abundant in disturbed habitats near agricultural fields or urban ponds [35]. Therefore the presence of environmental pollution could have an influence on the composition of local populations of these European greenfrogs. In addition, our study shows that larvae of *R. lessonae* greatly increased their growth rate and reduced their time to metamorphosis at low pH, whereas larvae of *R. esculenta* had a slightly reduced growth rate. *Rana lessonae* is found more frequently in habitats having a low pH [36], and this preference for more acidic ponds may also involve its use of such sites as refuges from less tolerant competitors or predators [33]. The hybrid species, *R. esculenta,* which is a strong competitor of *R. lessonae,* is less abundant in more acidic ponds and showed a tendency to reduce growth at low pH.

The cause of genetic differences between hemiclonal hybrids and the parental species, and between the two hemiclones, can be related to three mechanisms: (1) heterosis resulting from hybridity, (2) interclonal selection, or (3) some combination of the two. Although spontaneous heterosis is not universal among clonal hybrids [37], it has recently been demonstrated in the larval performance of newly generated F_1 hybrids of *R. esculenta* [38]. Nevertheless, because our study was performed using natural lineages of hemiclones subjected to many generations of selection, it is also likely that interclonal selection has acted upon life history traits of individuals to yield a subset of best-adapted coexisting hemiclones in populations [19,39]. Thus, any explanations of why the hybrid *R. esculenta* survived and performed better under many environmental conditions must consider both genetic heterosis and interclonal selection [14–16]. Although our results for both hemiclones of *R. esculenta* combined tend to support the general-purpose genotype model that assumes broad tolerances to environmental conditions [17], significant differences between the hemiclones in some performance traits weakens our support for this model by suggesting more than one adaptive genotype.

In conclusion, our study shows that environmental factors such as pH and environmental chemicals have a significant impact on growth and development of tadpoles and, in turn, could negatively affect important components of fitness [25,27]. Thus, we suggest that these factors may contribute to the variation in local frequencies of *R. lessonae* and *R. esculenta* in this hybridogenetic system as well as to the decline of populations of more sensitive amphibian species in general. In addition, our results support the idea that chemical stress is greater for those amphibian species that are more narrowly adapted, while greater tolerance can be observed for more broadly adapted amphibians [40].

Acknowledgement—We thank P. Beerli, G.-D. Guex, and H. Hotz for help in collecting and genotyping frogs, C. Rauter for helpful suggestions and advice using statistical software, A. Schymainda for the permission to use equipment, and especially to J. Hunn, who devoted time and patience during the GC analysis. E. Fioramonti also thanks B. Anholt for his support and other colleagues in the department for their help during his tenure. This study was funded by the Swiss National Fund Grants NF 31-28568.90 and NF 31-37579.93.

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