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Acute and chronic effects of Cr(VI) on *Hypsiboas pulchellus* embryos and tadpoles

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ABSTRACT: In the last few years there has been great concern about declines in the abundance of several species of amphibians around the world. Among amphibians, anurans have a biphasic life cycle, with aquatic tadpoles and generally terrestrial adults, and they have an extremely permeable skin, making them excellent indicators of the health of the environment. A number of different causes have been suggested for the global decline of anurans, the pollution of their habitat by chemical stressors being considered one of the major factors. Among chemical stressors, heavy metals are known for their high toxicity at very low concentrations. This study assessed short- (96 h, 'acute') and long-term (1272 h, 'chronic') exposure to Cr(VI) at lethal (3 to 90 mg l^{-1}) and sublethal concentrations (0.001 to 12 mg l^{-1}) on *Hypsiboas pulchellus* (previously called *Hyla pulchella*; see Faivovich et al. 2005) tadpoles (Fam. Hylidae) from central eastern Argentina. Fertilized eggs collected from a clean pond near La Plata (Buenos Aires Province) were used for acute and chronic toxicity testing. Assays were done under controlled laboratory conditions. Results of chronic exposure were used to assess the effect of factors such as toxicant concentration and age of organisms at the beginning of exposure on the response variables (growth, development and survival until metamorphosis). Results indicated a higher sensitivity to Cr(VI) of individuals first exposed as tadpoles than those first exposed as embryos during acute and chronic exposure. Exposure to the highest sublethal concentrations (6 to 12 mg l^{-1}) of the toxicant showed early inhibitory effects on growth of all treated organisms compensated at longer exposure periods with an increase in the growth rate compared to the control groups.

KEY WORDS: Anurans · *Hyla pulchella* · *Hypsiboas pulchellus* · Embryo · Larvae · Toxicity · Growth · Survival · Cr(VI)

INTRODUCTION

The presence of chromium in the environment has been mainly related to anthropogenic sources such as the tanning, electroplating, steel, chromate, cement and glass industries, and power plants (Abbasi & Soni 1984, Goyer 1996). Special attention has been given to this metal in the oxidation state Cr(VI) due to its higher toxicity, induction of genotoxicity, DNA damage and mutagenicity (Sugiyama et al. 1986, Zhitkovich et al. 1995, Gayatri et al. 1997, Stearns 2000, Zhitkovich et al. 2001, Quievryn et al. 2002). In relation to the effect of Cr(VI) on amphibians, the metal has been poorly characterized. Only a small set of toxicity data is available to evaluate potential effects (Linder & Grillitsch 2000). The LC_{50} vary widely, the embryos of *Gasthrophryne carolinensis* being a particularly sensitive species (Birge 1978). Differences in chemical species and test methodology tend to confound the interpretation of survival data. The reports indicate lethal Cr (VI) concentration generally >1 mg l^{-1} (Linder & Grillitsch 2000). Other reports also found inhibition of growth and development and teratogenicity, indicating sublethal effects at Cr (VI) concentrations <0.5 mg l^{-1} (Slooff & Canton 1983, Abbasi & Soni 1984).

The objective of the present study was to investigate the effects of long-term exposure to Cr(VI) on a Neotropical species of the family Hylidae, one of the most diverse and widely distributed frog families in the world. *Hypsiboas pulchellus* (previously called *Hyla pulchella*; see Faivovich et al. 2005) is the most representative species for the subtropical wetland area of

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South America (Cei 1980). The experimental design of the study aimed to assess short- and long-term exposure to Cr(VI) at lethal and sublethal levels on *Hypsiboas pulchellus* tadpoles. The 1200 individuals obtained from the same hatching were exposed to the toxicant at 2 different stages of development, and we assessed short-term lethal effects, inhibition of growth, and survival and development until metamorphosis.

MATERIALS AND METHODS

Test species and source of organisms. Anurans *Hypsiboas pulchellus* are slender and small (37 to 50 mm) with smooth and permeable skin. They lay their eggs in masses, which are attached to the submerged stems of aquatic plants and develop into aquatic larvae. The breeding season occurs in 2 well-defined periods, coinciding with the filling of temporary water pools after heavy rains and sharp temperature changes. The first and more conspicuous period corresponds to the beginning of the cold season in the southern hemisphere, between April and May, with larvae taking between 5 and 6 mo to reach metamorphosis. The second breeding period occurs during early spring, coincident with a sharp increase in temperature, with larval metamorphosis occurring ca. 2 to 3 mo later. This last event is also related to the drying of pools in December.

Adult frogs were collected from an unpolluted permanent pond located near La Plata, Province of Buenos Aires (35° 01′ S, 57° 51′ W) (Ronco et al 2001). Mating pairs were captured during amplexus and placed in 3 l glass jars containing water and vegetation from the site. The jar containing the pair was placed in the natural habitat until egg-laying was observed. The 1460 eggs obtained by this procedure were then transferred to the laboratory for bioassay studies. The staging of anuran embryos and larvae was done according to Gosner (1960).

Experimental design. Non-fertilized eggs were removed, and the remaining 1200 fertilized eggs were divided into 2 equal groups. One of the groups (16 h embryos, Gosner stage 8–10) was used immediately for acute toxicity testing. The second was maintained in the laboratory for 176 h until organisms reached Gosner stage 25, and were then exposed to the toxicant under the same conditions as the first group. Embryos and tadpoles were maintained in dechlorinated tap water (pH 7.6–8.3, hardness 250 mg $CaCO_3$ l^{-1}) with continuous aeration, at a temperature of 25 ± 1°C, and a 16:8 h light:dark cycle. Toxicity tests were done using 25 concentrations of Cr(VI), from 0.001 to 90.0 mg l^{-1}. Chromium concentrations of 3.0, 6.0, 9.0, 12.0, 18.0, 21.0, 24.0, 27.0, 30.0, 33.0, 36.0, 39.0, 60.0 and 90 mg l^{-1} were used to study acute, 96 h lethal effects. Individuals were removed after exposure and fixed in 10% v/v buffered formalin. Those exposed to Cr(VI) concentrations of 0.001, 0.005, 0.010, 0.050, 0.100, 0.250, 0.500, 0.750, 1.0, 3.0, 6.0, 9.0, 12.0 mg l^{-1} were used to assess chronic effects. Medium and conditions used for toxicity testing were the same as those for maintenance. Tests were performed in 0.5 l polypropylene test chambers with 5 individuals each. Medium was completely replaced every day for the 96 h acute exposure tests and once per week in the chronic tests. All tests included 4 replicates per concentration and negative controls. The individuals from the acute exposure conditions were not fed, and those under chronic exposure were fed once per week with blended lettuce ad libitum just before medium renewal. Final end point measurements were taken when the first individual of any group reached metamorphosis (Gosner stage 46).

Assessment of response variables. Mortality was determined every 24 h during acute exposure by visual observation of immobility after gently prodding the tadpoles with a glass rod. Assessed chronic exposure end points were growth, development and survival. Growth was registered once per week by measuring the body length with dial calipers to the nearest 0.01 mm, at the same time as survival assessment (ratio of live:dead organisms per test chamber). Additionally, on the last day of the experiment, the weight of all tadpoles was measured to the nearest 0.001 g using an electronic balance, in parallel with microscopic observations to determine development stage.

Statistical analysis of results. Acute toxicity data were analysed by the Probit method (Finney 1971). Chronic data was analysed to assess the effect of factors such as toxicant concentration and age of organisms at the beginning of exposure (age-group) on the response variables (survival, body length). Analyses included 3 types of hypothesis tests: 2-factor ANCOVA (on all end points measured during the experiment, using time of exposure and age of organisms as covariables), 2-factor ANOVA (to compare age-groups at each of the 7 measuring times of the experiment), and 1-way ANOVA with Dunnett's test (effect of exposure in each age-group) (Zar 1998). The median tolerance limit (time to reach 50% mortalities), and the 'no observed effect' and 'lowest observed effect' concentrations (NOEC and LOEC) for Cr(VI) were also estimated (USEPA 1989). The additional measured variables at metamorphosis (weight, development stage) were analysed using 2-factor ANOVA (to assess the 2 factors on weight), 1-way ANOVA with Dunnett's test (effect of exposure within each age-group) and Friedman and Kruskal-Wallis non-parametric tests (effect of the factors on development stage). Data of survival rate

per test chamber from the chronic exposure was angular transformed before statistical analysis of results (Zar 1998).

Quality controls. Test solutions of Cr(VI) were prepared from $K_2Cr_2O_7$ (Merck, analytical grade). Chromium concentrations in test solutions at the beginning of experiments and after exposure were confirmed by atomic absorption spectrophotometry using a Varian Spectra AA 300, with air-acetylene flame (APHA 1998). Quality controls included reagent blanks and a certified chromium solution (AccuTrace Reference Standard).

RESULTS

Lethal effects

Acute effect results on *Hypsiboas pulchellus* tadpoles after 96 h exposure, expressed as LC_{50}, were >60 mg Cr(VI) l^{-1} for the embryo age-group (Gosner stage 8–10), and 29.60 (27.46 to 31.94) mg Cr(VI) l^{-1} for the tadpole age-group (Gosner stage 25). Lethal median times for the embryo and tadpole age-groups exposed to a Cr(VI) concentration of 12 mg l^{-1} were 535 and 330 h, respectively.

ANCOVA analysis of chronic lethal effects on all tadpole data, considering time of exposure and age of individual as covariables, showed the influence of both studied factors on the mortality ($p < 0.001$) and the interaction between them ($p < 0.001$), though the survival of the tadpole age-group was lower than the embryo age-group ($p < 0.001$). The comparison between concentrations during the exposure time showed significant differences (Cr(VI) concentrations: 6, 9 and 12 mg l^{-1}, with respect to controls) in the survival of individuals.

Comparisons showed that the tadpole age-group had significantly higher mortalities than the embryo age-group ($p < 0.05$) during the exposure time interval 264 to 768 h. After that, no differences in mortality were observed between age-groups. The plots of mean survival proportion for different concentrations of the toxicant for both age-groups are shown in Fig. 1A,B, and indicate that the tadpole age-group was much more sensitive than the embryo age-group. There was higher mortality after the first days of exposure (Cr(VI) concentrations between 6 and 12 mg l^{-1}) in that age-group, and a more gradual response behaviour in the embryo age-group (Cr(VI) concentrations of 9 and 12 mg l^{-1}).

Results of NOEC and LOEC estimations with time using ANOVA and Dunnett's test for each age-group for the effect of Cr(VI) on survival can be seen in Fig. 2A,B. Both assessment end points follow the same tendency, with higher sensitivity for the tadpole age-group.

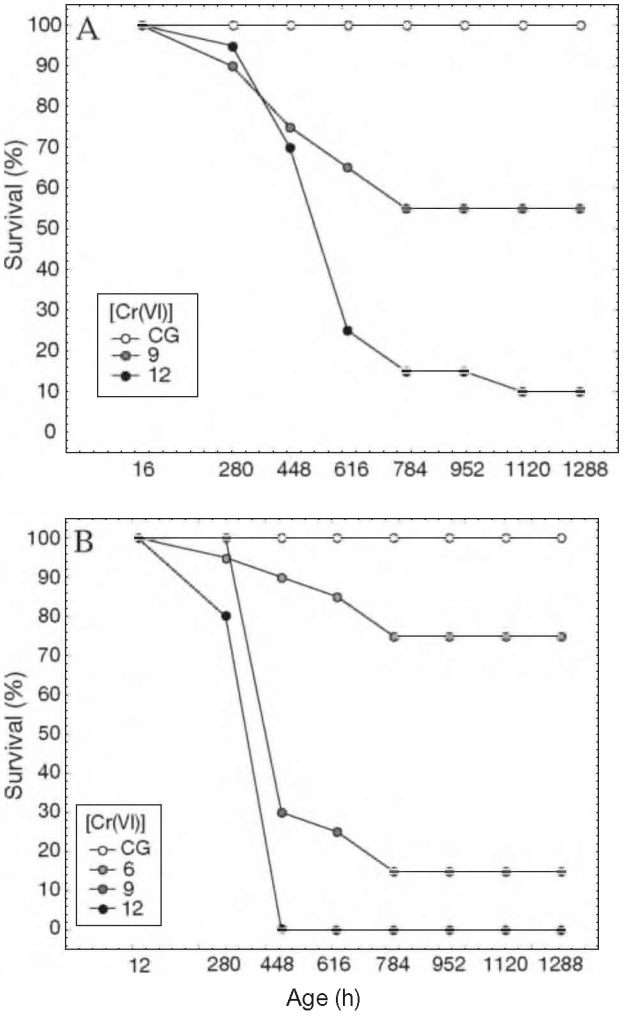

Fig. 1. *Hypsiboas pulchellus*. Mean survival proportion of (A) embryo and (B) tadpole age-groups at various Cr(VI) concentrations (mg l^{-1}). Only results for concentrations significantly different from control group (CG, 0 mg l^{-1}) are shown

Sublethal effects

ANCOVA analysis of growth found chronic sublethal effects of both studied factors ($p < 0.001$) but no interaction between them. Considering the factor age-group, the results indicate that the embryo age-groups had significantly higher growth and were less affected than the tadpole age-groups. The inhibition of growth was first detected at 3 and 6 mg l^{-1} of Cr(VI) for the tadpole and embryo age-groups, respectively. Results of the 2-factor ANOVA indicated that the embryo age-group had a significantly higher growth than the tadpole age-group ($p < 0.001$), for individuals of age 280 to 784 h. After that age, no significant differences in growth were detected. The effect of Cr(VI) on growth within experiments for each age-group according to the 1-way ANOVA and Dunnett's test is shown in Fig. 2A,B. The

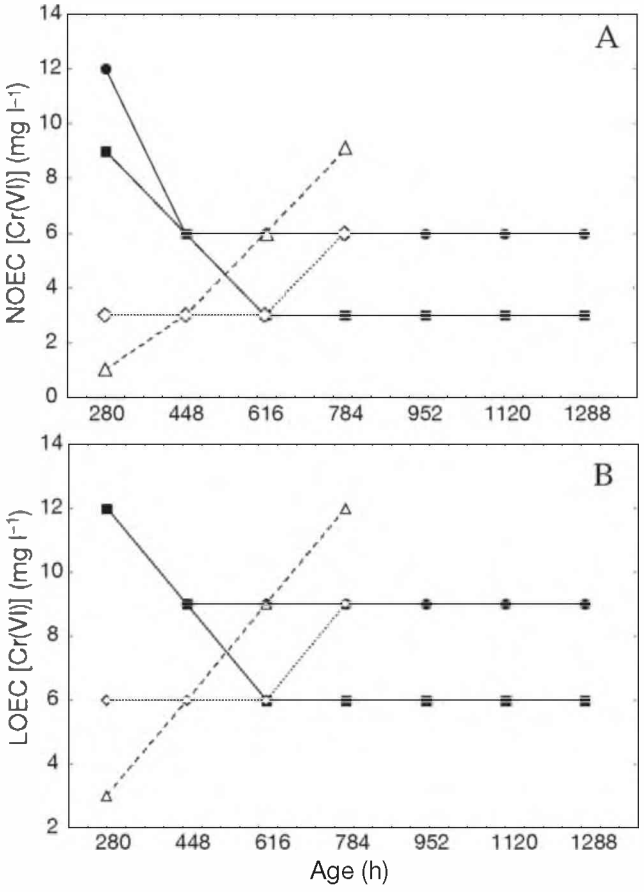

Fig. 2. *Hypsiboas pulchellus*. Effect of Cr(VI) on survival and growth for embryo and tadpole age-groups. Results of (A) NOEC and (B) LOEC estimations with time using ANOVA and Dunnett's test. ●: Embryo survival; ■: Tadpole survival; △: Embryo growth inhibition; ◇: Tadpole growth inhibition

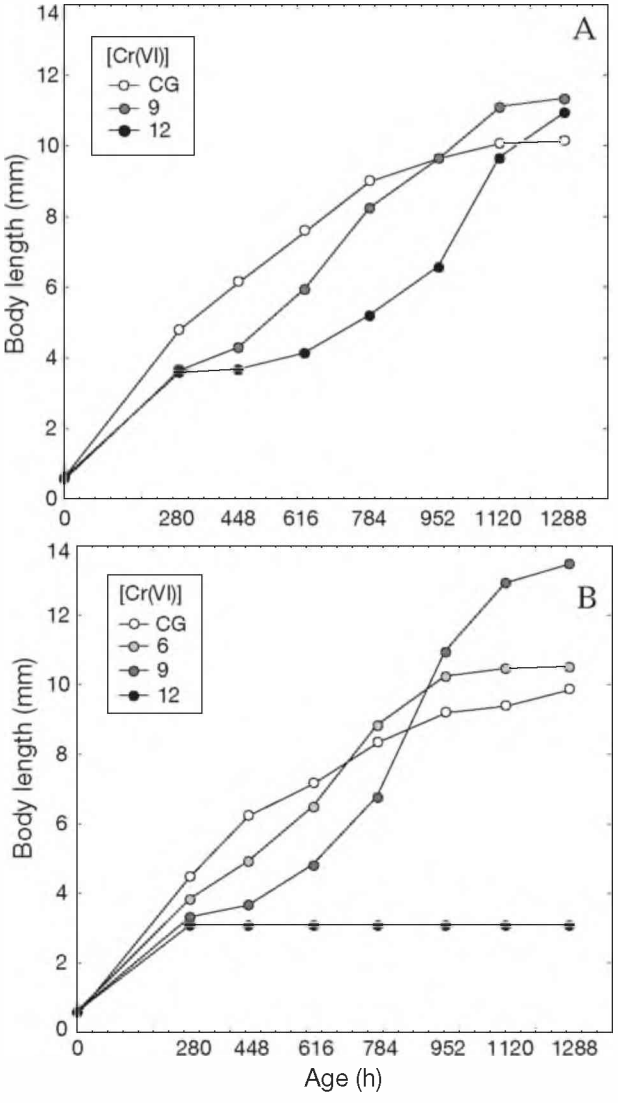

Fig. 3. *Hypsiboas pulchellus*. Growth curves (body length; mm) for (A) embryo and (B) tadpole age-groups during exposure to various Cr(VI) concentrations (mg l^{-1}). Only results for concentrations significantly different to the control group (CG; 0 mg l^{-1}) are shown

results showed that as exposure time (and also age of individuals) increased, higher concentrations of the toxicant were necessary to produce an effect on growth. Growth inhibition was detected at the 4 highest tested concentrations of Cr(VI) for individuals aged 280 to 784 h. Subsequently, measurements at age 952 h did not show inhibition and after 1120 h Cr(VI) concentrations of 6.0 and 9.0 mg l^{-1} enhanced growth with respect to controls for the tadpole age-group and Cr(VI) concentration of 9.0 mg $^{l-1}$ enhanced growth in the embryo age-group. This behaviour and the increase of the growth rate after 448 h exposure for both groups at the highest Cr(VI) concentrations that initially produced an inhibition on growth is shown in Fig. 3.

Taking into consideration the measured variables at the time of the first metamorphosis, the 2-factor ANOVA showed no significant differences in weight with respect to the factor age-group ($p = 0.71$), significant differences for the factor concentration of Cr(VI) ($p < 0.001$) and no interaction ($p = 0.02$). Regarding this variable, the 1-way ANOVA and Dunnett's test showed significant differences with respect to controls ($p < 0.005$). Higher weights were registered with the exposed individuals at concentrations of Cr(VI) of 6.0 and 9.0 mg l^{-1}. The effect of exposure on development stage did not show significant differences with respect to any of the 2 factors analysed with the Friedman ($p = 0.33$) and Kruskal-Wallis tests ($p = 0.22$ for the embryo age-group and $p = 0.15$ for the tadpole age-group).

DISCUSSION

The acute exposure experiments confirmed previous reported data for Cr(VI) toxicity in *Hypsiboas*

pulchellus (Natale et al. 2000) and also constituted a robust comparison parameter that supported the observations made here during long term exposure. Tadpoles are more sensitive than embryos. Apparently the egg coat, composed of a series of concentric mucoid capsules surrounding the ovum and vitelline membrane, protects the developing embryo and acts as a barrier that delays exposure to the toxicant. However, it has been reported that the membrane remains permeable and is subject to active and passive transport of chemicals throughout development (Westerman 1977, Duellman & Trueb 1994, Birge et al. 2000). The osmotic influx during imbibition may be one of the more chemically susceptible periods for the egg because of the relatively high permeability of the capsules at this time (Westerman 1977, Duellman & Trueb 1994). Birge et al. (2000) assumed that the concentration of a given chemical in surrounding media is at equilibrium with the concentration in the eggs; however, it is not known if the chemicals move freely from the environment through the egg coat and into the embryo, or if the egg coat provides some protection for the embryo. The present results indicated that the embryos were exposed to Cr(VI) despite the presence of the enveloping layers and jelly coat. It was also shown that a concentration 3 times higher in the media was necessary to produce an equivalent effect on mortality when compared to the tadpoles. Therefore, we cannot be sure whether embryos are more resistant than tadpoles or if there was a lower exposure associated with a protective action of any of the egg layers.

Comparing the sensitivity of amphibian embryo/larval acute LC_{50} values with rainbow trout using the chemical hazard index (CHI; amphibian LC_{50}:rainbow trout LC_{50} ratio) according to Birge et al. (2000) suggests that *Hypsiboas pulchellus* is much less sensitive than the benchmark species (CHI value >>1). In comparison to the Cr(VI) CHI values for other studied species of anurans, *H. pulchellus* is within the middle sector of the scale, according to the rank extracted from Birge et al. (2000), organized from the most sensitive to the least sensitive: *Gastrophryne carolinensis* > *Rana pipiens* > *Bufo fowleri* > *Rana tigrina* > *H. pulchellus* > *Bufo melanosticus* > *Rana hexadactyla*. The low sensitivity to chromium of most amphibian species tested does not reflect the sensitivity to other metals. Pooling CHI values for all metals and combining data for all taxa, amphibians show higher sensitivity than fish (in 67% of the 573 data registers). This underscores the unusually high susceptibility of amphibian species to metal pollution.

In our experiments, organisms subject to chronic Cr(VI) exposure gave results similar to those from the acute exposure experiments, in that the embryo age-group had better survival and growth rates than the tadpole age-group. These results indicate that although there is a longer exposure in the embryo group in comparison with the tadpole age-group, the former presents higher survival, indicating some type of adaptation from the exposure to sublethal concentrations of Cr(VI) at the embryonic stage. If we compare the survival between age-groups along the exposure time, results show higher mortality of the tadpole age-group during the first 2 wk (age 448 h), the lethal median time for the embryo age-group being nearly twice that for the tadpoles (Fig. 1). After that, a similar trend was observed for both age-groups. This result can be explained by adaptation of survivors, in accordance with general adaptation syndrome (Seyle 1973).

Also, at sublethal exposure concentrations to Cr(VI), effects on growth indicate that organisms exposed at the embryo stage had a higher growth rate, again indicating the same type of adaptation from the exposure to sublethal concentrations of Cr(VI) at this stage. Although daily growth comparisons showed higher growth rates during the first 5 wk (784 h) for the embryo age-group, comparisons between observed effects for both age-groups after 5 wk of exposure do not show the differences detected at earlier exposure times. The behaviour of the response to the higher tested sublethal concentrations of Cr(VI) is also interesting. During the first part of the experiment (280 to 784 h) the expected dose response behaviour was seen (significant growth inhibition with respect to controls), but this was followed by unusual growth (increment of the growth rate after 448 h exposure) yielding larger organisms than negative controls after 952 h. This type of response is also enhanced in the tadpole age-group as seen in Fig. 3B. The compensatory response to low toxicant concentrations (as the one observed here after 448 h of exposure) has been reported for other amphibians, fish and other organisms (Sprague 1971). During this process, differences in growth at the initial time of exposure are compensated by an increment in the rate of growth (Newman & Unger 2002). Therefore, a hormetic type of behaviour at long exposure to sublethal concentrations of Cr(VI) was observed for *Hypsiboas pulchellus*. Fig. 4 summarises the described behaviour showing the variation of the response to Cr(VI) during the first and last stages of exposure.

The analysis of the studied factors on the weight of tadpoles at the time of the first metamorphosis shows a similar result to that observed for body length, with no significant differences between age-groups. Taking these findings into account, the selection of a long exposure time such as the time to first metamorphosis (52 d in this case) provided a different and contrasting appreciation of the sensitivity of tadpoles when com-

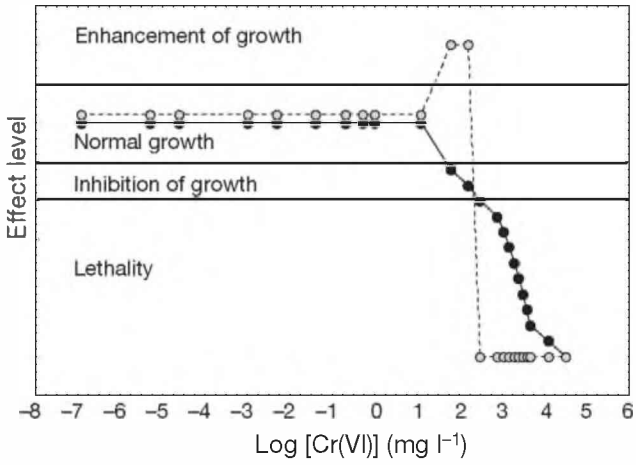

Fig. 4. *Hypsiboas pulchellus*. Variation in response of tadpoles to Cr(VI) exposure during the first stage (age 14 to 25 d, continuous line) and last stage (age 46 to 53 d, dotted line) of experiments

pared to the effects detected at earlier periods. If the present study had been carried out using the experimental conditions for amphibian embryos (14 d exposure) proposed in the standardized test used to evaluate chronic toxicity (AMPHICHRO), one of the tests from the AMPHITOX set (Pérez-Coll & Herkovits 1999a,b) or by other shorter chronic test criteria (e.g. USEPA 1989), this hormetic response would not have been apparent.

In our experiments, the stage of development at the time of the first metamorphosis (Day 53) did not show significant differences between any of the groups. Taking into account the early findings of Hensley (1993) and Beck (1997), this would suggest that age at metamorphosis becomes fixed on or before the development stage when the treatments were applied. Also, the results showing enhancement of growth rate are evidence that tadpoles modify their growth rate so as to maintain a fixed development rate. Both tested age-groups (embryo and tadpole) responded in a similar manner, accelerating the tadpoles' growth rate in the presence of sublethal high chromium concentrations. Individuals exposed to high sublethal Cr(VI) concentrations probably did not increase their development rate as a response to stress but rather metamorphosed as soon they reached the minimum size for metamorphosis (Wilbur & Collins 1973, Travis 1984, Hensley 1993).

The results from this study support the assumption that tadpoles have the ability to modify their growth rate as a response to stresses, independently of the time point at which they fix their development rate. According to Brakefield & Wijngaarden (2003) and Schwenk & Wagner (2003), this plasticity is apparently only available at specific larval developmental stages, such as those between stages 26 and 41: Environmental stresses acting at other times (e.g. embryonic development, stages 0–19, or during the metamorphic crisis after stage 42) would cause the death of the organisms, due to lack of plasticity at these stages.

The observed acute and chronic toxicity survival variability of all exposed organisms (considering they belong to the same hatch) at the different concentrations of Cr(VI) and exposure times, added to their plasticity, may act as a potential advantage to *Hypsiboas pulchellus* when faced with factors affecting the normal development of eggs, hatchlings and tadpoles.

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