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Julie Céline Brodeur [a]; Cynthia Melina Asorey [a]; Abelardo Sztrum [a]; Jorge Herkovits [a]
[a] Instituto de Ciencias Ambientales y Salud (ICAS), Fundación PROSAMA, Buenos Aires, Argentina

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Julie Céline Brodeur, Cynthia Melina Asorey, Abelardo Sztrum, and Jorge Herkovits
Instituto de Ciencias Ambientales y Salud (ICAS), Fundación PROSAMA, Buenos Aires, Argentina

The current study evaluated acute and subchronic toxicity of arsenite (As^{3+}) and zinc (Zn^{2+}) to stage 25 tadpoles of *Rhinella arenarum* in both single and joint laboratory exposures. LC50 values obtained for As^{3+} were elevated and remained within the range of 46 to 50 mg/L of As^{3+} between 4 and 17 d of exposure. Growth of tadpoles was completely inhibited with 30 mg/L of As^{3+}, demonstrating the presence of ecologically relevant sublethal effects at concentrations lower than those resulting in lethality. With respect to Zn^{2+}, a 96-h LC50 value of 2.49 mg/L was calculated in soft water. Contrary to results obtained for As^{3+}, LC50 values of Zn^{2+} gradually decreased with increasing exposure duration, from 2.49 mg/L at 96 h to 1.30 mg/L after 21 d. In joint exposures to both metals, the type of interaction observed between As^{3+} and Zn^{2+} was concentration dependent. Lethal effects of As^{3+} were mitigated, unaffected, or potentiated by 0.01, 0.1, and 1–2 mg/L of Zn^{2+}, respectively. However, although 0.01 mg/L of Zn^{2+} significantly reduced lethality of As^{3+}-exposed tadpoles, the same concentration of Zn^{2+} did not help to reverse the stunt growth of these animals. Further studies need to examine which are the lowest concentrations As^{3+} required to reduce growth and whether Zn^{2+} serves to antagonize growth effects in this range of concentrations.

In recent years, the growing trend observed worldwide to use water from underground sources has generated a global epidemic of cases of arsenic (As) poisoning in human populations (Pearce, 2003). The weathering of As-bearing rocks can locally elevate concentrations of As in groundwater, and As-contaminated groundwater has been reported in several countries (Mandal & Suzuki, 2002). The Chaco-Pampean Plain of Argentina is one of the largest regions of high As in groundwater to be known with an area of approximately 978,634 km^2 (Farias et al., 2003). While the highest concentration of As desirable in drinking water was set at 0.01 mg/L by the World Health Organization (WHO, 1971), measured groundwater concentrations of As commonly range between 0.05 and 0.1 mg/L in this region of Argentina, with peak values frequently reaching 0.6 mg/L and concentrations as high as 8 or 14.9 mg/L being sometimes reported (Farias et al., 2003; Gonzalez Uriarte et al., 2002; Bhattacharya et al., 2006; Bundschuh et al., 2004; Galindo et al., 2007).

While the negative impacts associated to the increased mobilization and dispersion of As-contaminated groundwater are well recognized in humans (Lin et al., 1998; Bernstam & Nriagu, 2000; Mandal & Suzuki, 2002), its potential effects on ecosystems and wildlife have been poorly documented. As-contaminated groundwater is, indeed, not only used as drinking water for humans but is also frequently given to livestock and used for the irrigation of crops (Gonzalez Uriarte et al., 2002). The elevated levels of As in vegetables, rice, and milk produced in regions of Bangladesh and Argentina possessing groundwater rich in As (Meharg & Rahman, 2003; Alam et al., 2003; Das et al., 2004; Perez-Carrera & Fernandez-Cirelli, 2005) provide evidence that As occurring in groundwater pumped for agricultural purposes makes its way into the biota.

The acute toxicity of As is dependent on its chemical form and its oxidation state, with trivalent compounds being generally more toxic than pentavalent compounds, and inorganic arsenicals being usually more toxic than organic forms (Hughes, 2002). The major As species found in surface freshwaters are usually As^{3+} and As^{5+}, which can be interconverted through redox and methylation reactions, but minor amounts of organic forms such as monomethylarsonic acid (MMA), and dimethylarsinic acid (DMA) can also be detected (Smedley & Kinniburgh,

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Julie Céline Brodeur and Jorge Herkovits are staff researchers of the "Consejo Nacional de Investigaciones Científicas y Técnicas" (CONICET), Argentina.
Current address for Cynthia Melina Asorey is Facultad de Ciencias del Mar, Universidad Católica del Norte, Larrondo 1281, Coquimbo, Chile.
Address correspondence to Julie Céline Brodeur, Instituto de Recursos Biologicos, Centro Nacional de Investigaciones Agropecvarias, INTA-Castelar, Casilla de Correo 77, CP 1712 Buenos Aires, Argentina. E-mail: julbrodeur@ hotmail.com

2002; Watt & Le, 2003). Trivalent inorganic As (arsenite or As^{3+}) is, therefore, considered the most toxic form of As to which freshwater organisms may be exposed. While the toxicity of As^{3+} was thoroughly investigated for many freshwater organisms, little information is available regarding the toxicity of As^{3+} to amphibians and existing data is limited to acute toxicity. Johnson (1976) reported 96-h LC50 values of 34.6, 54.8, and 71.0 mg/L of As^{3+} (in the form of sodium arsenite, $NaAsO_2$) in 1- to 2-wk-old tadpoles of the brown striped marsh frog (*Limnodynastes peroni*), the tusked frog (*Adelotus brevis*), and the giant toad (*Bufo marinus*), respectively. A similar 96-h LC50 value of 60 mg/L of As^{3+} (in the form of $NaAsO_2$) was also reported for embryos of *Xenopus laevis* (Bantle et al., 1999), while a lower 96-h LC50 value of 0.189 mg As^{3+}/L (in the form of arsenic trioxide, As_2O_3) was found for 2-cm-long tadpoles of *Rana hexadactyla* (Khangarot et al., 1985).

Given the current global declines in amphibian populations (Houlahan et al., 2000, Stallard, 2001, Stuart et al., 2004, Nystrom et al., 2007), it appears important to reach a better understanding of the risks posed to these animals by the pumping of As-contaminated groundwater. In this context, a first objective of the current study consisted in determining both acute and subchronic toxicity of As^{3+} to stage 25 tadpoles of the common South American toad, *Rhinella arenarum*, an anuran with a wide distribution in the Chaco-Pampean Plain of Argentina. For many years and until 2006, *R. arenarum* was called *Bufo arenarum*. However, the species saw its name changed on two occasions in the last few years, first to *Chaunus arenarum* and then to *Rhinella arenarum* (Frost et al., 2006; Chapparo et al., 2007). Additional objectives of the current study included evaluating acute and subchronic toxicity of zinc (Zn^{2+}) as well as its potential influence on As^{3+} toxicity. These objectives were based on various reports stating that Zn^{2+} is linked to decreased arsenic toxicity in mammals (Kreppel et al., 1994; National Research Council, 1999; Rabbani et al., 2003; Milton et al., 2004; Modi et al., 2005) and observations of a modulation of the toxicity of various other metals by Zn in *Rhinella arenarum* (Herkovits & Perez-Coll, 1995; Herkovits et al., 1997, 2000; Herkovits & Helguero, 1998).

MATERIALS AND METHODS

Embryos

Adults of the common South American toad, *Rhinella arenarum* (Hensel, 1867), weighing approximately 200–250 g, were captured in the wild in Lobos county fields, Buenos Aires Province, Argentina. Ovulation of female toad was induced by means of an intraperitoneal injection of homologous hypophysis suspended in 1 ml AMPHITOX solution (ASL) (Herkovits & Perez-Coll, 2003). Oocytes were fertilized in vitro using fresh sperm suspended in ASL. The resulting embryos were maintained in ASL at 20 ± 2°C until reaching stage 25 (Gosner, 1960). Tadpoles were offered boiled Swiss chard *ad libitum* when they began feeding at stage 24–25.

Protocol for Exposure to As^{3+} and Zn^{2+} Alone

A standard solution of sodium arsenite ($NaAsO_2$) was purchased from Merck (Darmstadt, Germany). Test solutions containing As^{3+} were prepared by adding the adequate volume of this standard solution to ASL. Nominal concentrations of As^{3+} tested were 10, 20, 30, 40, 50, 60, 70, or 80 mg/L. Zn^{2+} was added to ASL in the form of zinc chloride (Mallinckrodt, Phillipsburg, NJ). Nominal concentrations of Zn^{2+} assayed were 0.01, 0.1, 0.5, 1, 2, 5, 10, and 50 mg/L. Acute and subchronic toxicities of As^{3+} and Zn^{2+} were evaluated using the AMPHITOX toxicity tests (Herkovits & Perez-Coll, 2003). For each concentration tested, 10 tadpoles having recently reached stage 25 were placed in triplicate 10-cm-diameter glass petri dishes containing 40 ml ASL with or without (controls) the tested metal. Test solutions were entirely replaced every 48 h, and temperature was maintained between 20 ± 2ºC throughout the experiments, which lasted 17 d for As^{3+} and 21 d for Zn^{2+}. Dead tadpoles were removed and survival was evaluated every other day. A piece of boiled Swiss chard of approximately 2 cm^2 was added to the test vessels after changing the solution. Exposures to Zn^{2+} and As^{3+} were repeated two and five times, respectively, with tadpoles from a unique but distinct pair of parents being used on each occasion (i.e., Zn^{2+} and As^{3+} results were obtained with tadpoles from two and five breeding pairs, respectively).

Protocol for Joint Exposures to As^{3+} and Zn^{2+}

The influence of Zn on As^{3+} toxicity was evaluated by exposing stage 25 tadpoles of *R. arenarum* to As^{3+} and Zn^{2+} alone and in combination in a factorial experimental design. Two joint exposures were conducted. The first exposure lasted 7 d and included As^{3+} concentrations of 30, 40, or 50 mg/L, with Zn^{2+} concentrations of 0.1, 1, or 2 mg/L. The second joint exposure lasted 28 d and included As^{3+} concentrations of 30, 40, or 50 mg/L, and Zn^{2+} concentrations of 0.01, 0.1, 1, or 2 mg/L. Experimental procedures were as described earlier for single metal exposures. A control group exposed to ASL only was included in both exposures. Tadpoles from a single and distinct pair of parents were used in each exposure. Total length of each live tadpole was measured after 14, 21, and 28 d of exposure in the second joint metal exposure by transferring tadpoles one by one into a petri dish placed over millimetric paper and containing ASL.

Data Analysis

The concentrations of As^{3+} and Zn^{2+} resulting in the mortality of 10, 50, and 90% of tadpoles (LC10, LC50, and LC90, respectively) after diverse exposure durations were calculated by fitting a 4-parameter logistic regression equation to the survival data using the GraphPad Prism software version 3.02. LC values obtained for various exposure durations and their associated standard errors were compared using a one-way analysis of variance (ANOVA), followed by a Student–Newman–Keuls

test for multiple comparisons. As survival data from the 7-d joint metal exposure failed to present normality (even after transformation), data obtained for various concentrations of Zn^{2+} were compared within each concentration of As^{3+} tested (0, 30, 40, and 50 mg/L) using a Kruskal–Wallis one-way ANOVA on ranks. The ANOVA was followed by a Student–Newman–Keuls test for multiple comparisons when a significant difference was found. Survival data from the 28-d joint metal exposure obtained with various concentrations of Zn^{2+} were also compared within each concentration of As^{3+} tested (0, 30, 40, and 50 mg/L) using a two-way repeated-measures ANOVA with exposure duration (repeated) and the concentration of Zn^{2+} as factors. The ANOVA was followed by a Student–Newman–Keuls test for multiple comparisons when a significant effect was found. Finally, as data regarding the length of the tadpoles failed to present normality (even after transformation), data obtained with various concentrations of Zn^{2+} were compared within each concentration of As^{3+} tested (0, 30, 40, and 50 mg/L) and each exposure duration using a Kruskal–Wallis one-way ANOVA on ranks. As treatment group sizes were unequal due to differential mortality at distinct test concentrations, the ANOVA was followed by Dunn's test for multiple comparisons when a significant difference was found. All ANOVAs and multiple comparison tests were conducted using SigmaStat 3.11 statistical software (SPSS, Chicago). The criterion for significance was set at $p < .05$.

RESULTS

Exposure to As^{3+} and Zn^{2+}, Alone

The LC10, LC50, and LC90 calculated from the survival data obtained during exposures to As^{3+} alone are presented as a function of exposure duration in Table 1. This table shows that the concentration of As^{3+} necessary for reducing survival to 50% (LC50) did not change significatively and remained within the range of 46–50 mg/L between 4 and 17 d of exposure. Conversely, in exposures to Zn^{2+} alone, the calculated LC50 gradually decreased with increasing exposure duration, with the LC50 declining from 2.71 to 1.30 mg/L between 2 and 21 d of exposure, respectively (Table 2).

Joint Exposures to As^{3+} and Zn^{2+}

Results of the 7-d joint exposure to As^{3+} and Zn^{2+} are presented in Figure 1. Although none of the tested concentrations of Zn^{2+} significantly affected survival when applied individually (data not shown), the toxicity of all As^{3+} concentrations was greater when 2 mg/L of Zn^{2+} were added. This effect amplified gradually with increasing concentrations of As^{3+}, and became statistically significant with 50 mg/L of As^{3+}. For its part, 0.1 mg/L of Zn^{2+} slightly improved the survival of tadpoles exposed to 50 mg/L of As^{3+}, although the effect was not statistically significant.

TABLE 1
Lethal Concentrations (LC) 10, 50, and 90 Calculated for Stage 25 Tadpoles of *Rhinella arenarum* Exposed to As^{3+}

Days of exposure	LC10 (mg/L)	LC50 (mg/L)	LC90 (mg/L)
2	32.28[a] (27.86–37.41)	56.61[a] (53.37–60.05)	N.A.
4	27.73[a] (23.55–32.58)	50.04[a,b] (47.05–53.23)	N.A.
7	29.92[a] (27.16–32.96)	46.20[b] (44.43–48.04)	71.29[a] (65.01–78.34)
14	33.42[a] (28.91–38.63)	49.78[a,b] (46.81–52.94)	74.13[a] (63.83–86.30)
17	30.83[a] (25.18–37.76)	46.36[b] (42.62–50.42)	69.66[a] (57.02–85.31)

Note. Confidence intervals 95% (CI) are indicated in parentheses. Values with the same letter within each column are not significantly different ($p < .05$). N.A., not available because calculated value is outside the range of tested concentrations.

TABLE 2
Lethal Concentrations (LC) 10, 50, and 90 Calculated for Stage 25 Tadpoles of *Rhinella arenarum* Exposed to Zn^{2+}

Days of exposure	LC10 (mg/L)	LC50 (mg/L)	LC90 (mg/L)
2	1.69[a] (1.52–1.87)	2.71[a] (2.37–3.10)	4.03[a] (2.99–5.41)
4	1.69[a] (1.51–1.90)	2.49[a,b] (2.46–2.52)	3.66[a] (2.53–4.32)
7	1.79[a] (1.63–1.97)	2.60[a,b] (2.19–3.08)	3.76[a] (2.51–5.62)
14	1.34[a] (0.97–1.86)	2.11[b] (1.95–2.29)	3.32[a] (2.19–5.02)
17	0.85[b] (0.61–1.19)	1.71[c] (1.49–1.97)	3.44[a] (0.41–4.83)
21	0.84[b] (0.84–0.85)	1.30[d] (1.12–1.51)	1.24[b] (1.19–1.30)

Note. Confidence intervals 95% (CI) are indicated in parentheses. Values with the same letter within each column are not significantly different ($p < .05$).

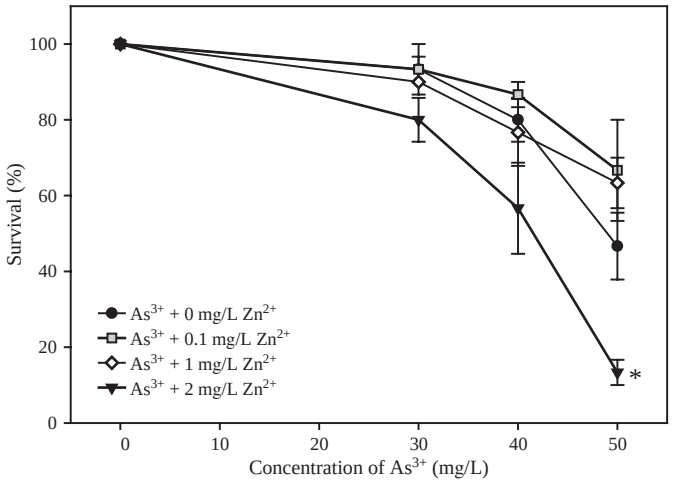

FIG. 1. Survival of stage 25 tadpoles of *Rhinella arenarum* after 7 d of exposure to Zn^{2+} and As^{3+}, alone and in combination. Asterisk indicates significantly different ($p < .05$) from group exposed to a similar concentration of As^{3+} without Zn^{2+}.

Figure 2 illustrates the survival data obtained during the 28-d joint metal exposure. Data confirm the tendency observed above in the 7-d experiment for Zn^{2+} to possess a dual effect on As^{3+} toxicity. Indeed, in joint exposures to As^{3+} and 1 or 2 mg/L of Zn^{2+}, the addition of these concentrations of Zn^{2+} repeatedly augmented the toxicity of As^{3+} (Figure 2, b–d). In contrast, when animals were exposed to 50 mg/L of As^{3+} in presence of 0.01 mg/L of Zn^{2+}, their survival was significantly improved compared to when 50 mg/L of As^{3+} was applied alone (Figure 2d). Interestingly, this antagonistic effect of 0.01 mg/L of Zn^{2+} could only clearly be observed with 50 mg/L of As^{3+}, possibly because the other concentrations of As^{3+} were not sufficiently lethal for an improvement of survival to be detected. Similarly, the synergistic effect of the higher concentrations of Zn^{2+} was more evidently seen with 40 mg/L of As^{3+}, possibly because of the intermediate level of toxicity this concentration generated (Figure 2c).

As regards the length data collected during the 28-d joint metal exposure, Figure 3a shows that exposure to 1 and 2 mg/L of Zn^{2+} without As^{3+} completely inhibited growth of the tadpoles, whereas 0.01 and 0.1 mg/L of Zn^{2+} did not exert any effect on this parameter. For their part, all three concentrations of As^{3+} tested completely inhibited growth when applied alone (Figure 3, b–d). The only occasion when the addition of Zn^{2+} altered the effect of As^{3+} occurred when 0.01 mg/L of Zn^{2+} was applied in conjunction with 40 mg/L of As^{3+}. Tadpoles subjected to this treatment were significantly larger then tadpoles exposed to As^{3+} alone when first measured after 14 d of exposure, although they did not present any further growth over the remaining of the exposure (Figure 3c).

DISCUSSION

The current study evaluated acute and subchronic toxicity of As^{3+} and Zn^{2+} to stage 25 tadpoles of *R. arenarum*, both alone and in combination. With respect to As^{3+}, the calculated 96-h LC50 value of 50.04 mg/L is comparable to the majority of previously reported values for amphibian embryos and larvae (Johnson, 1976; Bantle et al., 1999; Khangarot et al., 1985). Exposure to As^{3+} was continued for 17 d so as to provide the first subchronic toxicity data for an amphibian larva. The LC50 changed little with increasing exposure duration, with its value remaining within the range of 46–50 mg of As^{3+}/L between 4 and 17 d of exposure. These sustained high values of LC50 and the fact that 30 mg/L of As^{3+} produced less than 10% of mortality after 28 d of exposure in the second joint metals experiment indicate that stage 25 tadpoles of *R. arenarum* are fairly resistant to As^{3+}. However, the fact that growth was completely inhibited by 30 mg/L of As^{3+} is a clear indication that although tadpoles managed to survive the range of concentrations used for subchronic exposures, they nevertheless suffered important sublethal effects. This finding implies that future studies will need to determine which are the lowest concentrations of As^{3+} to inhibit tadpole growth before the influence of As-contaminated groundwater on these animals can be fully understood.

As regards Zn^{2+}, the 96-h LC50 values available in the literature vary widely, from 2.1 and 4.69 mg/L for *Rana hexadactyla* and *Hyla chrysocelis* to 19.86 and 28.38 mg/L for *Bufo melanostictus* and *Rana luteiventris*, respectively (Khangarot et al., 1985; Khangarot & Ray, 1987; Gottschalk, 1995; Leftcort et al., 1998). This wide range of data is principally due to the fact that toxicity of Zn ions is highly dependent on water hardness; the highest values of LC50 being observed when concentrations of calcium ions are at their highest (Skidmore, 1964). The 96-h LC50 value of 2.49 mg/L calculated in the current study using soft water (Ca^{2+}= 36 mg/L) is therefore comparable to values previously obtained in other species under similar conditions. As opposed to what was observed for As^{3+}, LC50 values of Zn^{2+} gradually decreased with increasing exposure durations, from 2.49 mg/L at 96 h to 1.3 mg/L after 21 d. This last value remains, however, considerably greater than concentrations normally observed in Argentine surface and groundwater, which normally range between 0.048 to 0.17 mg/L (Farias et al., 2003; Gonzalez Uriarte et al., 2002; Bhattacharya et al., 2006; Bundschuh et al., 2004). Furthermore, in contrast with As^{3+}, the only concentrations of Zn^{2+} to inhibit growth were the ones that also affected survival, and tadpoles exposed to concentrations of Zn^{2+} as high as 0.1 mg/L showed a growth rate similar to controls. Altogether, these observations converge to suggest that tadpoles of *R. arenarum* are unlikely to suffer Zn^{2+}-associated toxicity in the wild at normally occurring concentrations.

As regards the influence of Zn ions on As toxicity, results obtained in the current study demonstrate that the type of interaction observed between the two metals is concentration

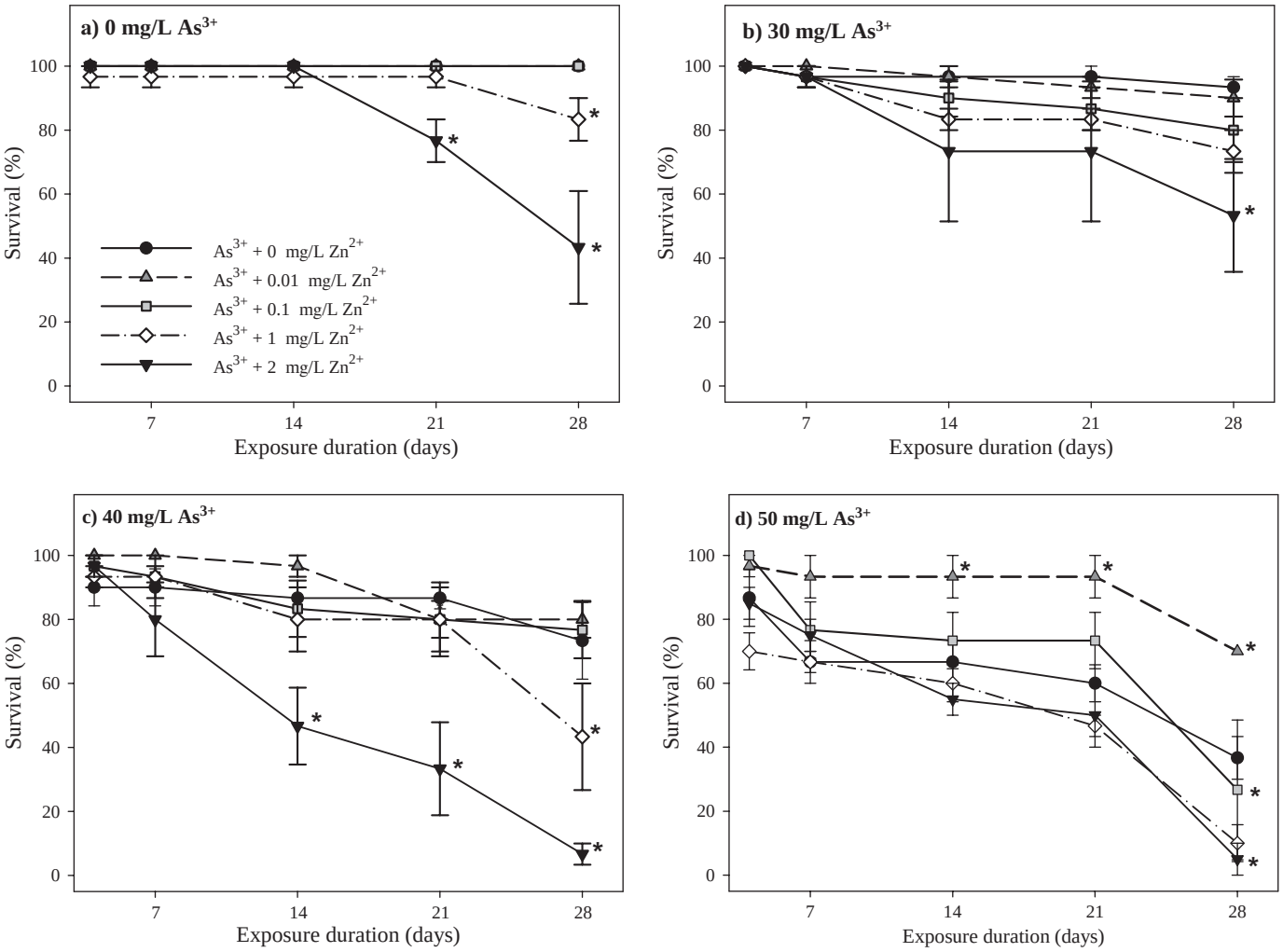

FIG. 2. Survival of stage 25 tadpoles of *Rhinella arenarum* after up to 28 d of exposure to Zn^{2+} and As^{3+}, alone and in combination. Asterisk indicates significantly different ($p < .05$) from group exposed to a similar concentration of As^{3+} without Zn^{2+}.

dependent. Indeed, although lethal effects of As^{3+} are mitigated by low concentrations of Zn^{2+} (0.01 mg/L), high concentrations of Zn^{2+} (1–2 mg/L) potentiate As^{3+}-induced lethality and intermediate concentrations (0.1 mg/L) exert no effect. These interactions between Zn^{2+} and As^{3+} are furthermore clearly observable only when given levels of As^{3+} are reached; synergism is more evidently observed with 40 mg/L of As^{3+} and antagonism is especially evident with 50 mg/L of As^{3+}.

Zinc is a biologically essential metal, which plays a key role in genetic expression, cell division, and growth and which is essential for the function of more than 200 enzymes (Salgueiro et al., 2000). Biologically essential metals are known to interact with toxic metals due to their similar physical and chemical properties, and Zn^{2+} was cited as a protective element against the toxicity of As and other metals in various physiological systems and species (Kudo et al., 1986; Kreppel et al., 1994; Joshi et al., 2004; Singh et al., 2006). In contrast, although previous reports do exist (Vanegas et al., 1997; Herkovits et al., 2000), it is much less common to observe synergism between Zn^{2+} and other metals, such as was the case here between As^{3+} and elevated concentrations of Zn^{2+}. In the case of amphibian embryos and larvae, the beneficial effects of Zn^{2+} thus far demonstrated include (1) protection from spontaneous malformations (Herkovits et al, 1989), (2) compensation of delayed development produced by Cd^{2+} (Herkovits & Perez-Coll, 1990), and (3) antagonism of lethal effects generated by Al^{2+}, Cu^{2+}, Ni^{2+}, and Cd^{2+} (Herkovits & Perez-Coll, 1995; Herkovits & Helguero, 1998; Herkovits et al., 1997, 2000).

Interestingly, results obtained demonstrate that although the addition of a low concentration of Zn^{2+} reduced mortality of As^{3+}-exposed tadpoles, growth of these tadpoles was not improved. Indeed, although survival of tadpoles exposed to both 30 and 50 mg/L of As^{3+} was improved with 0.01 mg/L of Zn^{2+}, this concentration of Zn^{2+} did not help reverse the stunted

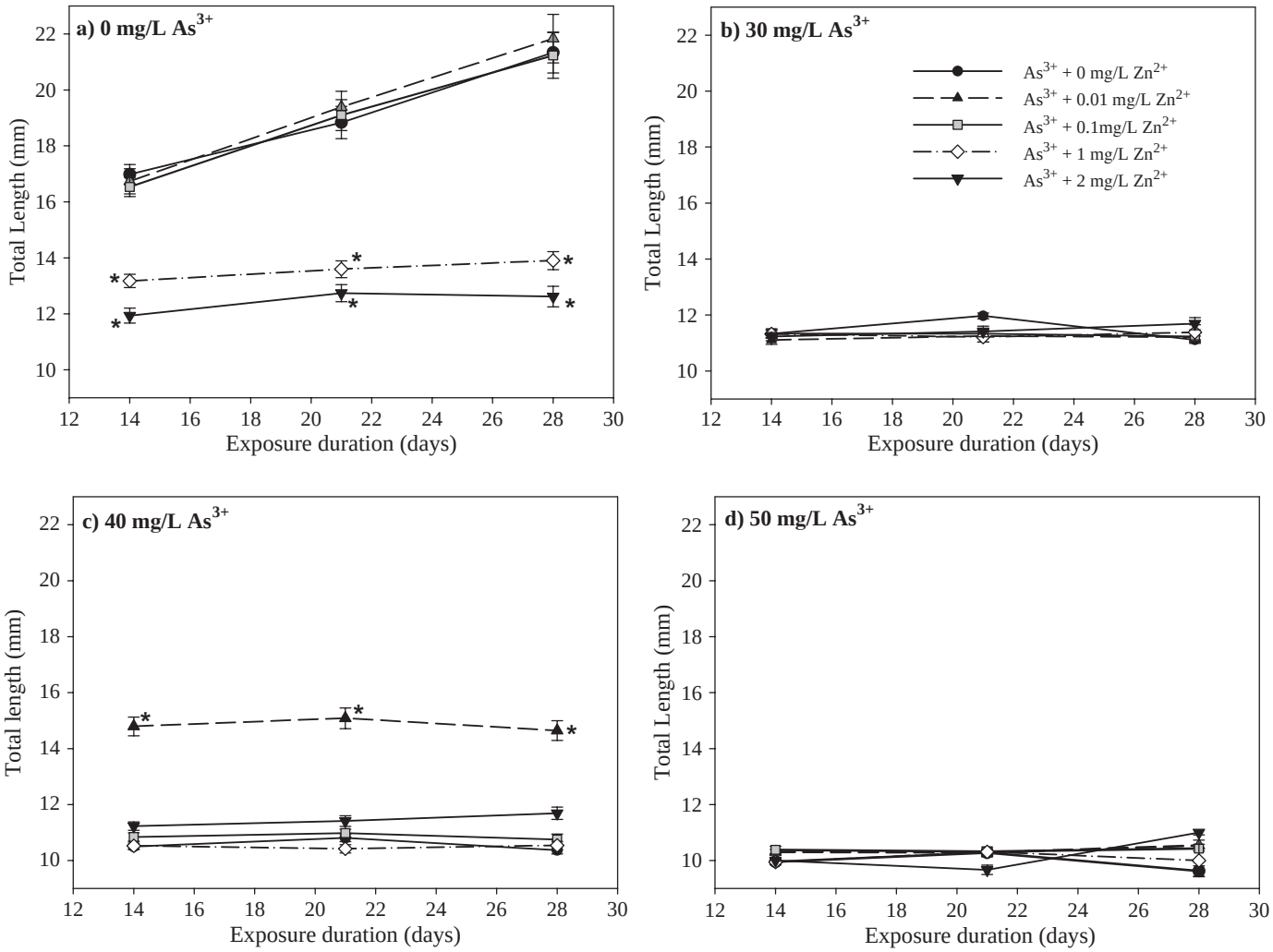

FIG. 3. Total length of stage 25 tadpoles of *Rhinella arenarum* after up to 28 d of exposure to Zn^{2+} and As^{3+}, alone and in combination. Asterisk indicates significantly different ($p < .05$) from group exposed to a similar concentration of As^{3+} without Zn^{2+}.

growth of these animals. Strangely, tadpoles exposed to a combination of 0.01 mg/L of Zn^{2+} and 40 mg/L of As^{3+} did present a quantitatively greater length than tadpoles exposed to the same concentration of As^{3+} without Zn. However, this is probably because the tadpoles were quantitatively larger from the beginning, given that they did not grow any further as the experiment proceeded. It thus appears that, within the range of concentrations tested, the improved survival of As^{3+}-exposed tadpoles in presence of low concentrations of Zn^{2+} is of limited ecological relevance given that tadpole growth (and possibly development) remains impaired.

In conclusion, although this study generated important information regarding single and joint toxicity of As^{3+} and Zn^{2+} to larval amphibians, further studies are needed before the potential impacts these metal ions may have in the wild are elucidated. More specifically, it would appear important to determine which concentrations of As^{3+} reduce growth and whether low concentrations of Zn^{2+} serve to antagonize growth effects at this range of concentrations.

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