Journal of Herpetology, Vol. 47, No. 4, 000–000, 0000

Suppressing Bullfrog Larvae with Carbon Dioxide

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ABSTRACT.—**Current management strategies for the control and suppression of American Bullfrog (*Lithobates catesbeianus* = *Rana catesbeiana* Shaw) and other invasive amphibians have had minimal effect on their abundance and distribution. This study evaluates the effects of carbon dioxide (CO_2) on pre- and prometamorphic Bullfrog larvae. Bullfrogs are a model organism for evaluating potential suppression agents because they are a successful invader worldwide. From experimental trials we estimated that the 24-h 50% and 99% lethal concentration (LC_{50} and LC_{99}) values for Bullfrog larvae were 371 and 549 mg CO_2/L, respectively. Overall, larvae that succumbed to experimental conditions had a lower body condition index than those that survived. We also documented sublethal changes in blood chemistry during prolonged exposure to elevated CO_2. Specifically, blood pH decreased by more than 0.5 pH units after 9 h of exposure and both blood partial pressure of CO_2 (pCO_2) and blood glucose increased. These findings study suggest that CO_2 treatments can be lethal to Bullfrog larvae under controlled laboratory conditions. We believe this work represents the necessary foundation for further consideration of CO_2 as a potential suppression agent for one of the most harmful invaders to freshwater ecosystems.**

The American Bullfrog (*Lithobates catesbeianus* = *Rana catesbeiana* Shaw) is native to Eastern North America but the pet, food, and bait trades have introduced Bullfrogs to four continents (Ficetola et al., 2007; Santos-Barrera et al., 2009). This species thrives in human-created or modified habitats because it is highly adapted to a range of environmental conditions and prey (Adams and Pearl, 2007), extremely mobile (documented dispersal distances >1.6 km; Ingram and Raney, 1943), and highly fecund (females can produce up to 40,000 eggs per clutch; Bury and Whelan, 1984). Tadpoles outcompete the larvae of some native amphibians (Lawler et al., 1999), adults may prey directly on native amphibians (Pearl et al., 2004; Wu et al., 2005), and both larvae and adults serve as a vector for the transmission of chytridiomycosis; this infectious disease is considered one of the causes of global amphibian decline and extinction (Garner et al., 2006; Pounds et al., 2006). For these reasons, Bullfrogs rank among the most harmful invaders to freshwater ecosystems and among the 100 most harmful invasive species in the world (Lowe et al., 2000; Beebee and Griffiths, 2005).

Rapid response to Bullfrog invasion is promoted widely because, once established, Bullfrogs are extremely difficult to eradicate (Adams and Pearl, 2007; Ficetola et al., 2007). While some techniques have aided in reducing Bullfrog densities, few approaches have eradicated entire populations successfully (Altwegg, 2002; Doubledee et al., 2003). Current Bullfrog suppression tools, such as trapping and shooting, have had limited success, so the development and evaluation of novel suppression tools are needed to prevent further loss of native taxa (Adams and Pearl, 2007).

Consideration of new chemical agents for invasive amphibian suppression is well documented. Snow and Witmer (2010) recently examined effects of chemical agents and found that caffeine, chloroxylenol, and rotenone produced 100% mortality when sprayed onto Bullfrogs. Similarly, Campbell (2002), Pitt and Sin (2004a), and Pitt et al. (2010) evaluated the toxicity of citric acid on Coqui Frogs (*Eleutherodactylus coqui*) to determine the lowest concentration needed to achieve 80% mortality. After confirmation of its effectiveness, citric acid was later tested on nontarget taxa including vascular plants (Pitt and Sin, 2004b; Pitt et al., 2008) and invertebrates (Pitt and Sin, 2004c). The compound has now been tested and used successfully to treat small but expanding populations of invasive Coqui in Hawaii (Beachy et al., 2011). This measured approach to identifying potential suppression agents of Coqui frogs was critically needed to 1) evaluate the effectiveness of individual compounds, 2) identify concentrations necessary to kill or stress target invaders, and 3) consider how a toxicant stresses or kills (or both) target organisms. Here we present findings from laboratory experiments to evaluate the potential of using carbon dioxide (CO_2) to suppress Bullfrog larvae.

CO_2 is a chemical agent that is likely to have lethal and sublethal effects on Bullfrog larvae at high concentrations. Dissolved CO_2 (dCO_2) can have lethal and sublethal effects on fish and aquatic invertebrates because it reduces blood or hemolymph pH (i.e., acidosis; Yoshikawa et al., 1988; Elzinga and Butzlaff, 1994; Gelwicks et al., 1998; Watten et al., 2005). In fish, high concentrations or prolonged exposure times can cause death (Gelwicks et al., 1998). Exposure to elevated dCO_2 can also cause narcosis, reduced condition factor, and reduced growth rate (Yoshikawa et al., 1991; Fivelstad et al., 1998; Gelwicks et al., 1998; Cech and Crocker, 2002). Although studies have been conducted to explore whether the skin of adult Bullfrogs is an effective mechanism for CO_2 uptake and elimination (Jackson and Braun, 1979), no studies have been conducted examining the effects of CO_2 on larval amphibians.

We tested the effects of prolonged exposure to elevated concentrations of dCO_2 on the survival and physiology of American Bullfrog larvae. Specifically our objectives were to 1) determine the 24-h 50% and 99% lethal concentration (LC_{50} and LC_{99}) of dCO_2 for Bullfrog larvae, and 2) document sublethal changes in blood chemistry and blood analytes. We believe that this study is the first to demonstrate the effects of elevated levels of dCO_2 on anuran larvae and the first to determine lethal concentrations of dCO_2 for a harmful, invasive amphibian. This latter point is critical for managers seeking to use CO_2 as a

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DOI: 10.1670/12-126

technique for the management of invasive amphibians including American Bullfrogs.

Materials and Methods

Animal Care and Handling.—We acquired 200 Bullfrog tadpoles from a licensed supply house (Connecticut Valley Biological Supply Company, Southampton, Massachusetts, USA). After receipt, animals were divided evenly among two fiberglass raceways (2.4 m × 0.6 m) in approximately 10 cm of water (total volume of 0.14 m^3) and allowed to acclimate to holding conditions for at least 72 h prior to experimentation. Raceways were supplied with flow-through spring water supplied at a flow rate of 0.6 L/min. The temperature of incoming waters was 17.5°C, dissolved oxygen (DO) concentration was 8.84 mg O_2/L, pH was 7.24, total gas pressure was 777 mm Hg, and conductivity was 634 μS/cm. Total alkalinity of these waters was 286 mg/L $CaCO_3$.

Bullfrog larvae were almost exclusively premetamorphic and prometamorphic, ranging from Gosner stage 26 to 42 (Gosner, 1960); however, >84% of tadpoles used in experiments were between Gosner stage 35 and 41. We fed the larvae ReptoMin (Tetra Werke, Blacksburg, Virginia, USA) ad libitum.

Experimental CO_2 System.—To test the effects of CO_2 on anuran larvae, we adapted the hyperbaric system from Watten et al. (2005); two identical, parallel systems were used. Each system included nine 3-L hyperbaric chambers (see Fig. 1). For both hyperbaric chamber systems we used a single water source (same flow rate) and CO_2 source (flow rate dependent on treatment) with separate carbonators. Source water was pumped from a nearby spring: Gray Spring, Kearneysville, West Virginia, USA. We introduced CO_2 from bulk storage and split the gas feed into two independent lines, each regulated by a mass flow meter adjusted until the flow rate reached the desired dCO_2 concentration (see below). We used a 1/3-hp pump, forcing water into a pressurized vertical column carbonator where source water was mixed with CO_2; the carbonator measured 10.2 cm internal diameter × 78.9 cm height and contained 1.6 cm NorPac plastic packing (Watten et al., 2005).

We monitored water flow rate through the hyperbaric chambers individually with variable area flow meters and adjusted the individual chamber flow control valves to maintain a constant flow rate of 1.5 L/min. We characterized concentrations of dCO_2 in the effluent waters at 4-h intervals using a head space unit as described by Pfeiffer et al. (2011) and Watten et al. (2004) that incorporated a portable infrared CO_2 analyzer (GD-888 Series, CEA Instruments, Inc., Westwood, New Jersey, USA). Head space readings allowed for calculation of dCO_2 in units of mg/L and as mm Hg tension, given local barometric pressure (Colt, 1984; Watten et al., 2004; Pfeiffer et al., 2011). In brief, the IR gas analyzer measures concentrations in head space CO_2 by measuring CO_2 concentrations in gas captured in a gas-liquid contacting chamber in the head space unit (Pfeiffer et al., 2011:fig. 1) that is vented through the headbox and ultimately interfaces with the IR analyzer through a gas sample port. Dissolved CO_2 in discharge water is calculated by converting percent by volume CO_2 levels measured in the gas liquid contacting chamber to P^G_{CO2} values using the following equation:

$$P^G_{CO_2} = BP(\%CO_2/100),$$

where BP is barometric pressure expressed in mm Hg. Using this design, CO_2 tension in head space gases (P^G_{CO2}) approximates CO_2 tension in liquid (P^L_{CO2}) and therefore supports the calculation of dissolved CO_2 (mg/L) in test waters as follows:

$$\text{Dissolved } CO_2(\text{mg/L}) = \beta CO_2 \left(\frac{P^G_{CO_2}}{.03845} \right).$$

In the above equation, β (from Henry's Law) is the Bunsen solubility coefficient (Colt, 1984), βCO_2 is in units of l gas/l water-atmosphere, and P^L_{CO2} is in units of mm Hg. The factor 0.3845 equals 760/(1,000k) and k represents the ratio of molecular weight to molecular volume of CO_2 gas (Colt, 1984).

We also measured conductivity (μS/cm) with a YSI 30 conductivity and temperature meter (Yellow Springs Instruments, Inc. [YSI], Yellow Springs, Ohio, USA), total gas pressure (TGP) with a total gas pressure meter (Point Four Systems, Inc., Coquitlam, BC, Canada), pH with a YSI pH100 (YSI, Inc.), and DO concentration, water temperature, and barometric pressure with a HQ40d Portable Meter (Hach Company, Loveland, Colorado, USA). All experiments took place in October 2011.

Experiment 1: Determination of LC_{50}.—We exposed 135 Bullfrog larvae to CO_2 treatments in each of nine 3-L cylindrical hyperbaric chambers (Fig. 1) for approximately 23 h. Within each chamber we held a randomly selected larva using a mesh basket (9 cm diameter × 28 cm height) covered with nylon stockings. To determine the 24-h LC_{50} and LC_{99}, we tested 27 larvae (9 individuals per trial; 3 trials for each concentration) in five concentrations of dCO_2: ambient (~75 mg CO_2/L), 150, 300, 450, and 600 mg CO_2/L. We selected these concentrations from preliminary work with this species (A. Ray, pers. obs.). We adjusted concentrations of dCO_2 by regulating gas feed rates into the carbonator using the system described previously. Because we had two separate hyperbaric chamber systems (see Fig. 1), we were capable of simultaneously running two independent treatments on 9 individuals during each 24-hr dosing period. The order for testing individual CO_2 treatments and the hyperbaric system used during each treatment was assigned randomly. The full complement of treatments (75, 150, 300, 450, and 600 mg CO_2/L) was completed for a trial before beginning subsequent trials. By using two hyperbaric systems, five CO_2 treatments could be completed in just 3 days. Accordingly, all testing was completed over a 9-day period. The pH meter did not function during two treatments (150 mg CO_2/L Trial 2 and ambient CO_2 Trial 2), so those pH values were omitted from our analyses.

After 23 h we removed larvae from the chambers, stored them in labeled 3.8-L, individually marked Ziploc bags filled with 1 liter of unamended source water. We suspended the bags in a 189-liter holding tank filled with the same source water. After a 24-h recovery period we checked larvae for mortality; individuals were classified as dead if they exhibited no response to a stimulus (light probing with a tongue depressor). Surviving larvae were euthanized in a 500 mg/L tricaine methanesulfonate (MS-222) solution. We measured total length (cm) and mass (g), determined survival, and assigned Gosner stage (Gosner, 1960) to all individuals; we calculated body condition index by dividing each larvae's mass by its total length after exposure to CO_2 (Karraker and Welsh, 2006).

Experiment 2: Sublethal Effects—Changes in Blood Chemistry.—We exposed Bullfrog larvae to approximately 450 mg CO_2/L in each of nine 3-L cylindrical hyperbaric chambers (described above) to examine the effects of prolonged exposure to elevated CO_2 on blood chemistry. Within each chamber we contained a randomly

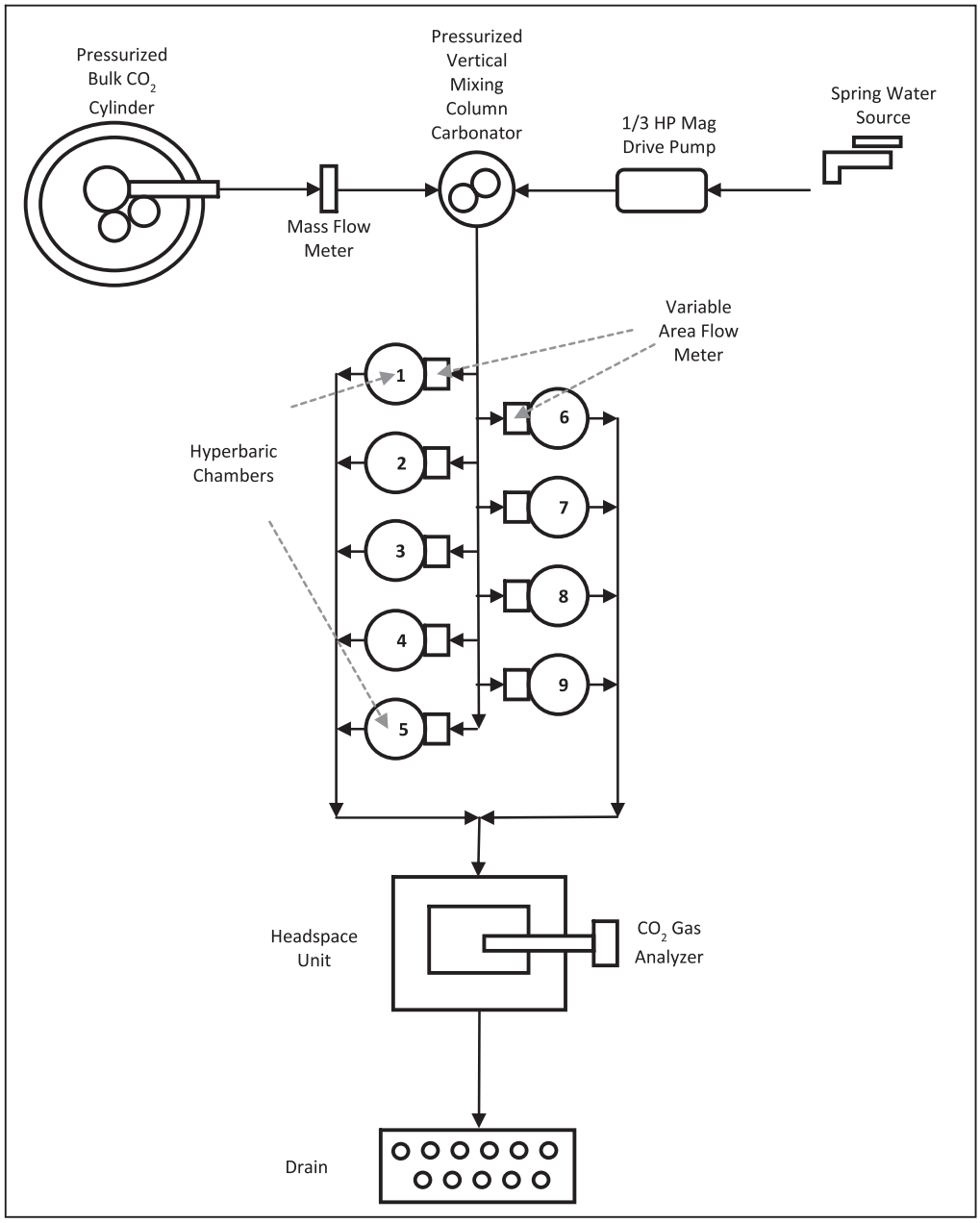

FIG. 1. Schematic of water flows for hyperbaric system used in CO_2 experiments. Complete description of system is provided in the Methods section. Hyperbaric system shown includes nine test chambers connected to a single water and CO_2 source.

selected individual using a mesh basket (9 cm diameter × 28 cm height) covered with nylon stockings. We measured total length (cm), total mass (g), assessed survival, and assigned Gosner stage (Gosner, 1960). Larvae were exposed to elevated CO_2 for one of four discrete time periods: 0, 3, 6, and 9 h. We handled larvae, including controls (0 h CO_2 exposure), in an identical manner (e.g., animals were captured, transported, introduced to, and removed from hyperbaric chambers) for comparison of blood chemistry parameters among treatments.

We anesthetized tadpoles with tricaine methanesulfonate (MS-222; 500 mg/L) for approximately 2 min to immobilize them immediately before blood was drawn. Approximately 100 µl of blood was drawn from the cardiac ventricle of tadpoles with a lithium-heparinized 28-gauge tuberculin syringe for immediate analysis with a portable VetScan iStat 1 blood analyzer with EC 8+ cartridges (Abaxis, Union City, California, USA). Analytes measured included glucose, blood gas concentration (partial pressure of CO_2 = pCO_2), and pH as an acid-base indicator.

Statistical Analyses.—We used a one-way analysis of variance (ANOVA) to test for differences in dCO_2 among treatments to confirm that we were subjecting larvae to statistically different dCO_2 concentrations. After confirming that our treatment levels of dCO_2 were unique, we used an ANOVA to test for differences in other water quality parameters (e.g., specific conductance and dissolved oxygen [DO]) among dCO_2 treatments. Simple linear correlations were used to characterize the associations (positive and negative) among measured water quality parameters to understand better how the introduction of CO_2 may influence source water characteristics.

We used one-way ANOVA to test for differences in survival among dCO_2 treatments. After detecting differences among

TABLE 1. Water quality summary for CO_2 treatments used in this study, where dCO_2 = dissolved carbon dioxide concentration (mg CO_2/L), BP = barometric pressure (mm Hg), temperature in °C, DO = dissolved oxygen concentration (mg O_2/L), TGP = total gas pressure (mm Hg), and conductivity in μS/cm. Values shown as mean ± 1 SD.

	dCO_2 (mg/L)				
	Ambient	150	300	450	600
dCO_2	70.8 ± 1.6	153 ± 4	300 ± 5	432 ± 5	591 ± 9
BP	752 ± 10	747 ± 7	753 ± 9	753 ± 7	756 ± 8
Temperature	14.5 ± 0.1	14.4 ± 0.1	14.5 ± 0.1	14.5 ± 0.1	14.5 ± 0.1
DO	10.5 ± 0.4	10.3 ± 0.3	10.6 ± 0.2	10.4 ± 0.2	9.5 ± 0.4
pH	6.93 ± 0.02	6.50 ± 0.07	6.24 ± 0.07	6.10 ± 0.06	5.89 ± 0.05
TGP	777 ± 9	787 ± 8	814 ± 8	826 ± 8	843 ± 22
Conductivity	629 ± 3	634 ± 4	630 ± 3	631 ± 1	632 ± 6

dCO_2 treatments, we used a logistic model to summarize the dose-mortality patterns documented over the range of dCO_2 treatments and to calculate the 24-h LC_{50} and LC_{99}. Logistic models, a common model for continuous responses to dosing experiments (Meister and van den Brink, 2000), were used to summarize the mortality of Bullfrog larvae for all trials (9 replicates per trial; 3 trials for each of five CO_2 concentrations). We selected a three-parameter log-logistic model:

$$y = \frac{a}{1+\left(\frac{x}{x_o}\right)^b}$$

where y = percent mortality and x = log-transformed dCO_2 concentration, because it was the best fitted (based on regression coefficient and root mean square deviations [RMSD]) and most parsimonious logistic model. Following this analysis we used a Student's t-test to test for body condition differences among animals that survived or died during CO_2 exposure, regardless of treatment concentration.

Differences in mean blood glucose, pCO_2, and pH levels among four exposure periods (0, 3, 6, and 9 h) were examined using one-way ANOVA. Before each ANOVA we first tested for equal variance using Levine's test of homogeneity of variance. If variances did not differ, we used Tukey-Kramer honest significance difference (HSD) post hoc tests to summarize pairwise differences among treatment groups. If variances were unequal ($P < 0.05$), we used a Kruskal-Wallace ranks test to test for significant differences among exposure periods. If a significant difference was detected using a nonparametric Kruskal-Wallace test, we performed pairwise Mann-Whitney U-tests to summarize differences among exposure periods. We performed all statistical analyses using SPSS (v. 12.0, Chicago, Illinois, USA).

RESULTS

Experiment 1: Determination of LC_{50}.—CO_2 introductions produced five distinct concentrations of dCO_2 in test waters (Table 1). There were differences in pH among treatments, and pH and dCO_2 were inversely correlated ($r = -0.950$, $P < 0.001$). Differences in DO levels among dCO_2 treatments were also detected and DO was inversely correlated with dCO_2 ($r = -0.583$, $P = 0.022$). Total gas pressure increased with increasing dCO_2 ($r = 0.907$, $P < 0.001$). Temperature and specific conductivity did not differ among treatments.

Larval mortality was a function of dCO_2 concentration: no individuals died in control or 150 mg CO_2/L treatments, but 11.1 ± 19.2% (mean percent mortality ± 1 SD) died at 300 mg CO_2/L; 81.5 ± 22.6% died at 450 mg CO_2/L; and 100 ± 0% died at 600 mg CO_2/L. Percent mortality differed between each of these dCO_2 treatments (Kruskal-Wallis: $H_4 = 12.798$, $P = 0.012$). A logistic model provided a good fit to the observed mortality data (Fig. 2) and explained nearly 95% of the variation in mortality (where $a = 101.7$; $b = -9.5$; $x_o = 372.7$; $R^2 = 0.948$, $P < 0.001$; RMSD = 39.531). Using the three-parameter logistic model described above and in Fig. 2, we calculated the 24-h LC_{50} and LC_{99} as 371 and 549 mg CO_2/L, respectively.

Eighty-three of 135 test larvae (61.5%) survived CO_2 treatments. Larvae that died had a lower body condition than those that survived ($t_{133} = 4.251$, $P = 0.001$; Fig. 3). Surviving larvae had a body condition factor of 1.35 ± 0.28 (mean body condition factor ± 1 SD). Larvae that died during the experimental process, regardless of the CO_2 treatment, had a condition factor of 1.14 ± 0.26.

Experiment 2: Sublethal Effects—Changes in Blood Chemistry.—There were statistically significant differences in blood pH levels ($F_{3,16} = 8.928$, $P = 0.001$), pCO_2 levels ($H_3 = 10.985$, $P = 0.012$), and glucose levels ($H_3 = 15.180$, $P = 0.002$) among exposure periods (Fig. 4). With increasing exposure, blood pH levels decreased while pCO_2 levels and glucose levels increased. Using median values, we detected a 3-fold increase in blood glucose

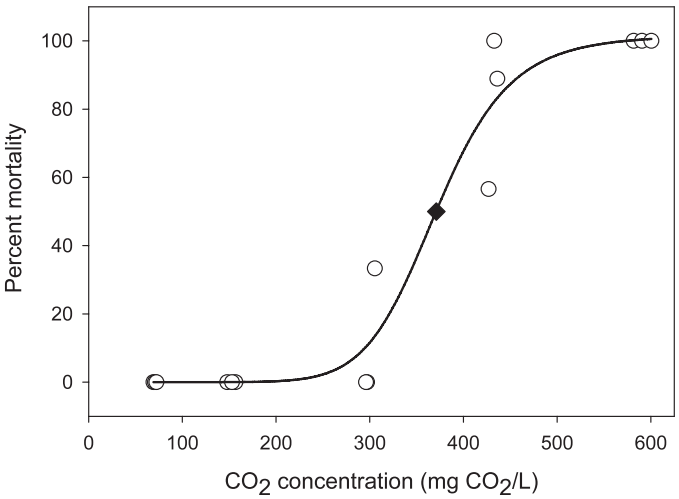

FIG. 2. Larval mortality (expressed as a percentage of individuals exposed for 24 h) as a function of dissolved CO_2 concentration. Each point represents the mortality documented using nine tadpoles per CO_2 concentration trial. The 24-h LC_{50} is denoted by a black diamond at 371 mg CO_2/L.

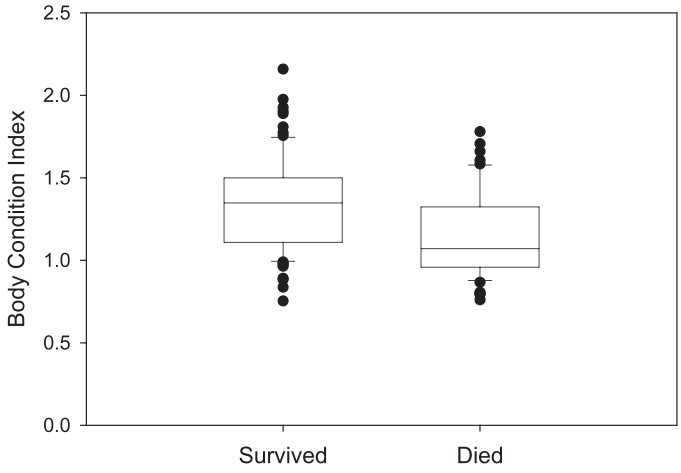

FIG. 3. A summary of the body condition index for tadpoles used in the 24-h LC_{50} experiments ($N = 135$). Tadpoles were lumped into two groups: those that survived ($N = 83$) or died ($N = 52$) during 24 h of CO_2 exposure, regardless of concentration.

levels, a 2-fold increase in blood pCO_2 levels, and a concomitant decrease of nearly 0.6 pH units following 9 h of CO_2 exposure.

DISCUSSION

We found that prolonged exposure to elevated levels of CO_2 is lethal to American Bullfrog larvae. Specifically, mortality increased sigmoidally as dCO_2 increased from 75 to 600 mg/L, and the 24-h LC_{50} and LC_{99} were 371 mg CO_2/L and 549 mg CO_2/L, respectively. During experiments we documented that dCO_2 concentrations could be elevated with little consequence to DO and that DO levels did not reach concentrations low enough to stress Bullfrog larvae (Table 1; McIntyre and McCollum, 2000; Rocha et al., 2010). Therefore, low DO levels did not drive tadpole mortality. We also could not attribute mortality to handling stress because we found no mortality in controls. Percent mortality was correlated with increased dCO_2, decreased pH, and increased total gas pressure. It is likely that increased CO_2 was stressful and ultimately lethal to Bullfrog larvae because it elevated blood pCO_2 and lowered pH levels (see below).

In this study individuals with a lower body condition, regardless of Gosner stage, had a higher mortality rate at elevated dCO_2 levels. Although our experiments were not explicitly designed to test whether a larva's susceptibility to CO_2 is influenced by its body condition, our results and those of Freda and Dunson (1985) indicate that body condition may be an important predictor of susceptibility to CO_2. Elzinga and Butzlaff (1994) reported increased mortality for smaller rather than for larger (estimated by shell length) zebra mussels (*Dreissena polymorpha* Pallas). These authors did not integrate body size and mass, but the results suggest that exposure to dCO_2 may have differential, size-based effects on mortality. Bullfrog larvae with lower body conditions may be more susceptible to high concentrations of CO_2 because they have a greater surface area to mass ratio and diffuse CO_2 at a greater uptake rate than individuals with higher body conditions. Future experiments should explicitly examine the role of larval body condition in response to CO_2 and other environmental stressors.

We do not know if our results are transferable across all Gosner stages of larval development in Bullfrogs. Bullfrog

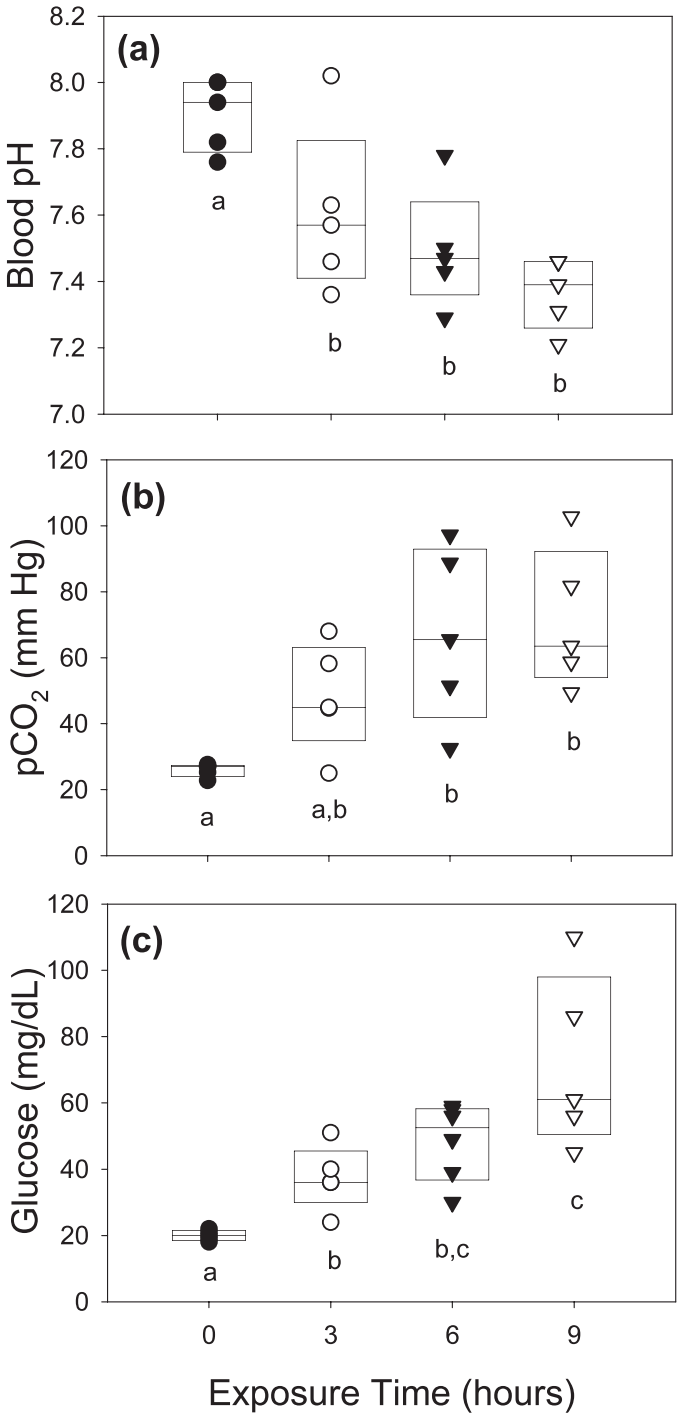

FIG. 4. Blood analyte summaries for tadpoles exposed to 450 mg CO_2/L for 0, 3, 6, or 9 h: (a) pH, (b) partial pressure of CO_2 (pCO_2 in mm Hg), and (c) glucose levels (mg/dl) . Different letters indicate statistical differences among treatments at the $P < 0.05$ level.

larvae rely heavily on cutaneous exchange of CO_2 (Burggren and West, 1982), as the lungs of larvae are not fully developed until Gosner stage 40–42. Larvae of Gosner stages 43 or higher will drown if not allowed to surface (McDiarmid and Altig, 1999). Larvae at Gosner stage 30–40 may take longer to be affected by elevated CO_2 due to partial pulmonary elimination of CO_2; larvae at Gosner stage 40+ require air for normal activity and so may be less susceptible to elevated dCO_2. That said, recent experiments with Nile Tilapia (*Oreochromis niloticus*)

TABLE 2. Summary of tadpole mortality (expressed as a percentage) at five experimentally induced dissolved carbon dioxide (dCO_2) concentrations. Ambient concentrations were approximately 75 mg CO_2/L and served as our unamended controls. Mortality is summarized by treatment and by trial. We conducted a total of three trials for each dCO_2 concentration. Average mortality represents the average mortality for all three trials.

	dCO_2 (mg/L)				
	Ambient	150	300	450	600
Trial 1	0.0	0.0	33.3	100.0	100.0
Trial 2	0.0	0.0	0.0	88.9	100.0
Trial 3	0.0	0.0	0.0	55.6	100.0
Average	0.0	0.0	11.1	81.5	100.0

hybrids in open tanks showed that fish still become narcotized by elevated CO_2, even when allowed to surface and gulp air. Once narcotized, these fish lost their ability to swim and ultimately died from prolonged CO_2 exposure (A. Ray, pers. obs.).

Sublethal Effects—Changes in Blood Chemistry.—Analyses of blood chemistry and blood analytes indicate that CO_2 has sublethal effects on Bullfrog larvae. Sublethal effects of prolonged CO_2 exposure documented here include acidemia (decreased blood pH), hypercapnia (increased blood pCO_2), and hyperglycemia (elevated blood glucose). Carbon dioxide diffuses across the skin from water and equilibrates rapidly and effectively (Burggren and West, 1982). It is likely that diffusion of CO_2 from water to the tadpoles' extracellular fluid is producing the hypercapnia and associated changes to acid-base status. Trends in blood pH and pCO_2 documented here support this hypothesis and demonstrate that median blood pH can decrease nearly 0.6 pH units following 9 h of exposure to elevated CO_2. In other studies, increased dCO_2 similarly caused a reduction in blood pH in fish (Eddy et al., 1979; McKenzie et al., 2002; Baker et al., 2009) including Common Carp (*Cyprinus carpio* Linnaeus; Yoshikawa et al., 1991).

We found that glucose levels were lowest in the control group and highest in individuals exposed to elevated dCO_2 concentrations for longer periods. These data indicate that the hyperglycemia among larvae was caused by prolonged CO_2 exposure and not by handling (see Rocha et al., 2010). In other studies Danley et al. (2005) and Ross et al. (2001) showed that blood glucose levels in fish increased with elevated dCO_2. Hyperglycemia among vertebrate species, including amphibians, is a general indicator of physiological stress (e.g., Broughton and deRoos, 1984).

The levels of blood glucose in this study are indicative of a state of acute stress. Further research is needed to determine whether elevated dCO_2 levels would likewise act as a sublethal chronic stressor over a more-extended exposure. Physiological effects of chronic exposure to CO_2 are distinct from the effects of acute exposure that we observed. Physiological impacts related to chronic stress among amphibians include alterations in growth and development (Denver, 1997), reproductive impairment (Licht et al., 1983), and potential immunosuppression (Carey et al., 1999). Among fish species, chronic stress associated with high dCO_2 levels has manifested as decreased growth rates, increased plasma sodium levels, increased plasma chloride levels, decreased oxygen consumption and affinity, nephrocalcinosis, and decreased condition factor (Yoshikawa et al., 1991; Fivelstad et al., 1998; Danley et al., 2005). Nephrocalcinosis or the mineralization-calcification of renal tissue in fish is thought to be associated with the mobilization of pH buffers (e.g., bicarbonate) due to acid-base disturbance (Eddy et al., 1977; Smart et al., 1979). This is a plausible scenario among Bullfrogs with chronic exposure to elevated dCO_2 and, more generally, amphibians are susceptible to nephrocalcinosis (Godfrey and Sanders, 2004).

Limitations and Next Steps.—Our study was based on work in pressurized chambers using a single water source; achieving target dCO_2 levels with other source waters and in unpressurized systems may produce slightly different 24-h LC_{50} and LC_{99} estimates or present challenges not addressed here. For example, total alkalinity in test waters was high in our spring water source and, as a result, the influence of CO_2 introductions on surface water pH may be even more exaggerated in waters with low alkalinity because of their reduced acid-buffering capacity. Therefore, LC_{50} and LC_{99} values for dCO_2 might be reduced in water with lower alkalinity. Results described here were also conducted in a controlled laboratory setting. Future work should carefully evaluate whether CO_2 introductions can be successfully used to uniformly elevate dCO_2 concentrations in larger, unpressurized systems and ultimately in field environments. Given the abundance of artificial ponds (e.g., golf course and detention ponds) in our contemporary landscape (Oertli et al., 2009), isolated ponds would provide field opportunities for replicated testing of this suppression strategy to control larval Bullfrogs (see Boone et al., 2008).

Ideal suppression agents for Bullfrog control should also be effective against multiple life stages. Because our initial investigations suggest CO_2 is toxic and ultimately lethal to Bullfrog larvae (Gosner stages 26–42), future experiments should consider the impacts of CO_2 on other aquatic life stages of Bullfrogs not addressed here. Late-stage larvae (Gosner 40+) may be able to withstand the increased dCO_2 due to pulmonary elimination of CO_2, while larvae whose lungs have not fully developed may still be susceptible to increased dCO_2 levels. Hibernating juvenile and adult Bullfrogs may also be susceptible to increased dCO_2.

Bullfrogs are an ideal species for testing the effects of elevated CO_2 because they are worldwide invaders (Lowe et al., 2000; Beebee and Griffiths, 2005; Adams and Pearl, 2007) and, therefore, successful suppression agents could have far-reaching implications. This research provides the groundwork to further consider CO_2 as a potential suppression agent for the aquatic life stages of Bullfrogs. We report critical baseline information to advance the use of CO_2 as a suppression agent. Our data can aid in 1) determining whether CO_2 concentrations can be elevated in different source water and in unpressurized environments, 2) justifying experiments designed to document the impact of elevated CO_2 on nontarget organisms, and 3) investigating whether CO_2-related mortality occurs in natural settings.

Acknowledgments.—We thank T. Tucker, USGS Leetown Science Center, for support and assistance with this research. Funding for this effort was provided by the BLM (IAA No. L11PG00079) and USGS's Aquatic Invasive Species Fund and the Northern Rocky Mountain Science Center. All work was approved by the Northern Rocky Mountain Science Center Institutional Care and Use Committee (authorization number: 110314-JG). Any use of trade, product, or firm names is for descriptive purposes only and does not imply endorsement by the U.S. Government.

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Accepted: 9 November 2012.