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Effects of dietary vanadium on growth and lipid storage in a larval anuran: Results from studies employing *ad libitum* and rationed feeding

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ABSTRACT

Vanadium (V) exerts a variety of effects related to metabolic function in vertebrates, including modifying glycolytic pathways and lipid metabolism. However, little is known about toxicity of V to wildlife in natural systems. We conducted parallel, independent studies to evaluate the effect of dietary exposure to vanadium on survival, metabolism, growth, and lipid storage of larval leopard frogs (*Rana sphenocephala*). In one study ("Ad lib") larvae were fed unlimited food and in the other ("Ration") larvae were fed a restricted diet. Experimental diets contained 10 ppm dw V_{total} (Control, "C"), 109 ppm (Low, "L"), and 363 ppm (High, "H"). In neither study did V affect metabolic rate or survival to forelimb emergence. However, in the Ration study, 31% of individuals in H that had initiated metamorphosis died prior to completion of metamorphosis, compared with 22% in M and 9% in C, however the difference was not significant at $\alpha = 0.05$. Mortality during metamorphosis in the Ad lib study was more variable, yet followed a similar trend (26, 14, and 12% mortality in H, L, and C). In both studies, individuals that died during the metamorphic period were smaller at the time of forelimb emergence than those that survived. In the Ration study, individuals in H also had significantly lower growth rates, delayed metamorphosis, and reduced lipid content at metamorphosis compared to C and L. Growth was not affected by V in the Ad lib study. Our results suggest that habitat contamination by V may present risks to amphibians during the metamorphic period attributable to energetic responses influencing size at the initiation of metamorphosis and body lipid stores. Furthermore, while we cannot make formal comparisons between the studies, our results imply that protocols that employ *ad libitum* feeding regimes could fail to capture the effects of contaminants as they may be expressed in natural situations in which resources are typically more limited.

1. Introduction

Increasing use of fossil fuels worldwide will bring increased release of associated contaminants to air, water, and soils. Fly ash produced following combustion of petroleum and coal is rich in numerous toxic trace elements which may by liberated to aquatic and terrestrial systems and the atmosphere through release of liquid or solid combustion wastes (Groen and Craig, 1994; Ghio et al., 2002; Rowe et al., 2003). Considerable research has been conducted to assess environmental risks of many fossil fuel derived elements, such as Hg, Se, and Cd (see Rowe et al., 2002). However, there is little information with which environmental risks may be assessed for many other inorganic contaminants associated with fossil fuel combustion, including vanadium (V; e.g. Woods et al., 2004) which recently has been implicated in having severe effects on wildlife when present in very high concentrations (Rattner et al., 2006).

Vanadium may enter aquatic systems through multiple avenues, including release of fly ash and via runoff from naturally V-rich soils and irrigated areas (e.g. Hamilton and Buhl, 1997; Rowe et al., 2002). It is accumulated by biota, sometimes to very high concentrations (for example; Hopkins et al., 2001; Rattner et al., 2006; Unrine et al., 2007). In the laboratory, dissolved V has been shown to be acutely or chronically toxic to fish and invertebrates, exceeding Se, Li, U, and B in toxicity (Hamilton, 1995; Hamilton and Buhl, 1997; Woods et al., 2004), and ingestion of V-contaminated food has also been found to presents risks of bioaccumulation and toxicity (Rattner et al., 2006).

Despite contamination of some habitats by V, very little is known regarding the potential adverse effects on wildlife that may result from chronic exposure. A recent, acute toxicity study conducted following a die-off of waterfowl in a petroleum fly ash disposal basin provided strong evidence that the presence of high

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concentrations of V in food was most likely the primary agent responsible for mortality observed in the waterfowl (Rattner et al., 2006). However, the V concentrations in this site were remarkably high (467,000 $\mu g\,L^{-1}$ dissolved, 57 and 226 $\mu g\,g^{-1}$ dw, in livers and kidneys from dead geese collected at the site; Rattner et al., 2006) relative to those observed in abiotic and biotic matrices in other V-contaminated systems (E.P.R.I., 1987; Bu-Olayan and Subrahmanyam, 1998; Metwally et al., 1997; Peterson et al., 2002; Unrine et al., 2007). While the study by Rattner et al. (2006) clearly showed that V may be a threat to wildlife in situations where contamination is exceptionally severe, these results cannot be extrapolated to systems that harbor much lower concentrations of V.

Research concerning relatively low concentrations of V has been almost exclusively conducted with mammals (including humans) in an effort to understand the element's interaction with metabolic processes, frequently exploring its potential efficacy for clinical use in the treatment of diseases such as type II diabetes (Tsiani and Fantus, 1997; Thompson, 1999; Crans, 2005). Vanadium is suspected of being an essential element for higher animals, although definitive proof is lacking (see Barceloux, 1999). The biological activities of V are extremely complex and vary with route and duration of exposure, the specific V derivative/oxidation state and concentration to which tissues are exposed, and species owing to species-specific differences in sensitivity (Crans et al., 2004; Goc, 2006). Vanadium, particularly the vanadyl ion, can substitute for phosphorous or complex with phosphorylated substrates and enzymes (NAD, ADP, GDP, glucose-6-phosphate, Na^+ K^+ ATPase, Ca^{2+} Mg^{2+} ATPase, glucose-6-phosphatase, acid and alkaline phosphatases, protein tyrosine phosphatases; Nechay et al., 1986; Crans et al., 1992; Nakai et al., 1995; Tsiani and Fantus, 1997; Mukherjee et al., 2004; Crans, 2005), replacing the phosphorylated compounds with vanadylated compounds in metabolic pathways, sometimes substantially increasing the rate of glucose oxidation in diabetic animals (see Crans, 2005). As the cellular insulin enhancer is a tyrosine protein kinase, inhibition of protein tyrosine phosphatase by V is likely responsible for its insulin-enhancing action on glycolysis and lipid metabolism (Ullrich et al., 1985; Tsiani and Fantus, 1997).

While insulin and V typically stimulate uptake of glycogen, protein, and triglycerides and inhibit catabolism/mobilization of energy stores, maintaining or increasing body weight, (see Thompson, 1999; Srivastava, 2000; Goc, 2006), at higher-than-therapeutic exposures V may inhibit glucose absorption across the intestine (Madsen et al., 1993), induce weight loss, decrease body lipid content, and lead to anorexia-like symptoms (Ousterhout and Berg, 1981; Srivastava, 2000; Goc, 2006). Recognition of the latter effects of V have led researchers to explore V as a potential agent in the treatment of obesity (Wang et al., 2001).

The reported influence of V on pathways regulating glucose absorption and lipid metabolism (Madsen et al., 1993; Srivastava, 2000; Goc, 2006) suggests that under natural conditions in which energy substrates are finite and often limiting, exposure to V could lead to reductions in somatic growth, developmental rate, or investment in energy stores (lipid storage). If severe, alteration of these bioenergetic processes may compromise fitness potential or survival of the individuals (Congdon et al., 2001). In amphibians for example, reduced growth and/or developmental rates can extend the duration of exposure to gape-limited predators, and may delay metamorphosis, increasing the risk of mortality due to drying of temporary nursery pools. Reduced production of energy stores (lipids) brings risks of mortality during non-feeding periods, such as during metamorphosis when the gut and feeding structures are being reorganized and during periods of low resource abundance (Scott, 1994; Rowe et al., 2003). Thus V, operating through regulation of metabolic processes, could ultimately induce changes at the organism-level that decrease fitness or survival.

In this study, we investigated bioenergetic responses (growth, lipid storage, metabolism) of an amphibian when chronically exposed to V using two laboratory studies conducted in parallel. We added V to the diet of larval southern leopard frogs (*Rana sphenocephala*) at concentrations near those observed in natural, fly ash contaminated systems (see Rowe et al., 2002). We also incorporated food availability into our protocols in order to examine whether resource availability would influence expression of bioenergetic responses. In one study, food was supplied *ad libitum*, whereas in the other study food was rationed to more closely approximate natural systems where food is a limiting resource. We predicted that V would increase metabolic rates (respiratory oxygen demands) and subsequently decrease tissue production evident as reduced growth rates and/or production of lipid stores. Furthermore, we predicted that a restricted diet would exacerbate effects on growth and lipid storage relative to an *ad libitum* feeding regime. Note that our intent was not to quantify molecular or cellular mechanisms by which V may affect the organisms. Rather, our study was designed to examine effects *in toto* as they may modify ecophysiological traits in wildlife, which were largely unknown.

2. Materials and methods

2.1. Protocol

We conducted two parallel studies to examine potential effects of dietary V on survival, growth, life history traits, respiration, and lipid content on larval southern leopard frogs over 120 d. The focal study (hereafter "Ration") was designed to test V effects under conditions in which feeding rations were controlled, intended to represent conditions in natural habitats in which resources are limited and thus contaminant-induced energetic demands may be expressed as changes in growth or survival (e.g. Hopkins et al., 2002). A smaller, parallel study (hereafter "Ad lib") was similar to the Ration study with the exception that food was provided *ad libitum*, typical of most laboratory studies of chronic contaminant effects. Both studies included three food types, two having been supplemented with V (below) and a control that had no supplemental V. The Ration study included four replicates per food treatment, whereas Ad lib included three. Our rationale for conducting independent studies rather than one larger, factorial study in which food availability was included as an additional variable was that different feeding regimes would be expected to result in different exposures to V in the diet (e.g. when fed food having the same concentration of V, individuals in Ad lib would have potentially greater exposure that those in Ration), precluding direct comparisons.

Southern leopard frog larvae were hatched from eggs collected from wetlands located on the Patuxent Naval Air Station, Lexington Park, Maryland, USA. Portions of seven egg masses were collected within approximately 12 h of oviposition. Eggs were held in the laboratory in a single, aerated tank filled with well water. Approximately 48 h after hatching, when larvae had attained stage 25–26, larvae were assigned to replicate tanks for the duration of the experiment (below). Initial wet mass of larvae was 8.17 ± 0.32 mg ($\bar{x} \pm 1$ S.E.) based upon 32 additional larvae weighed individually on a microbalance and excluded from the experiment.

For the Ration study we used 12 polyethylene tanks (4 replicates of each of 3 treatments; below) filled with 12 L of well water. For Ad lib we used nine glass aquaria filled with 30 L of well water. Tanks for both studies were maintained with constant aeration in a 22–24 °C laboratory under a 14:10 light cycle. We measured total ammonia calorimetrically (Chemets Test Kit, Fisher Scientific) twice during

the study in unfiltered composite samples from all tanks prior to water changes (range 0.15–0.20 ppm).

Larvae were fed a diet consisting of a mix of ground tadpole food (Carolina Biological Supply) and an aquacultural gelatin food (Aquatic Ecosystems) mixed with equal volumes of stock solutions. Stock solutions were prepared once at the beginning of the study and used throughout. Solutions were mixed using sodium metavanadate ($NaVO_3$) dissolved in deionized water to nominally achieve V_{Total} concentrations of 88 and 176 ppm dry mass (treatments "L" and "H", respectively). Control food ("C") was prepared by adding deionized water to the food mix in an equal volume to that used in preparing V-enriched food. Nominal concentrations reflected those measured in periphyton samples collected during a prior study that we conducted in a coal fly ash drainage swamp (88 ppm), or twice that value (Rowe et al., 2001; V concentrations however were not reported in that publication as it was focused on other contaminants). Actual concentrations of V in food were verified by chemical analyses. Larval densities were set at 1.3 individuals per liter of water in both studies. Early in the Ration study, one control tank was spilled resulting in three replicates for treatment C and four for L and H.

Larvae in both studies were fed *ad libitum* through Day 16 post-hatching, after which those in the Ration study were supplied every 2 d with a ration of 13% of average biomass per treatment, adjusted at 6 d intervals when tanks were cleaned and larvae were weighed. This initial *ad libitum* feeding regime was intended to avoid damage to the fragile hatchlings that would otherwise occur when weighing to calculate rations. The feeding and cleaning schedule was the same for Ad lib as for Ration. Little or no food remained after the 2 d feeding intervals in Ration suggesting that resource restrictions were imposed by this feeding regime. As food typically remained after the first 1 d following feeding, we presume that restrictions were not extremely severe.

Once during the larval period (Day 55 post-hatch), we measured standard metabolic rate (SMR) via microrespirometry (Micro Oxymax, Columbus Instruments) on two randomly selected larvae from each tank following protocols described by Rowe et al. (1998). We also measured SMR of 2–3 newly metamorphosed individuals from each tank within 1 d of metamorphosis (below). Measurements of SMR on metamorphs were conducted on individuals that metamorphosed at nearly the same time (Days 105–107) to minimize differences in duration of prior exposure to V. Results from Rowe et al. (1998) suggest that if substantial differences in metabolic rates exist, they may be detected even when relatively few animals are measured.

At the time of forelimb emergence, individuals were removed, weighed, and held individually in water from their respective tanks at in a 22–24 °C laboratory until metamorphosis. Individuals were classified as metamorphs when less than 1 mm of tail remained at which time they were weighed, and measured for snout to vent length (SVL). Metamorphs were immediately sacrificed by cervical dislocation, and frozen for analysis of non-polar lipids or tissue V concentration. We measured total non-polar lipids (e.g. storage lipids) in 2–4 metamorphs from each replicate that completed metamorphosis between Days 88 and 94. Whole body non-polar lipid content was measured gravimetrically via Soxhlet extraction with petroleum ether (described in Rowe, 2003). Individuals used for lipid analyses did not differ in average size among treatments (1.792 ± 0.082 g wet weight across treatments; $F_{2,29} = 0.47$; $P = 0.630$).

On Day 120 post-hatching we ended the study although several individuals in both studies had not yet initiated metamorphosis. As no metamorphs were observed from Days 110 to 120, we assumed that the remaining individuals were arrested in development and would not metamorphose. Thus, we calculated both survival to forelimb emergence and survival to the end of the study, excluding any larvae remaining on Day 120 which were classified as unlikely to survive.

2.2. Chemical analyses

We analyzed [V_{Total}] in two to five metamorphs from each replicate tank based upon availability. Guts were not removed prior to analysis because the animals were collected following suspension of feeding during the period preceding forelimb emergence (due to reorganization of the gut and craniofacial structures; see Rowe et al., 2003) and continuing through completion of metamorphosis (approximately 7 d). We analyzed two composite samples of each food type consisting of approximately equal portions of food. The composites were made from freshly mixed food collected and frozen at four feeding times during the experiment. We also determined the potential reduction in V concentrations in food that could have occurred due to dissolution during the 48 h period in which food remained in tanks between feeding events. To do so, we placed a sample of food (~1 g) from the composites above in each of six, 2.5 L jars and allowed it to remain for 24 or 48 h (three tanks each) prior to removal by fine mesh net followed by filtration of the water (0.45 μm).

Tissue and food samples were freeze dried for 48 h, crushed and microwave (Milestone Ethos EZ) digested. Approximately 0.5 g of material was placed in the quartz vessel inner sleeve with 8 mL of high purity nitric acid. In the outer Teflon sleeve, 4 mL of hydrogen peroxide was added. The samples were digested at 200 C for 30 min. Digestion was carried out in lots of 10, and included a blank and either an SRM (NIST 1566b Va = 0.577 $\mu g\,g^{-1}$ or IAEA-405 95 $\mu g\,g^{-1}$) or matrix spike. Matrix spike recoveries averaged 91 ± 4%. The SRM concentrations averaged $89 \pm 4\,\mu g\,g^{-1}$ for IAEA 405 (95 ± 5) and $0.70 \pm 0.1\,\mu g\,g^{-1}$ for NIST 1566b. Analysis of the extracts was performed using an HP-4500 ICP-MS using scandium as an internal standard.

2.3. Statistical analyses

For data analyses, tanks were considered independent replicates and analyses were conducted on mean responses per tank. Treatment-specific differences were identified using Tukey's LSD *post hoc* comparisons. Prior to all analyses, data were subjected to tests of model assumptions. Proportional data for survival were transformed by arcsine square root. Growth data were $\log_{10}$ transformed. Larval growth rates were analyzed until removal of individuals possessing forelimbs (Days 71 and 78 post-hatching in Ad lib and Ration, respectively). We used repeated measures Analysis of Variance (ANOVA) to quantify treatment-specific differences in larval growth based on size measurements made at 6-d intervals. Survival, size at forelimb emergence and metamorphosis, time to forelimb emergence, size of individuals that died during metamorphosis, and total non-polar lipid content were compared among treatments using ANOVA. Prior to analyses of SMR, data for O_2 consumption were transformed based upon empirically determined allometric relationships between $\log_{10}[O_2$ consumed] and $\log_{10}$[wet mass], prior to analysis by ANOVA (described by Manyin and Rowe, 2006). This technique is an alternative to analysis of mass-specific SMR, which is insensitive to allometric relationships between respiration and body mass (e.g. Beaupre and Dunham, 1995). However, for comparison with the majority of studies that report mass-specific rates, SMR data are presented in tables as $\mu L\,O_2/g\,min$. Within-treatment relationships between size at forelimb emergence and survival through metamorphosis were analyzed by binary logistic regression. Statistical analyses were conducted using Minitab version 13 statistical software (Minitab

Table 1
Concentrations of total V (ppm dry mass) in food samples at the time of feeding ("initial") or after 24 or 48 h of soaking in water

Time	Vanadium treatment		
	Control	Low	High
Initial	8.2	108.8	363.2
24 h	9.7	138.4	246.5
48 h	12.5	151.0	285.3

Concentrations were measured in single, composite samples from each treatment.

Table 2
Concentrations of total V (ppm dry weight) in carcasses of metamorphs in the Ad lib and Ration studies

Vanadium treatment	Feeding regime	
	Ad lib	Ration
Control	1.32 ± 0.208^a	1.31 ± 0.320^a
Low	2.38 ± 0.291^b	2.45 ± 0.162^a
High	3.12 ± 0.234^b	3.93 ± 0.335^b
	$P = 0.002$	$P = 0.004$

Values are means ± 1 S.E. Different superscripts represent treatments that differed within each study.

Inc., State College, PA).

3. Results

3.1. Chemistry

Actual concentrations of V in food were 8.2, 108.8, and 363.2 ppm dry mass ©, L, and H, respectively; Table 1). There was no evidence of substantial decline in V concentrations in food in C or L during the maximum period of time (48 h) that the food remained in the tanks, however the concentration of V in H declined by approximately 30% within 24 h (Table 1). Concentrations of V in tissues of metamorphs (Table 2) significantly varied among treatments in both studies ($P = 0.002$ in Ad lib, $P = 0.004$ in Ration). In the Ad lib study, metamorphs from C had lower burdens than L ($P = 0.0408$, from *post hoc* comparisons) and H ($P = 0.0014$), however H and L did not differ ($P = 0.1459$). In the Ration study, metamorphs from C had significantly lower body burdens than those in H ($P = 0.034$) but did not differ from L ($P = 0.087$), and concentrations in metamorphs from L were significantly lower than H ($P = 0.0235$). Qualitatively, body burdens in each treatment did not appear to differ substantially between the two studies.

3.2. Biological responses

In the Ad lib feeding study, there were no effects of V treatment on survival, size at forelimb emergence, or time to and size at metamorphosis (Table 3), nor were there effects on growth rate throughout the larval period (Fig. 1). There was a relationship between size at forelimb emergence and survival through metamorphosis such that smaller animals in treatments L and H experienced higher mortality rates than larger animals (Fig. 2).

In the Ration study, there was no effect of V on survival through the end of the study, despite higher mortality of V-exposed than unexposed individuals during metamorphosis (mortality in L and H during metamorphosis was 2.3 and 3.3 times greater than in C, respectively; $P = 0.073$; Table 4). Larval growth rates began to diverge significantly on approximately Day 47, such that larvae in treatment C were consistently larger than those in treatment H throughout the remainder of the larval period (Fig. 1). As a result, individuals from treatment H at the time of forelimb emergence

Table 3
Biological responses of leopard frogs measured in the Ad lib study

Treatment	Survival to Day 120, metamorphs + remaining larvae (%)	Survivors having initiated metamorphosis by Day 120 (%)	Time to forelimb emergence (d post-hatch)	Wet mass at forelimb emergence (g)	Wet mass at metamorphosis (g)	SVL at metamorphosis (mm)
Control	69.0 ± 8.1	90.9 ± 6.4	92.5 ± 1.7	2.712 ± 0.264	1.899 ± 0.050	26.8 ± 0.1
Low	66.7 ± 14.0	93.0 ± 5.0	94.3 ± 2.5	2.601 ± 0.104	1.750 ± 0.098	26.3 ± 0.7
High	76.0 ± 9.3	73.6 ± 11.5	92.4 ± 1.9	2.820 ± 0.240	1.800 ± 0.227	26.0 ± 1.0
	$F_{2,6} = 0.20$; $P = 0.823$	$F_{2,6} = 1.73$; $P = 0.256$	$F_{2,6} = 0.28$; $P = 0.764$	$F_{2,6} = 0.26$; $P = 0.779$	$F_{2,6} = 0.27$; $P = 0.772$	$F_{2,6} = 0.36$; $P = 0.715$
Treatment	Time from forelimb emergence to metamorphosis (d)	Mortality during metamorphosis (% of individuals having forelimbs by (Day 120)	Standard metabolic rate, larvae (μL O_2/g/min), 22 °C	Standard metabolic rate, metamorphs (μL O_2/g/min), 24 °C		Non-polar lipid content at metamorphosis (% dry mass)
Control	7.6 ± 0.7	12.4 ± 4.0	2.73 ± 0.26	2.64 ± 0.02		15.5 ± 1.1
Low	7.9 ± 0.3	14.2 ± 3.2	2.93 ± 0.22	3.25 ± 0.44		10.8 ± 1.5
High	8.1 ± 0.1	25.9 ± 13.4	2.69 ± 0.13	3.69 ± 0.34		11.5 ± 3.1
	$F_{2,6} = 0.35$; $P = 0.720$	$F_{2,6} = 0.79$; $P = 0.496$	$F_{2,6} = 0.01$; $P = 0.998$	$F_{2,6} = 2.27$; $P = 0.174$		$F_{2,6} = 1.48$; $P = 0.301$

Values are means of replicates ± 1 S.E., from 3 replicates per treatment. Different superscripts denote those treatments that differed at $P < 0.05$ following *post hoc* multiple comparisons.

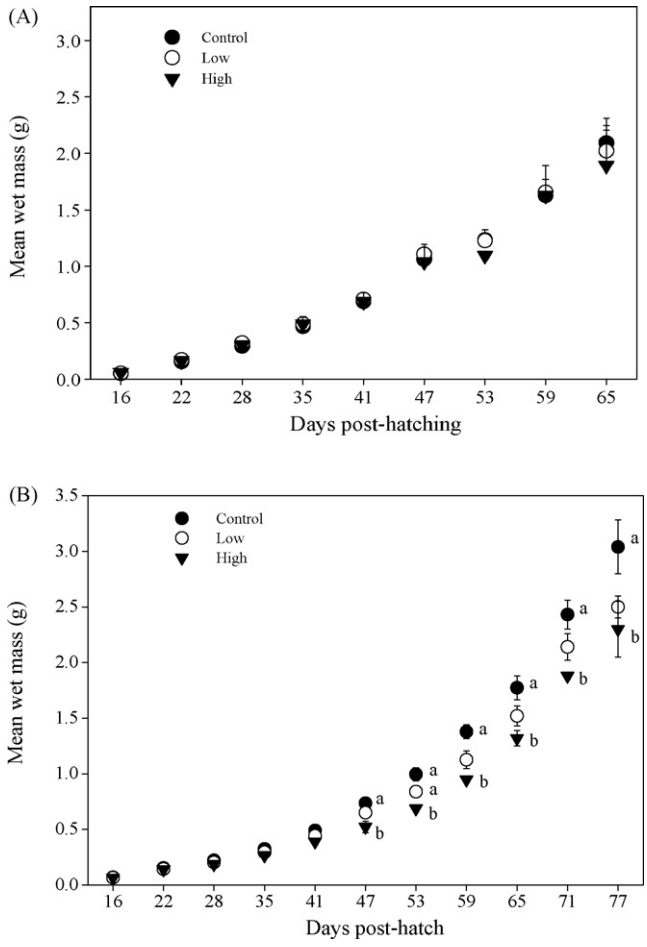

Fig. 1. Larval growth rates in the Ad lib (Panel A) and Ration (Panel B) studies. Metamorphosis in the Ad lib study began prior to the larval growth measurements on Day 71, thus larval growth rates were calculated only through the Day 65 measurement. Metamorphosis in the Ration study began following the Day 77 measurement. Values are means ± 1 S.E. Different superscripts represent statistically significant differences ($P < 0.05$) among treatments on a given day.

were about 16% smaller than those in C or L (Table 4). However, these differences were not sustained through completion of metamorphosis, likely due to high mortality rates of the smallest individuals in treatment H during metamorphosis (as was observed in Ad lib as well; Fig. 2). The duration of the larval period was significantly longer in treatment H than in L in the Ration experiment, although the difference was only 3 d (Table 4). There was no difference in time elapsed between forelimb emergence and metamorphosis among treatments in either experiment.

Total non-polar lipid content of metamorphs was not influenced by V exposure in the Ad lib experiment (Table 2). However, in the Ration study total non-polar lipid content was reduced by approximately one-third in metamorphs from treatment H relative to C (Table 4). There was no effect of V exposure on metabolic rates of larvae or metamorphs during the Ad lib study, but metabolic rates of metamorphs in the Ration study were approximately 7% lower ($P = 0.076$) in treatment H relative to the others (Tables 3 and 4).

4. Discussion

Exposure to dietary V reduced larval growth rates, survival during the metamorphic period, and the capacity of individuals to store energy in the form of lipids. These effects were expressed only under feeding regimes that simulated natural situations in which

Table 4
Biological responses of leopard frogs measured in the Ration study

Treatment	Survival to Day 120, metamorphs + remaining larvae (%)	Survivors having initiated metamorphosis by Day 120 (%)	Time to forelimb emergence (d post-hatch)	Wet mass at forelimb emergence (g)	Wet mass at metamorphosis (g)	SVL at metamorphosis (mm)
Control	81.3 ± 18.8	74.7 ± 16.0	90.3 ± 0.8	2.650 ± 0.020[a]	1.744 ± 0.044	25.6 ± 0.4
Low	75.0 ± 10.8	71.3 ± 4.8	88.8 ± 1.2[a]	2.662 ± 0.106	1.836 ± 0.092	26.1 ± 0.6
High	79.7 ± 14.3	87.1 ± 5.4	93.0 ± 0.5[b]	2.218 ± 0.024[b]	1.633 ± 0.065	25.3 ± 0.4
	$F_{2,8} = 0.29$; $P = 0.752$	$F_{2,8} = 1.18$; $P = 0.355$	$F_{2,8} = 5.81$; $P = 0.028$	$F_{2,8} = 13.64$; $P = 0.003$	$F_{2,8} = 2.02$; $P = 0.194$	$F_{2,8} = 0.72$; $P = 0.518$
Treatment	Time from forelimb emergence to metamorphosis (d)	Mortality during metamorphosis (% of individuals having forelimbs by Day 120)	Standard metabolic rate, larvae (μL O_2/g min), 22 °C	Standard metabolic rate, metamorphs (μL O_2/g min), 24 °C	Non-polar lipid content at metamorphosis (% dry mass)	
Control	7.0 ± 1.0	9.4 ± 6.3	2.99 ± 0.39	4.02 ± 0.30	15.2 ± 0.1[a]	
Low	7.1 ± 0.2	21.5 ± 5.1	2.99 ± 0.24	3.69 ± 0.70	13.1 ± 1.2	
High	6.9 ± 0.2	30.9 ± 6.7	2.67 ± 0.08	2.52 ± 0.35	10.1 ± 0.2[b]	
	$F_{2,8} = 0.25$; $P = 0.784$	$F_{2,8} = 3.62$; $P = 0.076$	$F_{2,8} = 0.76$; $P = 0.500$	$F_{2,8} = 3.63$; $P = 0.076$	$F_{2,8} = 11.12$; $P = 0.005$	

Values are means of replicates ± 1 S.E., from 3 replicates for Control and 4 replicates for Low and High V concentrations. Different superscripts denote those treatments that differed at $P < 0.05$ following *post hoc* multiple comparisons.

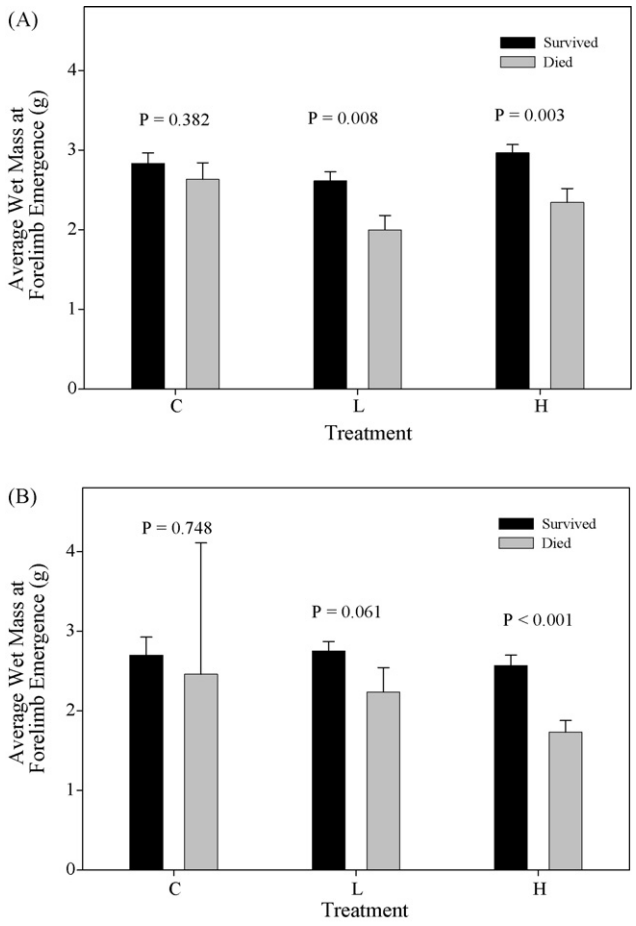

Fig. 2. Relationships between size at forelimb emergence and survival through completion of metamorphosis in the Ad lib (Panel A) and Ration (Panel B) studies. Values are means ± 1 S.E. *P*-values are derived from within-treatment analysis by logistic regression of mass versus fate (survived or died).

resources were somewhat restricted but were largely absent when food was provided *ad libitum*.

The strong effect of dietary V on larval growth rate and size at forelimb emergence in the Ration study did not translate to effects on average mass or length of metamorphs. This apparent contradiction likely reflected the relationship between size at forelimb emergence and survival through the metamorphic period; individuals initiating metamorphosis at a small size had much higher mortality rates than larger individuals, particularly in H (Fig. 2). However, the effect of V in reducing size at metamorphosis combined with higher mortality rates of the smallest individuals did not reduce average survival to the end of the study relative to unexposed individuals, likely reflecting within-treatment variability in survival overall (Table 4). As well, in the Ration study, mortality during metamorphosis was 2.3–3.3 times greater for individuals exposed to V relative to controls ($P = 0.076$), yet did not influence average survival rates to the end of the study. It is interesting to note that average size of individuals at the time of forelimb emergence in the Ad lib study was similar to that of animals in the control treatments in the Ration study. This, as well as similar body burdens of V in metamorphs in both studies, suggests that food restrictions in the Ration study were not severe.

While there was no discernible relationship between V exposure and size of metamorphs, lipid content of metamorphs in the Ration study was severely reduced in individuals from treatment H relative to other treatments. With respect to recruitment to the adult population and reproductive fitness of amphibians, both overall size and energy stores at metamorphosis may play a crucial role. Several studies have shown that size at metamorphosis is correlated with reproductive fitness (see review by Wilbur, 1980). Furthermore, as many amphibians experience periods of non-feeding or elevated activity costs during dispersal across relatively resource-poor habitat types or during seasonal periods of limited resource availability, energy stores are vital to survival (Rowe et al., 2003). For example, Scott (1994) demonstrated that body lipid content of metamorphs of the marbled salamander (*Ambystoma opacum*) correlated strongly with survival time under non-feeding conditions simulating reduced winter abundance of food. Thus, we speculate that the lower lipid content of metamorphs exposed to V may result in a carry-over effect of larval habitat quality to performance during later life stages (Wilbur, 1980; Scott, 1994).

While the pattern in lipid content relative to V exposure was similar in both Ration and Ad lib, there was much greater variability in the latter. This difference does not appear to be related to differences in experimental protocol (three replicates in Ad lib and four in Ration), as the control treatment in Ration contained only three replicates due to loss of one early in the study, yet the variation in lipids was much less than that observed in results for Ad lib. We are unable to speculate on the cause for such differences in variation in results between the studies.

Metamorphosis entails a period of non-feeding during reorganization of the gut and feeding structures (Rowe et al., 2003), and thus energy stored in somatic tissues must be reallocated to satisfy metabolic demands. While we did not measure lipid content in individuals prior to metamorphosis, we postulate that reductions in lipid stores may be related to the relatively high rates of mortality during metamorphosis in treatment H. On the other hand accumulated V may have been remobilized from somatic stores, thus entering circulation and being transported to sites of toxic action. For example, Snodgrass et al. (2003) observed lower concentrations of V in field collected larval frogs relative to metamorphs, suggesting that stored V was mobilized and eventually lost during metamorphosis. Whether the mortality that we observed was due to energetic constraints due to reduced lipid stores or remobilization of V and subsequent toxicity remains a question.

When food availability was restricted, dietary exposure to V delayed metamorphosis, yet these effects were not expressed when unlimited food was available. Delayed metamorphosis can increase an individual's probability of encountering aquatic predators, and can increase probability of mortality due to early drying of the nursery site (Wilbur, 1980). However, the highest V exposure increased the larval period only by an average of 3 d (3.3%) relative to controls. While we cannot rule out that in some situations even such a slight delay in metamorphosis could be important to recruitment success, we question its broad significance given the great variability in community composition and hydroperiods characteristic of natural breeding sites (e.g. Rowe and Dunson, 1993).

Theoretically, energetic investment in production should be regulated by metabolic expenditures, representing a trade-off between current costs of survival and activity and production of somatic and reproductive tissues (Congdon et al., 2001). Numerous studies have demonstrated that contaminant exposure can increase metabolic expenditures (Rowe et al., 1998; Hopkins et al., 1999), in some cases correlating with reduced growth/development, lipid content, and/or reproductive investment (e.g. Manyin and Rowe, 2006). Yet in the current study, there was little evidence of effects of V on metabolic rates, contrary to our expectations. We hypothesized that V exposure would modify metabolic rate

based upon documented action of V on metabolic enzyme activity and expression in tissue culture and diabetic rodent models (see reviews by Nechay et al., 1986; Crans et al., 2004; Mukherjee et al., 2004; Crans, 2005). However, biochemical properties of V are extremely complex, many of which remain to be identified (Crans, 2005). Thus, there are numerous mechanisms by which V exposure may have affected growth, developmental rate, and energy storage while not substantially altering metabolic rates of individuals.

5. Conclusions

While severe cases of environmental contamination by V present risks of mortality to wildlife (e.g. Rattner et al., 2006), there remains a paucity of information regarding the ecological ramifications of chronic, sublethal exposure to V in less extreme situations. Our experiments demonstrated that, at concentrations much lower than those known to induce mortality in heavily contaminated habitats, dietary V reduces growth of larval frogs and may reduce survival of juveniles during periods of resource limitation when lipid stores are critical energy sources. Growing industrialization and use of fossil fuels to satisfy global energy demands suggests that V contamination of the environment will continue to increase, and thus an understanding of V effects on wildlife may be important to assessing environmental risks of these activities. As well, further research addressing individual-level effects of V could prove valuable in establishing the relative contributions of specific enzymatic and metabolic pathways to fitness of individuals via their influences on bioenergetic traits.

Perhaps of broader significance, our study provides evidence that the ultimate expression of contaminant effects can be context-dependent. Here, a feeding regime more closely resembling that in natural systems provided results that contrasted with those obtained when food availability was unnaturally high, as is typical in many laboratory studies. While we cannot formally compare the two experiments, it appears that provision of a typical, *ad libitum* diet may have masked the effects of V that emerged when resources were more restricted, suggesting that protocols employing unlimited feeding may provide very conservative assessments of the effects of contaminants in natural systems.

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