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Metabolic measures of male southern toads (*Bufo terrestris*) exposed to coal combustion waste

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Abstract

Southern toads (*Bufo terrestris*) are found in coal fly ash collection basins associated with coal-burning electrical power plants. These basins contain large amounts of trace metals and organisms found in these basins are known to accumulate large quantities of metals. Studies on a variety of organisms exposed to trace metals found that they experience a significant increase in standard metabolic rate. We experimentally exposed southern toads to metal-contaminated sediment and food and measured changes in standard and exercise metabolic rates as well as changes in body, liver and muscle mass, blood glucose, and corticosterone. We found that toads exposed to trace metal contamination gained significantly less mass (18.3 %) than control toads (31.3%) when food was limited and experienced significantly decreased RQ after exercise. However, contaminated toads did not experience changes in standard ($\overline{x}_{control}=0.114\pm0.016$ mL O_2 g^{-1} h^{-1}; $\overline{x}_{ash}=0.109\pm0.013$ mL O_2 g^{-1} h^{-1}, $p=0.08$) or exercise metabolic rates ($\overline{x}_{control}=0.53\pm0.06$ mL O_2 g^{-1} h^{-1}; $\overline{x}_{ash}=0.44\pm0.04$ mL O_2 g^{-1} h^{-1}, $p=0.47$) plasma glucose levels ($p=0.6$), and hepatic or muscle percentage indices ($p>0.2$ in all cases) whether food was limited or not.

Keywords: Toad; Metabolic rate; Oxygen consumption; Metabolism; Trace metals; Coal fly ash

1. Introduction

Pollutants are ubiquitous components in many environments and their effects, along with their roles as stressors, may be a cause of wildlife mortality (Carey and Bryant, 1995). Pollutants have also been associated with amphibian population declines (Carey and Bryant, 1995; Rowe et al., 2001) and have been linked to changes in energetic demands in numerous organisms, including amphibians (Calow, 1991; Rowe et al., 1998). Some of the most wide-spread and common pollutants in the United States are trace metals. Metal pollution is a byproduct of many industrial processes including coal-burning electricity plants. These power plants are numerous, providing more than half of all the electricity used in the United States (EPA, 1997). The byproduct of burning coal is coal fly ash, which contains a variety of trace metals, such as arsenic, lead, mercury, selenium, cadmium as well as others, known to cause deleterious physiological effects (Cherry and Gutherie, 1977). Coal fly ash is usually mixed with water and pumped into a series of settling basins for ultimate disposal. The ash forms a layer of silt at the bottom of these basins (Cherry and Gutherie, 1977; Gutherie and Cherry, 1979). Coal fly ash settling basins often serve as habitat for aquatic organisms, including a number of invertebrates, turtles, fish, alligators, frogs, and toads (Gutherie and Cherry, 1979). Animals accumulate trace metals throughout their bodies and show deleterious effects associated with metal exposure (Chang, 1996). Ash basin environments may cause an elevation in plasma corticosterone levels and an increase (18–40%) in the standard metabolic rates of fish, reptiles, and larval anurans (Hopkins et al., 1999a, 1997; Rowe, 1998; Rowe et al., 1998). These responses can lead to a myriad of ancillary physiological and behavioral effects associated with chronic stress, including changes in blood proteins (e.g., increases in heat-shock proteins and metallothioneins), changes in the immune system, liver fibrosis and kidney necrosis, and a decrease in sex steroids (Nelson, 2000).

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Several recent studies have focused on the physiological effects of coal fly ash in a population of southern toads (*Bufo terrestris*) in South Carolina, USA. Southern toads migrate into breeding areas (which include ash basins) in the spring, reside there throughout the breeding season, and then migrate out of these areas once breeding has ceased in the fall. Toads in the ash basins accumulate metals throughout their bodies (Hopkins et al., 1998) and experience increases in stress hormones and acyclic changes in sex steroid concentrations (Hopkins et al., 1997, 1999b). Toads found in ash basins also have decreased larval recruitment (Rowe et al., 2001). Although changes in metabolic measures have been documented for organisms that are permanent residents of ash basins, such as shrimp, fish and water snakes (Hopkins et al., 1999a, 2002b; Rowe, 1998), these responses have not been examined in organisms that are only transient in this environment, such as the southern toad. When toads migrate into ash basins, they begin to accumulate trace metals that initiate physiological changes associated with increases in concentrations of the metabolically active stress-hormone, corticosterone.

The purpose of this study was to document how southern toads respond metabolically to coaly fly ash exposure during the breeding season. We utilized techniques to measure exercise and standard metabolic rates, plasma corticosterone and glucose concentrations as well as weight gain over two years with and with out food restriction.

2. Materials and methods

2.1. Animal capture

Male southern toads (*B. terrestris*, mean mass 15.0±0.2 g) were captured by hand at the Savannah River Site, Aiken, SC, USA, in March and April, 2001 (n=48), 2002 (n=170), and 2003 (n=46). Toads were collected from the ash basin area (ash) or a control site (control), ~15 km from the ash basin. Mass was measured on a digital balance (0.01 g accuracy) and an initial blood sample was taken (see *Blood sampling*). All toads were then given an individual identification number by toe clip.

2.2. Housing and experimental design

Toads were transported to Auburn University, Auburn, AL, USA, within 2d of capture and transferred to microcosms constructed of 208 L plastic containers (Rubbermaid®, Fairlawn, OH, USA) modified with screen lids. The bottom of each microcosm was covered with 70% sediment (coal fly ash collected from the capture site or sand) and 30% water to a depth of ~6 cm. All microcosms were located outdoors under a shade-cloth tent and exposed to ambient conditions. There was a total of 60 microcosms (30 ash and 30 sand controls), each housing three randomly selected toads.

In all years, toads were acclimated for 3–4 wk in microcosms containing their capture sediment before sampling began. Toads were assigned to one of two groups based on their site of capture: control or ash. Control and ash toads were housed in microcosms with sand or ash sediment, respectively. Toads were fed weekly with crickets (*Acheta domesticus*, ≈10 crickets per toad) raised from 6.5 mm to adulthood (≈3 weeks) on either a control diet of dry cat food or dry cat food contaminated with coal fly ash (50/50 mixture by volume), depending on treatment sediment. Crickets raised on cat food diets containing metals, are not known to have different nutrient content, than crickets raised on uncontaminated cat food.

This microcosm setup is known to elevate whole body trace metals in toads (Ward, Hassan and Mendonça, submitted). Toads maintained on ash exhibited elevated levels of all assayed metals compared with controls. Eleven of the 18 trace metals assayed were elevated significantly including Al, As, Cd, Co, Cs, Ni, Rb, Se, Tl, U, and V. The seven metals that did not change significantly were; Ba, Cr, Cu, Fe, Pb, Sr, and Zn. Increases in metal content ranged from 47.5% for Pb to 5431% for As (Ward, Hassan and Mendonça, submitted).

Toads collected in 2001 were monitored for changes in blood glucose, mass gain and plasma corticosterone. In 2002 and 2003, toads were monitored for changes in mass and standard and exercise metabolism. The average May to September temperatures for the three years of the approximately 4 mo experiment were 23.4 °C ranging from 10.6 to 35.0 °C in 2001, 24.6 °C ranging from 7.2 to 36.1 °C in 2002, and 24.3 °C ranging from 9.4 to 33.3 °C in 2003 (AWIS, 2005).

2.3. Year 1 (2001)

After acclimation, two groups of toads (control and ash, n=24 per group) were weighed every 2 wk for 5 mo on a digital balance. Blood samples were collected on a 3 wk cycle. Each of the two experimental groups of toads was randomly assigned to one of two subgroups (n=12 per group) from which blood samples were taken on consecutive weeks. Samples were not taken on the third week. This sampling regime resulted in each toad being bled only once every 3 wk.

All blood samples (0.1 mL) were taken via cardiac puncture using a 26.5 gauge heparinized needle. Blood samples were stored on ice (<2 h) until they were centrifuged. The plasma was then removed by pipette and frozen at −4 °C for no more than 6 mo.

Glucose concentrations were measured enzymatically in 10 μL plasma samples using a glucose hexokinase assay kit and standard (#GAHK-20 and #635-100, Sigma Aldrich, St. Louis, MO, USA). Inter-assay variation for glucose was ≈9.6% (Chard, 1995).

Plasma corticosterone levels (n_{ash}=72, $n_{control}$=36) were quantified using a standard competitive binding radioimmunoassay as described in Mendonça et al. (1996). Anhydrous diethyl ether was used to extract steroid hormones from 10 μL plasma sample. All samples were tested in duplicate. Corticosterone antibodies were purchased from Esoterix (Calabasas Hills, CA, USA). A standard charcoal-dextran separation technique was used. Intra-assay variation is ≈3.71% and inter-assay variation is ≈13.0% for corticosterone (Chard, 1995). Baseline corticosterone in this study ranged between 7 and 10 ng/mL; published baseline corticosterone concentrations for this species are 7.67±1.3 ng/mL (Hopkins et al., 1997).

2.4. Year 2 (2002)

All toads in 2002 were provided a limited diet (fed ≈10 crickets every 10 days) because toads in 2001 almost doubled in size and a limited diet can accentuate toxicant effects in fish (Hopkins et al., 2002b). After acclimation, subgroups of toads collected from the ash basin ($n=7$) and the control site ($n=7$) were brought into the laboratory for testing of exercise metabolism (see *Exercise metabolic measures* below). The remaining toads ($n=77$ per group) were maintained in the microcosms and used for the change of mass experiment (see above).

In the laboratory, toads were housed in ≈1.5 L (15×30×11.25 cm) plastic containers with either coal fly ash or sand and maintained at 23±2 °C and a photoperiod of 14 : 10 (L : D) throughout the experiment. Toads were acclimated to these conditions for 2 wk prior to testing following the 1 mo acclimation to the sediment. All toads, laboratory and microcosm housed, were given free access to water and fed ≈10 crickets every 10 d (raised as stated previously).

2.5. Year 3 (2003)

In 2003, 24 ash toads and 22 control toads were brought into the laboratory for standard metabolic measures and housed as in 2002. (see *Standard metabolic rate* below).

2.6. Standard metabolic rate

O_2 consumption and CO_2 production rates were measured using closed system respirometry on two mornings in August 2003, 3 mo after the toads had been introduced into the microcosms. Food was withheld 5 d before measurement and the bladder of each toad was emptied prior to measure by applying gentle pressure to the abdomen. Toads were then weighed on a digital balance and placed into individual 133 mL glass canning jars (Ball Co., Broomfield, CO, USA). The toads were acclimated in their individual jars, for 2 h, in a darkened room at 23 °C (Sievert and Bailey, 2000). The jars were flushed with CO_2-free, dry air (150 mL/min) for 5 min and then sealed. Toads were incubated for 50 min at 23 °C in the dark (this time interval was determined experimentally for optimal O_2 depletion and CO_2 enrichment in the chambers). Data from any toad that moved >10 s during the 50 min incubation period were excluded from the analysis.

A 3 mL air sample was removed from each chamber and a 1 mL sub-sample was injected into a Sable Systems TR-3 respirometry system (Sable Systems, Henderson, NV, USA) to determine O_2 depletion and CO_2 enrichment. Briefly, the air sample was injected into an air stream from which CO_2 and water vapor had been removed. The air stream and sample were drawn through a base lining system, a Li-Cor CO_2–H_2O analyzer (LI-6262: LiCor Inc., Lincoln, NE, USA), a Sable Systems FC-1 Oxygen analyzer, and a Side-Track® mass flow meter (Sierra Instruments Inc., Monterey, CA, USA) with a gas pump (Gast Mfg. Corp., Benton Harbor, MI, USA) at 150 mL/min (STP) (Vogt and Appel, 1999).

Data were recorded using DATACAN V software (Version 5.2; Sable Systems, Henderson, NV, USA). The exact time of incubation (from sealing the chamber to sampling) was recorded for each toad. Rates of O_2 consumption and CO_2 production were calculated as the volume of the gas divided by the mass of the toad, and the total incubation time. This resulted in $\dot{V}_{O_2}$ and $\dot{V}_{CO_2}$ in units of mL g^{-1} h^{-1} (Lighton, 1991).

2.7. Exercise metabolic measures

In May 2002, 4 wk after exposure to ash, toads ($n=7$ for both control and ash treatments) were tested for O_2 consumption and CO_2 production during exercise. A rotating metabolic chamber, which consisted of a clear plastic cylinder (8.44 cm internal diameter) sealed on both ends with large rubber stoppers (see Seymour, 1973 for details). Aquarium tubing (0.5 cm diameter) was used as the inflow and outflow for the flow-through system and connected to an air pump and Sable Systems TR-3 respirometer (as described previously). The chamber was flushed with dry, CO_2-free air for 10 min at 250 mL/min. An individual toad was prepared for testing as described above and placed in the chamber. The metabolic chamber was sealed and the toad acclimated in the chamber for 45 min while the chamber was again flushed with dry, CO_2-free air.

After a 45 min acclimation period, resting metabolic rate was measured for 15 min. Then the chamber was rotated (~1 m min^{-1}) so that the toad walked continuously without stumbling until maximum O_2 consumption was observed (≈20 min), indicated by a constant rate of O_2 consumption. The chamber was then stopped, and we continued to measure O_2 consumption and CO_2 production until both gases returned to previous resting levels for at least 5 min.

Data were recorded and analyzed with DATACAN V software. Plots were made for each gas, O_2 consumption and CO_2 production, during initiation and cessation of exercise. A 2nd order polynomial equation, $y=a+bx+cx^2$, where a is the y intercept, b is the rate of at which oxygen consumption or carbon dioxide production increases/decreases over time and c is the coefficient of the curve, was fit to each curve.

Care was taken during all metabolic measurements to keep stress to a minimum. All toads were handled as little as possible (<1 min) and allowed to acclimate to their environment before measurements began. Blood samples were not taken from toads in the metabolism study to avoid additional stress. Values for *B. terrestris* metabolism in other studies mirror previously published values.

2.8. Liver and muscle mass

All toads in 2001 and 2002 were sacrificed at the end of the experiment (October, 5.5 mo after exposure). Toads were euthanized by immersion in 300 ppm MS222, and the liver and right quadriceps muscle group were removed and weighed.

2.9. Statistical analysis

Changes in body mass were corrected for capture weight and liver and muscle mass were corrected by body mass. All percentage data (percent change in mass and liver and muscle percent body mass) were tested for a normal distribution with a

Kolmogorov–Smirnov test. Distributions that were not normal (change in mass 2002, muscle percent body mass in 2001, and 2002) were transformed (mass was square root transformed and muscle percentage indexes were arcsin transformed) (Sokal and Rohlf, 1995). Non-transformed data are presented in the figures. Changes in corrected body mass and glucose concentrations over time in different experiment treatments were analyzed by repeated measures ANOVA. Corrected liver and muscle mass was analyzed by ANOVA.

For standard metabolic rate measures with closed system respirometry, we used linear regression to determine significant effects of mass on $\dot{V}_{O_2}$ and $\dot{V}_{CO_2}$; the slope of the regression was 1.3 ± 0.28 ($R^2 = 0.301$). Since this value is not significantly different than 1 (t test, $p = 0.4$), data were analyzed isometrically with ANOVA. Respiratory quotient (RQ) was calculated as the ratio of $\dot{V}_{CO_2}$ to $\dot{V}_{O_2}$. Exercise metabolic data were fit with polynomial equations (see Exercise metabolic measure) using DAN (Version 5.2; Sable Systems, Henderson, NV, USA). The polynomial equations, as well as the average rates during exercise and rest, were then compared using ANOVA.

Corticosterone data were homogenous by a F max test ($p < 0.01$) and, thus, untransformed ANOVAs were used for statistical analysis (Sokal and Rohlf, 1995). Corticosterone concentrations among treatments for each time period were analyzed with a one-way ANOVA. Total comparisons among treatments for all time periods were analyzed with a two way ANOVA. Comparisons within a group between time periods were analyzed with a t test. All post hoc tests utilized a Fisher's PLSD. All statistics were calculated using Statview statistical package v. 5.0.1 (SAS institute, Cary, NC, USA, 1998). All data were compared statistically within years. We did not statistically compare data between years due to differences in outdoor temperatures, experimental timing, and sampling methods.

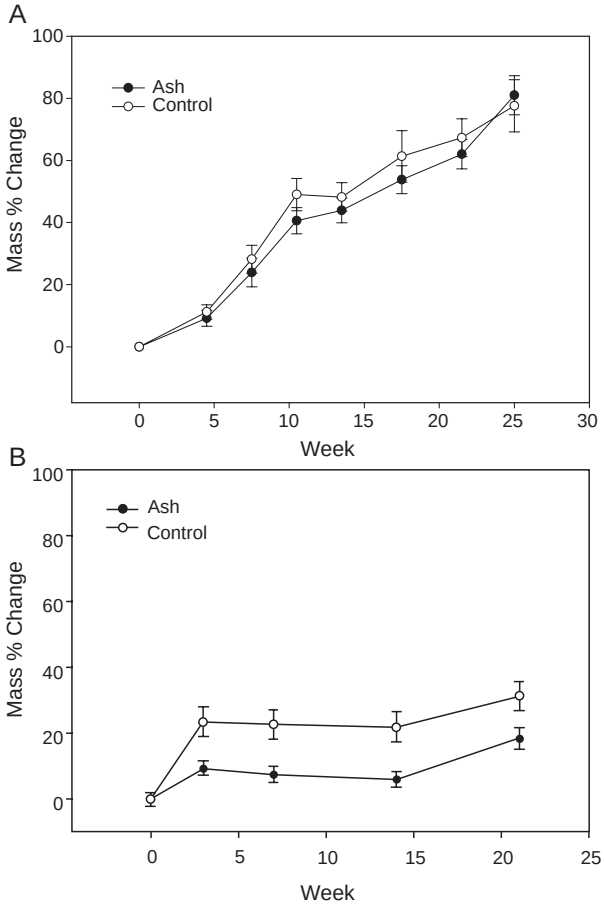

Fig. 1. Percentage increase in mass gain over time for 2001 (a) and 2002 (b). Ash animals ($n_{2001} = 24$, $n_{2002} = 77$) are represented by closed circles and control animals ($n_{2001} = 24$, $n_{2002} = 77$) are represented by open circles. Values are expressed as means ± 1 SE.

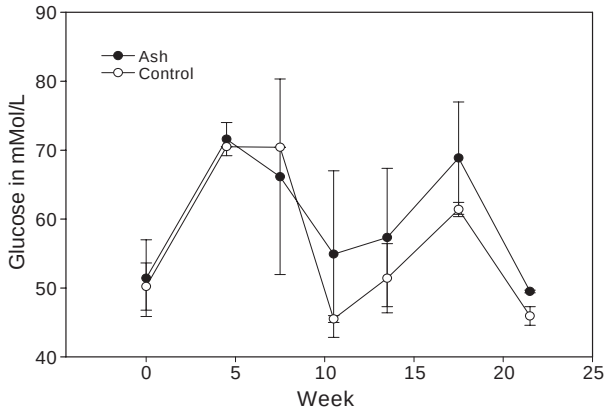

Fig. 2. Plasma glucose concentrations over time in mg/100 mL for 2001. Ash animals ($n = 24$) are represented by closed circles and control animals ($n = 24$) are represented by open circles. Values are expressed as means ± 1 SE.

3. Results

3.1. Mass

In 2001, mass at capture did not differ significantly between ash and control toads (ash $\bar{x} = 15.1 \pm 2.7$ g, control $\bar{x} = 15.0 \pm 2.5$ g, $p = 0.9$, $df = 42$, $F = 0.02$). In 2001, with increased food, control toads did not gain significantly more mass than ash animals after 24 wk of exposure. In October, ash toads had

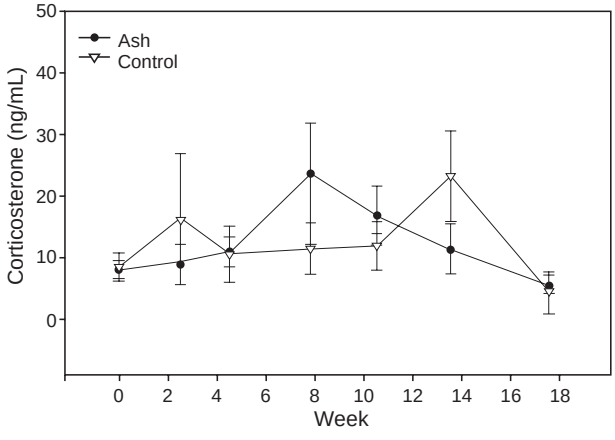

Fig. 3. Circulating corticosterone concentrations of male Southern Toads in 2001 moved from control (C) or ash (A) environments to control (C) or ash (A) mesocosms, A→A (closed circle) and C→C (open triangle). Values are expressed as means ± 1 SE.

increased in mass 81±6.3% while control toads increased 77.6 ±8.4% (p=0.5, df=43, F=0.05) (Fig. 1a).

In 2002, when food was limited to once every 10 d, percentage change in mass differed significantly between the two sediments. Control toads gained significantly more mass than ash animals over the 5 mo (31.3±4.4% and 18.3±3.3%, for control and ash respectively; p=0.007, df=392, F=4.9) (Fig. 2b) even though control toads (16.4±3.8 g) weighed significantly more than ash toads (14.1±2.4 g) at capture (p=0.0005, df=111, F=13.0).

3.2. Glucose

In 2001, glucose concentrations (mMol/L) did not differ between ash-exposed and control toads over the 5 mo of the experiment (p=0.6, df=5, F=0.8) (Fig. 2). Plasma glucose concentrations at capture also did not differ significantly (p=0.9, df=17, F=0.03).

3.3. Corticosterone

Plasma corticosterone concentrations did not differ between control ($\bar{x}$ =9.9±2.3 ng/mL) and ash ($\bar{x}$ =12.5±3.4 ng/mL) sites at capture (p=0.32, df=34, F=0.98). Corticosterone concentrations in ash and control exposed toads did not significantly differ for any time point during the experiment (p-values ranged from 0.13 to 0.99, df=12, F-values ranged from 0.005 to 1.08) (Fig. 3).

3.4. Standard metabolic rate

In August 2003, neither $\dot{V}_{O_2}$ (p=0.8, F=0.06, df=37) nor $\dot{V}_{CO_2}$ (p= 0.8, F= 0.06, df=37) differed significantly between control ($\dot{V}_{O_2}$, $\bar{x}$ =0.114±0.016 mL g^{-1} h^{-1}) and ash exposed toads ($\dot{V}_{O_2}$, $\bar{x}$ =0.109±0.013 mL g^{-1} h^{-1}). RQ ($\bar{x}_{control}$= 0.793±0.044, $\bar{x}_{ash}$=0.780±0.038, p=0.8, F=0.05, df=37) also did not differ significantly after 3 mo of exposure to coal fly ash.

3.5. Exercise metabolism

$\dot{V}_{O_2}$ resting metabolic rate (RMR, $\bar{x}_{control}$=0.22±0.03 mL g^{-1} h^{-1} ranging from 0.081 to 0.32 mL g^{-1} h^{-1}, $\bar{x}_{ash}$=0.13± 0.04 ranging from 0.032 to 0.27 mL g^{-1} h^{-1}), maximum walking metabolic rate (WMR, $\bar{x}_{control}$=0.53±0.06 mL g^{-1} h^{-1} ranging from 0.26 to 0.85 mL g^{-1} h^{-1}, $\bar{x}_{ash}$=0.44±0.04 ranging from 0.26 to 0.73 mL g^{-1} h^{-1}), and post-walking metabolic rate (PMR, $\bar{x}_{control}$=0.090±0.03 mL g^{-1} h^{-1} ranging from 0.007 to 0.18 mL g^{-1} h^{-1}, $\bar{x}_{ash}$=0.082±0.01 ranging from 0.037 to 0.153 mL g^{-1} h^{-1}) were compared between both groups during the fourth week of exposure. Ash animals and control animals did not differ significantly during WMR, PMR, and RMR for oxygen consumption (Table 1). RMR and PMR did not differ significantly in either group (ash: p=0.9, control: p=0.3); however RMR differed significantly from WMR in both groups (ash: p=0.007, control: p=0.14) (Fig. 4 illustrates a typical exercise O_2 recording).

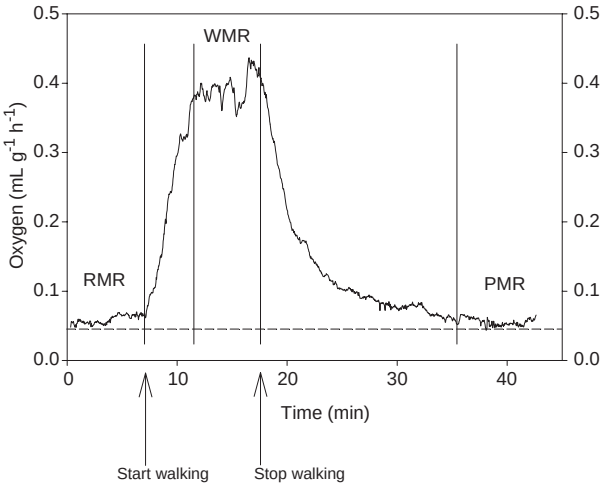

Fig. 4. Representative metabolic profile for the oxygen consumption of one toad. Graph represent resting metabolism (RMR), increase in oxygen consumption, maximum walking metabolic rate (WMR), decrease in oxygen consumption, and post-walking metabolic rate (PMR). Data for a 24.51 g toad recorded at 749 mm Hg, 23 °C, and 250 mL/min air flow.

The RQs for the exercise metabolic rates were also calculated. There was no significant difference in the RQ of RMR (p=0.83) or WMR (p=0.44) of both treatment groups. The RQ for PMR differed significantly (p=0.02) between the treatment groups (Table 2).

The rate of increase of $\dot{V}_{O_2}$ during exercise up to WMR ($slope_{control}$=0.096±0.03, $slope_{ash}$=0.107±0.021) and the rate of decrease to PMR ($slope_{control}$=0.042±0.007, slope $\bar{x}_{ash}$= 0.03±0.013) did not differ between ash and control groups (acceleration: p=0.78, df=9, F=0.08, deceleration: p=0.46, df=9, F=0.58).

3.6. Liver and muscle mass

The proportion of organ-to-body mass between ash exposed and control toads for either liver or muscle in 2001 or 2002 did not differ significantly (Table 3). However, liver and muscle

Table 1
Resting metabolic rate (RMR), walking metabolic rate (WMR), and post-walking metabolic rate (PMR), for ash and control exposed toads in 2002

Metabolic rate	Ash (mL O_2 g^{-1} h^{-1})	Control (mL O_2 g^{-1} h^{-1})	p	df	F
RMR	0.13±0.04	0.22±0.03	0.123	9	2.91
WMR	0.50±0.07	0.58±0.20	0.467	9	0.576
PMR	0.08±0.01	0.09±0.03	0.533	9	0.438

All means are expressed in mL O_2 g^{-1} h^{-1}±1 SE.

Table 2
Respiratory quotients (RQ) for resting metabolic rate (RMR), walking metabolic rate (WMR), and post-walking metabolic rate (PMR), for ash and control exposed toads in 2002

RQ	Ash	Control	p	df	t
RMR	0.79±0.17	0.75±0.12	0.83	9	0.22
WMR	0.79±0.07	0.84±0.05	0.44	9	−0.82
PMR	0.58±0.03	1.01±0.18	0.02	6	−3.09

All means are expressed±1 SE.

Table 3
Liver and quadriceps muscle mass for ash and control exposed toads in 2001 and 2002

Year	Tissue	Ash	Control	p	df	F
		% body mass	% body mass			
2001	Liver	5.20±0.23	5.26±0.22	0.87	43	0.03
	Muscle	1.34±0.04	1.35±0.02	0.85	43	0.04
2002	Liver	4.02±0.35	4.36±0.37	0.54	26	0.008
	Muscle	1.04±0.06	1.18±0.09	0.17	26	2.0

All means are expressed as percent total body mass±1 SE.

masses as percentage of body mass between years ($p=0.03$, $df=26$, $F=5.1$, for both liver and muscle) did differ significantly.

4. Discussion

When food was limited (2002), toads exposed to ash gained significantly less mass than control toads (Fig. 1). Many studies show that animals exposed to either a single or a mixture of trace metals exhibit mass loss or a decreased rate of mass gain (Vijayan and Leatherland, 1988; Camardese et al., 1990; Clark et al., 1994; Wilson et al., 1994). There are two hypotheses as to why mass decreases or growth slows. One is that animals exposed to ash experience increases in energetic costs, specifically, an increase in standard metabolic rate (Calow, 1991; Hopkins et al., 1999a; Rowe, 1998). The second hypothesis is that toads exposed to ash exhibit a decrease in appetite and increased anorexic behavior or food aversion (Wilson et al., 1994).

Studies that examined standard metabolic rates of animals exposed to trace metals report increased standard metabolic rates (from 18% to 40% increase in oxygen consumption) [e.g., freshwater shrimp (*Palaemonetes paludosus*) (Rowe, 1998), bullfrog larva (*Rana catesbeiana*) (Rowe et al., 1998), banded water snakes (*Nerodia fasciata*) (Hopkins et al., 1999a) and rainbow trout (*Oncorhynchus mykiss*) (McGreer et al., 2000)]. An increase in standard metabolic rate could be attributed to an increase in production of protective proteins, such as metallothionein or heat shock proteins (Calow, 1991), changes in ion balances and hydration (Gatten, 1987), or changes in metabolically active hormone levels (Vijayan, 1997).

The parameter most often measured in studies of animals exposed to contaminants is standard metabolic rate. In our study, however, we did not observe a difference in standard metabolic rate in ash exposed verses control toads. It is possible that our methods did not provide the sensitivity necessary to measure a slight difference in standard metabolic rate. However, published values of changes in standard metabolic rate in other organisms were ≥18 and our equipment can easily detect changes of this magnitude. Rather, our values were consistent with those of other studies on non-exposed southern toads. For example, the mean standard metabolic rate measured for all toads was 0.111 mL O_2 g^{-1} h^{-1} at 23 °C, which is virtually identical to the average standard metabolic rate of southern toad (0.113 mL O_2 g^{-1} h^{-1}) at the same temperature (Hutchinson et al., 1968). Therefore, it is much more likely that the toads in our study were not experiencing changes in standard metabolic rate.

We also did not find any significant difference in walking metabolic rate between animals exposed to different treatments. Published measurements for maximum oxygen consumption during exercise in southern toads do not exist. Exercise measure studies are available for other *Bufo* species: *B. americanus* [0.56 to l.l2 mL O_2 g^{-1} h^{-1} (Taigen and Beuchat, 1984; Taigen et al., 1982; Taigen and Pough, 1981; Wells and Taigen, 1984; Gatten, 1987)] and *B. woodhousii* [1.09 to 1.21 mL O_2 g^{-1} h^{-1} (Sullivan and Walsberg, 1985; Walsberg, 1986; Walton, 1988; Walton and Anderson, 1988) which are similar in mass (10–20 g) to southern toads. Our average WMR value was 0.54 mL O_2 g^{-1} h^{-1}, which is slightly lower than the published results for toads in this group, but approximately double their SMR. It is possible that the difference is due to differences in mass scaling, where the amount of energy required per gram of toad or the speed at which the toads were walked differed [see Gatten et al. (1992) for review of amphibian metabolism]. We did however detect a significant difference in RQ after exercise. Toads exposed to ash had an RQ of 0.58±0.32 which indicates protein digestion, while control toads had an RQ of 1.01±0.18 which is indicative of carbohydrate metabolism. It is likely that toads exposed to ash in 2002, because they gained significantly less mass than control toads, do not have similar glycogen stores as control toads. Therefore with prolonged exercise, while control toads are mobilizing and metabolizing glycogen sugar stores, ash toads are forced to resort to breaking down protein for energy. This would have long-term performance and physiological consequences over time, especially during breeding and emigration from the breeding area when energy demands are high.

Organisms exposed to toxicants also experience an increase in plasma concentrations of the metabolic-stress hormone, corticosterone (Hopkins et al., 1997). Corticosterone changes metabolic parameters such as mobilization of glycogen stores in the liver and can increase standard metabolic rate (Nelson, 2000). Toads in this experiment did not experience significant changes in corticosterone as a result of coal fly ash exposure (Fig. 3) (Ward and Mendonça, submitted). Our data also indicates that neither standard metabolic rate, nor metabolic rate during exercise as a result of exposure, increased. However, our data (i.e., differing increases in mass and differing RQs during exercise) do suggest a difference in the amount of available energy in ash and control toads.

In the field, however, toads experience a different corticosterone profile. Toads collected in coal fly ash basins experience a prolonged elevation in corticosterone (up to 70 ng/mL) greater than toads from control environments (10 ng/mL) (Hopkins et al., 1997). It is likely that the difference in corticosterone levels between field collected and laboratory-housed animals is due to the availability of food. Toads in the field have to expend energy to seek and catch prey, which could exacerbate physiological effects of toxicants, while toads housed in the laboratory are able to eat until satiated (even when food is limited to once every 10 d). This disparity in food availability and the energy required to capture food is most likely the cause of the mass increase in toads in laboratory experiments and probably contributes to the negative results observed in laboratory metabolic experiments.

We do not have any empirical data to support the hypothesis that toads exposed to ash exhibit a decrease in appetite and increased anorexic behavior, but numerous other studies have reported that stress can reduce feeding (Vijayan and Leatherland, 1988; Camardese et al., 1990; Woo et al., 1993; Wilson et al., 1994). Our data, however, do not indicate any significant difference in blood glucose levels between ash and control toads in 2001. If one group of toads was eating more than another, it is likely that glucose concentrations would be increased in the good eaters or that, the good eaters would have a larger liver, or greater muscle mass, none of which were observed. It is also interesting to note that in 2002, when food was more limited, toads had significantly smaller livers and less muscle mass per gram of toad than 2001 toads. Another possible explanation for the differences we observed in weight gain is the role metals may have on digestive efficiencies. Metals may decrease the amount of nutrients absorbed in the intestines, which would lead to a loss of mass, or a decrease in mass gain. This may also explain why weight gain and PMR–RQ differed in 2002. Toads exposed to ash, although consuming the same amount of food, could be less able to absorb nutrients through the gut (Chang, 1996). In 2001 toads may have been able to consume enough food to mask this effect.

No change in metabolic rate of laboratory-housed animals exposed to toxicants is a common result when food is unlimited (De Boek et al., 2001; Hopkins et al., 2002a; Kramarz and Kafel, 2003). However, when standard metabolic rate is measured in animals collected from the field, where trace metals are elevated, exposed and unexposed individuals often differ significantly (Hopkins et al., 1999a; Rowe, 1998; Rowe et al., 1998). For example, Hopkins et al. (2004) determined standard metabolic rates of field collected banded water snakes (*N. fasciata*) from the Savannah River Site ash basins and found that snakes from the ash basins had significantly greater standard metabolic rates (32%) compared with controls. However, when Hopkins et al. (2002b) raised neonate *N. fasciata* on an ash-contaminated diet in the laboratory, metabolic rate between groups did not differ significantly. It is possible differences in standard metabolic rates only become apparent when food is limited or if an animal has to actively search for prey. Our data support this hypothesis. In the second year, when food was more limited (feeding once every 10 d vs. once a week), the difference in mass gain between the two groups was much greater (Figs. 1 and 2). Hopkins et al. (2002b) also found that growth, survival, and metabolic effects were more pronounced in ash-exposed fish when food was limited.

It is clear from these data and the myriad of responses found in other studies that more research needs to be done on how exposure to toxicants affects energy balance. This study also highlights the point that care needs to be taken when interpreting results of metabolic studies on laboratory maintained animals. The amount of food provided to captive animals and the low amount of energy these animals expend to capture their provided prey may very likely mask subtle changes in metabolic measures.

Food and its availability are important factors in how an animal responds metabolically to a toxicant. Other important factors to consider are the type of toxicant and how an animal reacts to it. For example, metals stimulate the production of metallothionein and heat shock proteins and some pesticides stimulate p450 enzyme production, all of which can be energetically costly (Calow, 1991). The compartmentalization, storage, and excretion of toxicants may also have a cost (Calow, 1991). Metal ions may also alter digestive uptake of nutrients and water balance. Understanding the energetic cost of toxicant exposure and how this change in energetic cost affects other facets of physiology and behavior may elucidate other potential costs associated with exposure. If less energy is available, food demands may increase and make an animal more vulnerable to predation by increasing foraging time.

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References

AWIS, 2005. AWIS Weather Service. AWIS <http://www.awis.com> Accessed 2005 April 20.

Calow, P., 1991. Physiological costs of combating chemical toxicants: ecological implications. Comp. Biochem. Physiol. C 100, 3–6.

Camardese, M.B., Hoffman, D.J., LeCaptain, L.J., Pendelton, G.W., 1990. Effects of arsenate on growth and physiology in mallard ducklings. Environ. Toxicol. Chem. 9, 785–795.

Carey, C., Bryant, C.J., 1995. Possible interrelations among environmental toxicants, amphibian development and decline of amphibian populations. Environ. Health Perspect. 103, 13–18.

Chang, L.W., 1996. Toxicology of Metals. Lewis Publishers, Boca Raton.

Chard, T., 1995. An Introduction to Radioimmunoassay and Related Techniques. Elsevier, New York.

Cherry, D.S., Gutherie, R.K., 1977. Toxic metals in surface waters from coal ash. Water Resour. Bull. 13, 1227–1236.

Clark, J.T., Jimenez, B., Evans, S.L., Barrow, R., Winfree, M., Mortek, J.J., 1994. Cadmium-induced sexual dysfunction does not involve increased hepatic metabolism of testosterone nor increased circulating levels of corticosterone. Physiol. Behav. 56, 975–981.

De Boek, G., Vlaeminck, A., Balm, R.H.M., Lock, R.A.C., De Wachter, B., Blust, R., 2001. Morphological and metabolic changes in common carp, *Cyprinus carpio*, during short-term copper exposure: interactions between Cu^{2+} and plasma cortisol elevation. Environ. Toxicol. Chem. 20, 374–381.

EPA, U. S., 1997. Profile of the Fossil Fuel Electric Power Generation Industry. EPA Office of Compliance Sector Notebook Project.

Gatten, R.E., 1987. Activity metabolism of anuran amphibians: tolerance to dehydration. Physiol. Zool. 60, 576–585.

Gatten, R.E., Miller, K., Full, R., 1992. Energetics at rest and during locomotion. In: Feder, M.E., Burggren, W.W. (Eds.), Environmental Physiology of the Amphibians. University of Chicago Press, Chicago, pp. 314–377.

Gutherie, R.K., Cherry, D.S., 1979. Trophic level accumulation of heavy metals in a coal ash basin drainage system. Water Resour. Bull. 15, 244–248.

Hopkins, W.A., Mendonça, M.T., Congdon, J.D., 1997. Increased circulating levels of testosterone and corticosterone in southern toads, *Bufo terrestris*, exposed to coal combustion waste. Gen. Comp. Endocrinol. 108, 237–246.

Hopkins, W.A., Congdon, J.D., Rowe, C.L., Mendonça, M.T., 1998. Elevated trace element concentrations in southern toads, *Bufo terrestris*, exposed to coal combustion waste. Arch. Environ. Contam. Toxicol. 35, 325–329.

Hopkins, W.A., Rowe, C.L., Congdon, J.D., 1999a. Elevated trace element concentrations and standard metabolic rate in banded water snakes (*Nerodia fasciata)* exposed to coal combustion wastes. Environ. Toxicol. Chem. 18, 1258–1263.

Hopkins, W.A., Mendonça, M.T., Congdon, J.D., 1999b. Responsiveness of the hypothalamo–pituitary–interrenal axis in an amphibian (*Bufo terrestris*) exposed to coal combustion wastes. Comp. Biochem. Physiol. C 122, 191–196.

Hopkins, W.A., Roe, J.H., Snodgrass, J.W., Staub, B.P., Jackson, B.P., Congdon, J.D., 2002a. Effects of chronic dietary exposure to trace elements on banded water snakes (*Nerodia fasciata*). Environ. Toxicol. Chem. 21, 906–913.

Hopkins, W.A., Snodgras, J.W., Roe, J.H., Staub, B.P., Jackson, B.P., Congdon, J.D., 2002b. Effects of food ration on survival and sublethal responses of lake chubsuckers (*Erimyzon sucetta*) exposed to coal combustion wasters. Aquat. Toxicol. 57, 191–202.

Hopkins, W.A., Roe, J.H., Philippi, T., Congdon, J.D., 2004. Standard and digestive metabolism in the banded water snake, *Nerodia fasciata fasciata*. Comp. Biochem. Physiol. A 137, 141–149.

Hutchinson, V.H., Whitford, W.G., Kohl, M.A., 1968. The relation of body size and surface area to gas exchange in anurans. Physiol. Zool. 41, 65–85.

Kramarz, P., Kafel, A., 2003. The respiration rate of the beet armyworm pupae (*Spodoptera exigua*) after multi-generation intoxication with cadmium and zinc. Environ. Pollut. 126, 1–3.

Lighton, 1991. Insects: measurements. In: Payne, P.A. (Ed.), Concise Encyclopedia of Biological and Biomedical Measurement Systems. Pergamon Press, New York, pp. 201–208.

McGreer, J.C., Szebedinsky, C., McDonald, D.G., Wood, C.M., 2000. Effects of chronic sublethal exposure to waterborne Cu, Cd, or Zn in rainbow trout. 1: iono-regulatory disturbance and metabolic costs. Aquat. Toxicol. 50, 231–243.

Mendonça, M.T., Chernetsky, S.D., Nester, K.E., Gardner, G.L., 1996. Effects of gonadal sex steroids on sexual behavior in the big brown bat, *Eptesicus fuscus*, upon arousal from hibernation. Horm. Behav. 30, 153–161.

Nelson, R.J., 2000. Stress. An Introduction to Behavioral Endocrinology. Sinauer Associates, Inc., Saunderland, pp. 557–592.

Rowe, C.L., 1998. Elevated standard metabolic rate in a freshwater shrimp (*Palaemonetes paludosus*) exposed to trace element-rich coal combustion waste. Comp. Biochem. Physiol., A 121, 299–304.

Rowe, C.L., Kinney, O.M., Nagle, R.D., Congdon, J.D., 1998. Elevated maintenance costs in an Anuran (*Rana catesbeiana*) exposed to a mixture of trace elements during embryonic and early larval periods. Physiol. Zool. 71, 27–35.

Rowe, C.L., Hopkins, W.A., Coffman, V.R., 2001. Failed recruitment of southern toad (*Bufo terrestris*) in a trace element-contaminated breeding habitat: direct and indirect effects that may lead to a local population sink. Arch. Environ. Contam. Toxicol. 40, 399–405.

Seymour, R.S., 1973. Physiological correlates of forced activity and burrowing in the spadefoot toad, *Scaphiopus hammondii*. Copeia 1973, 103–115.

Sievert, L.M., Bailey, J.K., 2000. Specific dynamic action in the toad, *Bufo woodhousii*. Copeia 2000, 1076–1078.

Sokal, R.R., Rohlf, F.J., 1995. Biometry. W.H. Freeman and Company, New York.

Statview, Vers. 5.0.1. Statistical software. SAS Institute Inc., 1998. Cary, NC, USA.

Sullivan, B.K., Walsberg, G.E., 1985. Call rate and aerobic capacity in Woodhouse's toad (*Bufo woodhousei*). Herpetologica 41, 404–407.

Taigen, T.L., Beuchat, C.A., 1984. Anaerobic threshold of anuran amphibians. Physiol. Zool. 57, 641–647.

Taigen, T.L., Pough, F.H., 1981. Activity metabolism of the toad (*Bufo americanus*): ecological consequences of ontogenetic change. J. Comp. Physiol. 144 B, 247–252.

Taigen, T.L., Emerson, S.B., Pough, F.H., 1982. Ecological correlates of anuran exercise physiology. Oecologia 52, 49–56.

Vijayan, M., 1997. Metabolic responses associated with confinement stress in tilapia: the role of cortisol. Comp. Biochem. Physiol. 116C, 89–95.

Vijayan, M.M., Leatherland, J.F., 1988. Effects of stocking density on the growth and stress-response in brook charr, *Salvelinus fontinalis*. Aquaculture 75, 159–170.

Vogt, J.T., Appel, A.G., 1999. Standard metabolic rate of the fire ant, *Solenopsis invicta* Buren: effects of temperature, mass and caste. J. Insect Physiol. 45, 655–666.

Walsberg, G.E., 1986. Comparison of two techniques for estimating the maximum aerobic capacity of amphibians. Herpetologica 42, 389–394.

Walton, M., 1988. Relationships among metabolic, locomotory, and field measures of organismal performance in the Fowler's toad (*Bufo woodhousei fowleri*). Physiol. Zool. 61, 107–118.

Walton, M., Anderson, B.D., 1988. The aerobic cost of saltatory locomotion in the Fowler's toad (*Bufo woodhousei fowleri*). J. Exp. Biol. 136, 273–288.

Wells, K., Taigen, T.L., 1984. Reproductive behavior and aerobic capacities of male American toads (*Bufo americanus*): is behavior constrained by physiology? Herpetologica 40, 292–298.

Wilson, R.W., Bergman, H.L., Wood, C.M., 1994. Metabolic costs and physiological consequences of acclimation to aluminum in juvenile rainbow trout (*Oncorhynchus mykiss*). 1: acclimation specificity, resting physiology, feeding and growth. Can. J. Fish. Aquat. Sci. 51, 527–535.

Woo, P.T.K., Sin, Y.M., Wong, M.K., 1993. The effects of short-term acute cadmium exposure on blue tilapia, *Oreochromis aureus*. Environ. Biol. Fish. 37, 67–74.