Tadpoles, Temperatures, and Toxicants: Understanding Patterns of Growth and Development in an Era of Warming Climate and Persistent Organic Pollutants

By

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THESIS ABSTRACT

Declines in amphibian populations result from many stressors often acting synergistically. Thus, understanding the interplay between environmental contaminants and warming climates on amphibians was my research focus. Exposure of biota to contaminants is mediated by kinetics of uptake, biotransformation, and elimination, which are temperature dependent. However, no study to date had focused on toxicokinetics of contaminants, in an amphibian, at multiple temperatures. Thus, Chapter 1 measured PCB and PBDE elimination rates in *Lithobates pipiens* tadpoles reared at two temperatures. We exposed tadpoles to diets containing either PCBs or PBDEs at different temperatures during an accumulation phase, followed by an elimination phase where tadpoles were fed food sans contaminants. Both PCB and PBDE were eliminated faster at higher temperatures. Tissue concentrations of PCBs and PBDEs were linearly related to dietary concentrations as expected for first order kinetics, with no effect of rearing temperature. Lack of an effect of rearing temperature on tissue residue levels suggests faster elimination at warmer temperature was approximately balanced by faster uptake. We propose that temperature

dependence of elimination is similar to that for uptake, and thus exposure to toxicants may change very little in tadpoles living at different temperatures.

Depression of growth due to PBDE exposure has been documented in birds, mammals, amphibians, and fish. Yet, there are no studies to our knowledge, of how PBDE negatively influences growth and development of tadpoles under different temperature regimes. Therefore, in Chapter 2 we used a simple energy budget to address hypotheses regarding effects of PBDEs on growth in leopard frog tadpoles; namely that reductions in growth could be linked to either increased respiratory costs, reductions in digestive performance, or differences in body composition. Our results indicated that reductions in feeding rate were responsible for retarded growth rates in PBDE exposed tadpoles.

We also developed and validated an inert marker method to assess digestive performance of leopard frog tadpoles that compares well with dry matter digestibility based on measurement of food intake and excreta production (Appendix 1), and we documented for the first time changes in an ectotherm's gut microbial community at two different rearing temperatures (Appendix 2).

GENERAL INTRODUCTION

Amphibian declines

Amphibian populations have been in declining globally for decades, capturing the attention of the public and stimulating scientific research into the causative reasons behind said decline (Biek et al., 2002; Blaustein & Kiesecker, 2002). Not the least of these are effects resulting from our changing climate. Climate change has been implicated in many deleterious effects on amphibians, including altering breeding timing (Blaustein et al., 2001), the incidence of disease outbreaks (Bosch et al., 2007), and survivorship and reproductive success (Reading, 2007). Additionally, climate change rarely acts alone in influencing amphibian health, which calls for increased studies regarding the effects of multiple stressors

(Hof et al, 2011). Pollutants are a leading cause of declines here in the Midwestern United States, second only to habitat loss and degradation (IUCN 2011). Thus, understanding the interplay between environmental contaminants and changing climates on amphibians is an area desperately in need of research (Landis et al., 2014; Noyes et al., 2009). The collected chapters in this dissertation advance physiological knowledge about digestion, energetics, and toxicology of a common frog species reared at different temperatures.

Choice of study species, Lithobates pipiens

Choice of study species - We focus on the northern leopard frog, *Lithobates pipiens* they are common in the Great Lakes region and because their populations have been declining in recent years (personal obs.) (Mossman et al., 1998). Additionally, we have considerable experience working with *L. pipiens* and are able to rear them in a laboratory setting where they thrive (Gleason et al., 2016). Additionally, *L.* pipiens have been an important wild species in frog ecotoxicology and physiological research (e.g., (T. Cary & Karasov, 2012; Freitas, Brown et al., 2016; Kohl et al., 2015)).

Choice of toxicants

Choice of PBDEs –We chose a purified pentabromodiphenyl mixture that is a commercial formulation used in the U.S. with the trade name DE-71™. We chose this formulation as it is representative of congeners measured in Great Lakes biota samples (Stapleton & Baker, 2003), and has been measured in Lake Michigan plankton in levels as high as 0.72 ug/g lipid (Kuo et al., 2010). The major BDE congeners are as follows: BDE-47 (30.8%), BDE-99 (48.1%), BDE-100 (8.8%), BDE-153 (6.6%), BDE-154 (4.4%) (Rayne & Ikonomou, 2002). The congeners present in the DE-71 mix have different structural configurations as well as levels of bromination, which should afford a range of toxicities. Additionally, previous studies done in our laboratory have used the same commercial mix, which allowed for further comparisons between studies (Cary Coyle & Karasov, 2010; T. L. Cary & Karasov, 2013; T. L. Cary, et al., 2014).

Choice of PCBs –To study the temperature dependence of PCB exposure kinetics (rates of uptake and elimination; bioaccumulation; Chapter 1) we chose a PCB mixture of non-coplanar PCB-70 and coplanar PCB-126 (AccuStandard®, New Haven, CT) because they likely act via different pathways (Huang & Karasov, 2000; Jofré & Karasov, 2008), and could exhibit different elimination rates. In a study done on green frogs and leopard frogs, elimination rates of different PCB congeners differed by over an order of magnitude (Leney et al., 2006).

Chapter 1

In a review of 66 species of freshwater animals (Mayer & Ellersieck, 1986), acute toxicity of contaminants generally increased with increased temperature. Exposure of biota to contaminants is mediated by the kinetics of toxicant uptake, biotransformation, and elimination, all three of which are temperature dependent. However, no study (to our knowledge) had been done focusing on the toxicokinetics of a contaminant, in an amphibian, with respect to the increased temperatures associated with climate change. Thus, the objective of chapter one was to establish the temperature dependence of PCB and PBDE elimination rates in *L. pipiens* tadpoles. We first exposed free-swimming *L. pipiens* tadpoles for 14 days to foods containing either PCBs or PBDEs at different temperatures during an accumulation phase, followed by an elimination phase where tadpoles were not fed contaminants. Both PCB and PBDE were eliminated faster at higher temperatures, as expected. For both PCBs and PBDEs, tissue concentrations of exposed tadpoles were linearly related to dietary concentrations as expected for first order kinetics, with no significant effect of rearing temperature.

Chapter 2

Depression of growth due to PBDE exposure has been documented in birds, mammals, amphibians, and fish at single temperatures (Cary Coyle & Karasov, 2010; Chen et al., 2010; Fernie et al., 2006; Viberg et al., 2008). Yet, until our work in chapter 2, there were no studies, to our knowledge, of how PBDE negatively influences growth and development of tadpoles under different temperature regimes. Therefore, we used a simple energy budget to address hypotheses regarding effects of PBDEs on growth in leopard frog tadpoles; namely that reductions in growth could be linked to either increased respiratory costs, reductions in digestive performance, or differences in body composition. Our results indicated that ultimately reductions in feeding rate were responsible for the retarded growth rates associated with PBDE exposed tadpoles.

Additional publications

In the appendices are two publications that I co-authored while pursuing my PhD at the University of Wisconsin-Madison. The first paper validates and develops methods to assess the digestive performance of leopard frog tadpoles reared on a common laboratory diet. We measured the apparent digestive efficiency and tested the use of putative inert markers to calculate digestibility. Ash content in digesta dissected from the distalmost portion of the tadpoles' intestine was compared with the ash content in food. Our results compared well with a dry matter digestibility value based on our direct measurement of food intake and excreta production, giving us confidence in our results and allowing us to use these methods to address hypotheses developed in chapter 2.

The final publication appended investigated the effects of environmental temperature on the gut microflora in tadpoles. Although, studies on gut microbial communities are increasing,

this was the first study, to our knowledge, that looked at changes in an ectotherm's gut microbial community at two different rearing temperatures.

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WARMER TEMPERATURE INCREASES TOXICOKINETIC ELIMINATION OF POLYCHLORINATED BIPHENYLS AND POLYBROMINATED DIPHENYL ETHERS IN NORTHERN LEOPARD FROG LARVAE (*Lithobates pipiens*)

ABSTRACT

The increased temperatures associated with climate change could result in increased impacts of persistent organic pollutants (POPs) on wildlife, but no study has been done that focuses on the toxicokinetics of a POP in an amphibian at different temperatures. We studied the temperature dependence of elimination rates of polychlorinated biphenyls (PCBs) and polybrominated diphenyl ethers (PBDEs) in leopard frog (*Lithobates pipiens*) tadpoles, and measured tissue concentrations after prolonged exposure at different temperatures. We first exposed free-swimming *L. pipiens* tadpoles for 14 days to foods containing either PCBs or PBDEs at different temperatures during an accumulation phase, and then during an elimination phase of 14 days we provided food sans toxicants and measured the decline of toxicants in their tissue. Following 14 days of accumulation mean tissue residue levels did not differ for tadpoles reared at 18 or 27°C. Both PCB and PBDE were eliminated faster at the higher temperature, as expected. Using Arrhenius' equation along with published data for tadpoles at 23°C, we calculated that the Q_{10} for PBDE elimination (rate change for 10°C change in temperature) was 3.3; a similar analysis for PCB elimination yielded a Q_{10} of 3.8. We next raised tadpoles a month or more at different temperatures on diets with either PBDE or PCBs, each at several different toxicant concentrations, and compared tissue concentrations as a function food concentration and rearing temperature. For both PCBs and PBDEs, tissue concentrations of exposed tadpoles were linearly related to dietary concentrations as expected for first order kinetics, with no significant effect of rearing temperature. The lack of an effect of rearing temperature on tissue residue levels

suggests that faster elimination at warmer temperature was approximately balanced by faster uptake. We propose that the Q_{10} for toxicant elimination is similar to that for toxicant uptake, and thus exposure to toxicants (indexed by tissue residue levels) may change very little in tadpoles living at different temperatures.

INTRODUCTION

Declining amphibian populations have stimulated research seeking to identify their causative factors, which include climate change (Alford & Richards, 1999; Collins & Storfer, 2003; Houlahan, Findlay, Schmidt, Meyer, & Kuzmin, 2000). Climate change has been implicated in many deleterious effects on amphibians, including alterations in breeding timing (Blaustein et al., 2001), the incidence of disease outbreaks (Bosch, Carrascal, Duran, Walker, & Fisher, 2007), and reduced survivorship and reproductive success (Reading, 2007). Recognition that climate change may not act alone in influencing amphibian health has stimulated research regarding the effects of multiple stressors (Hof, Araujo, Jetz, & Rahbek, 2011). Pollutants are a leading cause of amphibian declines in midwestern North America, second only to habitat loss and degradation (IUCN 2011). Thus, understanding the interplay between environmental contaminants and changing climates on amphibians is an area greatly in need of research (Landis et al., 2014; Noyes et al., 2009).

Persistent organic pollutants, or POPs are a class of pollutants that is a global concern due to their abilities to move readily via wind and water, resist environmental degradation, and bioaccumulate in organisms and biomagnify in food chains (EPA 2009). Two POPs of particular concern are: polychlorinated biphenyls (PCBs) and polybrominated diphenyl ethers (PBDEs), both of which are lipophilic compounds with high octanol-water partition coefficients (log K_{ow} values between 5 and 10; (Cary Coyle & Karasov, 2010; Leney, Drouillard, & Haffner, 2006)).

Although the industrial and other uses of these compounds has declined as regulatory restrictions have increased, their global distribution and persistence in the environment creates an ongoing need for environmental monitoring of these compounds. Both are routinely found in biota of the Great Lakes watershed and are present at levels potentially harmful to wildlife (Sullivan & Delfino, 1982).

The increased temperatures associated with climate change could result in increased impacts of POPs on wildlife (Noyes et al., 2009). In a review of 66 species of freshwater animals (Mayer 1986), acute toxicity of contaminants generally increased with increased temperature. The underlying mechanism(s) are unclear but might involve either or both increased exposure at warmer temperatures or increased target site sensitivity. Exposure of biota to contaminants is mediated by the kinetics of toxicant uptake, biotransformation, and elimination, all three of which are temperature dependent. Increased temperatures are known generally to increase physiological rates (McDiarmid & Altig, 2000). Bioenergetic rates of metabolism, food intake, digestion and elimination all link to rates of uptake and elimination of toxicants that are taken up in food. In a study done on amphipods in the Great Lakes, temperature was positively correlated with rates of uptake and elimination of PCBs (Landrum, 1988). However, no study (to our knowledge) has been done that focuses on the toxicokinetics of a POP in an amphibian with respect to the increased temperatures associated with climate change.

Therefore, an objective of the present study was to establish the temperature dependence of PCB and PBDE elimination rates in *L. pipiens* tadpoles. Our *a priori* expectation for the effect of temperature was based on the common observation that many physiological rates increase according to a temperature coefficient Q_{10} of 2 to 3 (i.e., a rate increase by a factor of 2-3 for each 10-degree Celsius increase in temperature) up until some maximal rate, after which rates

and function decline at even higher temperatures (Hill & Wyse, 1989). In order to determine the elimination rates of PCBs and PBDEs we first exposed free-swimming *L. pipiens* tadpoles for 14 days to foods containing either PCBs or PBDEs at different temperatures during an accumulation phase of the experiment (Fig. 1A). Then, during an elimination phase of 14 days we provided food sans toxicants and measured the rate of decline in their levels of toxicants (Fig. 1A). Based on a model assuming a single compartment and first order elimination kinetics (see Methods), as shown previously for PBDEs and PCBs in tadpoles (Tawnya L. Cary & Karasov, 2013; Leney, Balkwill, Drouillard, & Haffner, 2006), we predicted faster elimination at warmer temperature, assuming a typical Q_{10} in the range of 2 to 3.

A second objective of the present study was to relate the toxicant elimination rates, and their temperature dependence, to actual exposure differences, which we take to be the toxicant tissue concentrations. We raised tadpoles a month or more at different temperatures on diets with different toxicant concentrations and compared tissue concentrations as a function food concentration and rearing temperature. Tissue concentrations could be fairly independent of rearing temperature if warmer temperature increases the rate of uptake to the same extent as it increases the rate of elimination, as in Fig. 1B or in Fig. 1C, the latter offers an alternative way to depict tissue toxicant concentration when kinetics are first order (Tawnya L. Cary & Karasov, 2013; Gibaldi & Perrier, 1982). Alternatively, tissue concentrations could vary with rearing temperature if temperature change has a different effect on the kinetics of toxicant uptake than on the kinetics of toxicant elimination (as in Figures 1E or 1F; see also (Honkanen & Kukkonen, 2006) for an empirical example of this). Our null hypothesis was that the Q_{10} for uptake would be similar to that for elimination, resulting in data depictions like those in Figures 1B and 1C rather than 1E and 1F. Results from this study will help shed light on the temperature

dependence of exposure kinetics that could result from the increasing temperatures projected to occur in climate change scenarios.

METHODS

Choice of toxicants, temperatures, and interpretive model

We used a commercial mixture of PBDEs (Great Lakes mixture DE-71™; Wellington Laboratories Inc., Ontario, Canada) to facilitate comparisons with our earlier studies with this mixture (Cary Coyle & Karasov, 2010; Tawnya L. Cary & Karasov, 2013). For PCBs, we chose to use a mixture of non-coplanar PCB-70 and coplanar PCB-126 (AccuStandard®, New Haven, CT) because they likely act via different pathways (Huang, Melancon, Jung, & Karasov, 1998; Jofré & Karasov, 2008). In addition, congeners in both mixtures could exhibit a range of elimination rates (Leney et al., 2006).

Our highest target test temperature (27 °C) was chosen to be close to the projected increase to 28˚C for water temperatures in the Great Lakes region of North America due to impacts of climate change (Veloz et al., 2012). Our lowest test temperature (18 °C) was chosen to maximize rate changes as a function of temperature difference, and because this water temperature occurs during early spring breeding of leopard frogs in Wisconsin (Mossman et al., 1998). However, based on results from our first experiments we decided that key rates at 18 °C were slower than desired and that the study's findings would be improved if subsequent experiments used 23 °C as the lower temperature.

The design, analysis and interpretation of this study are based on a few pharmacokinetic and thermodynamic principles that are best outlined at the outset. Our study assumes tissue toxicant concentration on any given day (C_t, ng/g mass) is modeled using an open, single-compartment model with first-order toxicant elimination (Ritschel, 1998):

$$C_t = (I \bullet C_{food} \bullet a) \bullet (1\text{-}e^{-k_e \bullet t})/(M * k_e) \quad \text{(eq. 1)}$$

where t is the day(s), I is a constant daily food intake rate (g wet mass/d), C_{food} is the toxicant content of the food (ng/g wet mass), a is the proportion of toxicant absorbed, M is body mass (g), and k_e, is the rate constant for elimination (d^{-1}). We believe this to be an appropriate model for tissue toxicant burden for several reasons. Previous studies have shown that elimination of both PCBs and PBDEs by tadpoles is best described by a single compartment and first order elimination kinetics (Tawnya L. Cary & Karasov, 2013; Leney, Drouillard, et al., 2006). PCB's and PBDE's have been shown to bioaccumulate in the sediment of Great Lakes waterways (Samara et al. 2006), where tadpoles tend to feed. Also, an earlier study in captivity found that PBDE congeners were not detected in water samples, which supports minimal leaching into the water and that the primary route of PBDE exposure to tadpoles was dietary (Cary Coyle & Karasov, 2010). Our model predicts that C_t will rise with increasing days eating toxicant-laden food up to an asymptotic value of steady state (C_{ss}) where rate of uptake is balanced out by the rate of elimination:

$$C_{ss} = (I \bullet C_{food} \bullet a)/(M * k_e) \quad \text{(eq. 2)}$$

If the animal is switched to a toxicant-free food, then C_t declines exponentially daily with e^{-k_e}.

The temperature dependence of these kinetics is modeled by increasing I and k_e by factors representative of how physiological rates increase with temperature. Our *a priori* expectation for the effect of temperature is based on the observation that many physiological rates increase according to a temperature coefficient Q_{10} of 2 to 3 (i.e., a rate increase by a factor of 2-3 for each 10-degree Celsius increase in temperature). However, Q_{10} can differ over different ranges of temperatures (Hochachka & Somero, 2002). Therefore, to integrate and interpret our experiments and results across the various temperature ranges, we used Arrhenius' equation,

which gives the dependence of rate constants for chemical reactions on the absolute temperature (K) (Laidler 1984).

In the following sections two types of experiments are described (Table 1). Toxicant elimination rate constants (k_e) were measured in experiments 1 & 2, where tadpoles were first fed diet with toxicant at a relatively high concentration for 14 days to achieve high tissue concentrations (accumulation phase in Fig. 1A), followed by 14 days of feeding sans toxicant (elimination phase) that would terminate with still measurable tissue concentrations (C_t). In experiments 3 & 4, we fed tadpoles for longer periods of time diets with environmentally relevant levels of toxicants to test for sub-chronic toxicity at several concentrations (results presented elsewhere), but the more prolonged exposure times (> 5 weeks) could yield higher tissue concentrations that are equal to or at least closer to C_{ss} (Fig. 1).

Animals, husbandry, and temperature control

Procedures for this study were approved by the UW-Madison College of Agricultural and Life Sciences Institutional Animal Care and Use Committee (Protocol number A01336). *Lithobates pipiens* embryos were purchased commercially from Nasco© (Fort Atkinson, WI) on the day fertilization took place (Gosner stage [GS] 1). Immediately upon arrival, embryos were randomly aliquoted into 92 0.5-L Nalgene containers (40 embryos/container) containing filtered, dechlorinated municipal water, and placed in temperature-controlled rooms at either 18°, 23°, or 27°C (±1°C). Water within the containers was changed daily to ensure proper dissolved oxygen content (all measures > 6 mg O_2/L) as well as to minimize bacterial and fungal growth; non-viable or dead embryos were removed immediately when found.

Once the embryos developed into free-swimming tadpoles (GS 25; approximately 5-8 d post fertilization [dpf]), they were transferred into 40 18.9-L glass aquaria (25 tadpoles/tank)

with air stones in 12 L of water in temperature-controlled racks. The racks had a system to circulate temperature-controlled water around each aquarium. By flowing temperature-controlled water around the aquaria, as well as maintaining a constant temperature in the animal rooms, we ensured that tadpoles were maintained at their target temperatures, 18°, 23°, and 27° C (+/- 1˚C). Static renewal of the aquaria water (> 80% water change) occurred every other day, and water quality was monitored weekly for pH, nitrites, ammonia, and dissolved oxygen according to IACUC's standards and were never found to be outside of an acceptable range for any of the aforementioned parameters (pH = 8 ± 0.2; nitrite < 1.0 mg/L; total NH_3 < 1mg/L; dissolved oxygen > 6.0 mg/L). Light/dark cycles were maintained at 14L/10D, via ambient florescent lighting from the ceiling as well as full spectrum light fixtures (Reptisun 5.0 UVB, ZOO MED Laboratories, Inc., San Luis Obispo, CA), which were suspended directly above the aquaria. Tadpoles were fed *ad libitum* a diet that consisted primarily of rabbit chow (Harlan Teklad, catalog 2030) suspended in gelatin/agarose mixture, prepared according to Cary Coyle et al.(Cary Coyle & Karasov, 2010). Wet mass of food provided daily was 20 - 25% of summed tadpole mass in the tank, and left over food and feces were syphoned every day prior to feeding. Tanks were checked every morning and any dead or metamorphosing (Gosner stage 42) tadpoles were removed from tanks and excluded from the study.

To create diets containing contaminants, specified concentrations of PCB-70, PCB-126, and DE-71™ in acetone were mixed with ground rabbit chow and stirred for 15 minutes to ensure proper adsorption of each toxicant into the chow. Control diet (0 ng DE-71/g) was prepared with the same volumes of toluene and acetone sans PCB and PBDE. The mixture was then thinly spread out in a fume hood overnight to allow the acetone to evaporate. The rabbit chow containing toxicants was then mixed with agarose, gelatin, and water and heated, stirring

continuously, until boiling for 1 minute. Then the mixture was cooled down to form a jelly-like consistency, similar to that of the control diet. The final diet contained: 18.5% rabbit chow, 1.5% agar, 1% gelatin and 79% water (see Gleason et al (Gleason, Yahn, & Karasov, 2016) for diet nutrient composition).

Experiments 1 and 2 to measure elimination rate constants

We designed these experiments, which ran concurrently, so that exposure and subsequent elimination would be completed before tadpoles entered metamorphosis (i.e., prior to Gosner stage 42), a developmental stage that could have a different elimination rate constant than the tadpole stage (Tawnya L. Cary & Karasov, 2013; Leney, Drouillard, et al., 2006). We also designed it to ensure exposure and elimination would occur when tadpoles were near their asymptotic size in order to minimize growth dilution (which is the reduction in tissue toxicant concentration due to accretion of new tissue rather than elimination of toxicant from tissue). Consequently, we fed the tadpoles diets without PCB or PBDE until the tadpoles approached their asymptotic size (45 dpf in 18°C room and 33 dpf in 27°C room), at which point their diets were switched to diets containing a PCB mixture (31 ng/g PCB-70 + 29 ng/g PCB-126; Accu Standard®,New Haven, CT) or PBDE mixture (753.9 ng/g DE-71™; a commercial mixture of PBDE congeners; Wellington Laboratories Inc., Ontario, Canada). The exposure dosages and duration (2 weeks) were chosen in order to yield a measurable accumulation of toxicant yet not cause any large toxicological consequences (*personal communication with Cary, TL*).

After two weeks of dietary exposure, tadpoles were switched back to the diet sans toxicant for two weeks to allow for toxicant elimination. Measures of tissue toxicant concentrations and elimination rates are described in subsequent sections.

Experiments 3 and 4 to measure C_t close to C_{ss}

Experiment 3 using PBDE was done concurrently with experiments 1 and 2 whereas experiment 4 using PCBs was performed 12 months later. In experiment 3, tadpoles were fed diets containing different concentrations of PBDE (6.11, 16.81, 39.9, or 81.96 ng/g DE-71™) and reared at either 18 or 27 °C. In experiment 4 tadpoles were reared at 23 or 28 °C and were fed different concentrations of single PCB congeners (25 or 170 ng/g PCB-70 or 3.5 or 7 ng/g PCB-126). Exposure levels were chosen to yield ecologically relevant concentrations based on samples from the Great Lakes (Stapleton & Baker, 2003). Tadpoles were fed *ad libitum* from the free-swimming stage (Gosner stage 25) until they reached metamorphic climax (Gosner stage 42).

Tissue collection and chemical analysis

Premetamorphic tadpoles used for measurement of tissue toxicant concentration were first euthanized with buffered 1% MS-222. Individual tadpoles yielded too little tissue for analysis, and so measures were made on pools of tadpoles ($n = 2$ pools per dietary treatment at each temperature, with each pool containing at least 7 grams tissue). Mass and Gosner stage were recorded for every tadpole in a pool, averaged, and used to correct for mass in our calculation of elimination rate constants (see the next subsection). Each tadpole's digestive tract containing any remnant diet was removed to avoid confounding the chemical analysis of tissue concentration. Diet and tissue samples were sent to ALS Environmental, ALS Group USA (Kelso, WA, USA) for PCB and PBDE detection and quantification. Chemical analyses of the concentrations of many congeners of PCBs and PBDEs as well as the concentrations of the sum totals of all PCBs and PBDEs were performed according to the laboratory's NELAP-approved methods and quality assurance program (K1413603; www.alsglobal.com). The method reporting limit (MRL) for

PCB-70 and PCB-126 is 1.9 ng/g wet tissue mass, and the MRL for PBDE is 0.21 ng/g wet tissue mass.

Data and statistical analyses.

Previous studies have shown that elimination of both PCBs and PBDEs is best described assuming a single compartment and first order elimination kinetics (Tawnya L. Cary & Karasov, 2013; Leney, Drouillard, et al., 2006), which allows us to use only two data points to determine the apparent elimination rate constant (Klaassen, 2007).

The elimination rate constants were estimated using linear regression based on the 1st order kinetics model:

$$k_e = \frac{(lnC_{t_{14}} - lnC_{t_{28}})}{t_{28} - t_{14}} \quad \text{(eq. 3)}$$

where C_{t14} and C_{t28} are the tissue residue levels measured at the beginning and end of the elimination phase, respectively (Fig. 1) and $t_{28} - t_{14}$ is the duration of the elimination phase, 14 days. The elimination rate constants were corrected for body size differences using the equation:

$$k^*{}_e = \frac{k_e}{M^{-1/4}} \quad \text{(eq. 4)}$$

where k^*_e is the mass-corrected rate constant and where M is the mean wet mass of the tadpoles that were pooled for the analysis (Riviere, 2011).

The Q_{10} value for elimination was calculated using the adjusted elimination rate constants:

$$Q_{10} = \left(\frac{(k^*{}_e)_{T_1}}{(k^*{}_e)_{T_2}}\right)^{10/(T_1 - T_2)} \quad \text{(eq. 5)}$$

where $(k^*{}_e)_{T_1}$ is the mass-corrected rate constant measured at temperature T_1, and $(k^*{}_e)_{T_2}$ is the mass-corrected rate constant measured at T_2 (note $T_1 > T_2$).

All statistical analyses were performed using R version 3.1.2 (R Foundation for Statistical Computing). All averages presented in results and elsewhere are followed by the standard error of the mean.

In experiments 1 and 2, tadpole masses in each pool were compared using two-way ANOVA, with temperature and diet treatment as factors, along with post-hoc Tukey HSD comparisons. Elimination rate constants were determined by using linear regression, calculating the rate of change in tissue concentrations at the two collection time-points as per eq. 3. Multiple linear regression with an additional variable designed to qualitatively distinguish data points was used to determine if elimination rate constants at different temperatures were significantly different from one another (Draper, 1981). Q_{10} confidence intervals (CI) were obtained by simulating 5,000 values of k_e at each of T_1 and T_2, based on their respective means and standard deviations, and calculating Q_{10} values from randomly drawn paired values of k_e.

In experiments 3 and 4, analysis of covariance (ANCOVA) was used to compare the linear relationships of $\ln C_t$ *vs*. $\ln C_{food}$ at two different rearing temperatures (as in Fig. 1B). Ratios of C_t/C_f were compared as a function of exposure duration and temperature using two-way ANOVA with post-hoc Tukey HSD comparisons.

RESULTS

Experiments 1 and 2 to measure elimination rate constants

Tadpole mortality across all temperature and toxicant treatments was less than 1% during the exposure and elimination phases (data not shown). A goal in experiments 1 and 2 was to minimize growth and associated growth dilution of tissue toxicant concentrations. In the pools of tadpoles used to measure toxicant concentrations at the beginning and end of the elimination phase ANOVA revealed no significant difference in average mass of tadpoles over time ($F_{1,49}$ =

0.67, $P > 0.4$), or between toxicants ($F_{1,49} = 0.12$, $P > 0.7$) (Figure 2 A and B). However, animals reared at 18˚C were significantly larger than those reared at 27˚C ($F_{1,49} = 83.18$, $P < 0.001$), which was anticipated as ectotherms tend to be larger when reared at cooler temperatures (Karasov & Martínez del Rio, 2007).

In Experiment 1, following two weeks of dietary exposure to PBDE, a one-way ANOVA indicated that mean tissue residue level of tadpoles reared at 18˚C (total PBDE 689 ± 146 ng/g wet mass) did not differ significantly from mean tissue residue levels of tadpoles reared at 27˚C (1197 ± 305 ng/g) ($F_{1,2} = 4.5$, $P > 0.05$) (Figure 3A; Table 2). After two weeks of elimination, tadpoles reared at 27˚C had mean tissue residue levels (total PBDE 168 ± 9 ng/g) significantly lower than tadpoles reared at 18˚C (553 ±48ng/g) ($F_{1,2} = 125$, $P < 0.01$). The changes in PBDE levels over time were not due to growth dilution, because the average mass of tadpoles in analyzed tissue pools did not differ significantly (Fig. 2A). Hence, PBDE elimination was certainly much faster at the warmer temperature, as expected. Multiple linear regression showed that the slopes (i.e. the k_e) for the two temperatures differed significantly for total PBDE elimination ($F_{1,4} = 49.3$, $P < 0.001$). Additionally, tadpoles exposed to PBDE at 18˚C, showed no significant decline in tissue residue level from day 14 to day 28 ($F_{1,2} = 1.6$, $P > 0.05$), but the reported k_e for 18˚C is clearly lower than that for 27˚C. The elimination rate constant for total PBDE (k_e; eq. 3) was 9.3 times higher at 27˚C than at 18˚C (Table 3), though once the differences in body size were taken into account (eq. 4), k_e^* for PBDE was 7.6 times higher at 27˚C than at 18˚C (Table 3).

Q_{10} values for PBDE congeners (Table 3), calculated using eq. 5, were much greater than the expected values of 2-3. Due to the very slow PBDE elimination in animals exposed at 18˚C, we were not able to obtain statistically significant k_e values (i.e., not different from zero).

Consequently, the Q_{10} values reported for PBDE, ranging from 8.5 to 36.5 among all congeners, are crude and arguably unreliable. In Discussion we offer an alternative analysis for the effect of temperature on PBDE elimination rate constants.

In Experiment 2, following two weeks of dietary exposure to PCBs, a one-way ANOVA indicated that mean tissue residue level of tadpoles reared at 18˚C (total PCB 47 ± 1 ng/g wet mass) did not differ significantly from those of tadpoles reared at 27˚C (75 ± 9 ng/g; $F_{1, 2} = 8.29$, $P = 0.103$) (Fig. 3B, Table 4). After two weeks of elimination, tadpoles reared at 27˚C (6.75 ± 0.7 ng/g) had significantly lower mean tissue PCB levels than tadpoles reared at 18˚C (26 ± 1 ng/g; $F_{1,2} = 261$, $P < 0.01$). As expected PCB elimination was much faster at the warmer temperature. Multiple linear regression showed that the slopes, i.e. k_e values or elimination rates, for the two temperatures differed significantly for total PCB ($F_{1,4} = 117$, $P < 0.001$). The elimination rate constant for total PCB was 4.1 times higher at 27˚C than at 18˚C (Table 4), though once the differences in body size were taken into account, k_e^* for PCB elimination was 3.4 times higher at 27˚C than at 18˚C (Table 5). Q_{10} values for PCBs (Table 5), calculated using eq. 5, were a little higher than the expected values of 2-3. Their confidence intervals do not overlap with the Q_{10} values for PBDEs (Table 3), suggesting a lower sensitivity to temperature for elimination of PCBs than PBDEs.

Experiments 3 and 4 to measure C_t close to C_{ss}

In experiment 3, tadpoles reared at 18 and 27 °C for 12-13 weeks on diets containing different levels of PBDE, $\log_{10}$ tissue concentrations were linearly related to $\log_{10}$ diet concentrations (ANCOVA, $F_{1,6} = 222$, $P < 0.001$) as expected for first order kinetics (Gibaldi & Perrier, 1982), but neither temperature ($F_{1,4} = 4.234$, $P = 0.11$) nor the interaction of temperature and PBDE diet concentration ($F_{1,4} = 2.81$, $P = 0.17$) were significant (Fig. 4a). Notably, the

tissue concentration data for tadpoles in experiment 1 reared only 2 weeks on diet with PBDE fell near the extrapolated regression line despite the much shorter rearing time on toxicant-containing diet (Fig. 4a). Considering the apparent first-order kinetics over the entire concentration range in both longer and shorter duration trials, we normalized the total PBDE in tissue to that in the diet by calculating their quotient (ratio tissue/diet) (Gibaldi & Perrier, 1982). Ratios in tadpoles reared for 12-13 weeks at either 27 ºC (1.74 ± 0.2, $n = 4$) or 18ºC (1.47 ± 0.04, $n = 4$) did not differ (ANOVA $F_{1,6} = 1.3$; $P = 0.29$; Fig. 4b), and their mean value (1.60 ± 0.12, $n = 8$) is arguably close to the steady-state concentration. Judging by a significant interaction between study duration and temperature ($F_{1,8} = 5.74$, $P = 0.043$) and the relative magnitudes of the mean ratios (Fig. 4b) it appears that the tadpoles at 18 ºC fed toxicant just 2 weeks had not reached steady state.

In experiment 4, tadpoles reared at 23 and 28ºC for 5 weeks on diets containing PCB congeners 70 or 126, $\log_{10}$ tissue concentrations were linearly related to $\log_{10}$ diet concentrations (ANCOVA, $F_{1,14} = 159$, $P < 0.001$; Fig. 5A) as expected for first order kinetics (Gibaldi & Perrier, 1982), but no other factors such as temperature ($F_{1,8} = 0.81$, $P > 0.3$), the particular congener (i.e., PCB 126 *vs.* 70; $F_{1,8} = 0.26$, $P > 0.6$), or any of the interactions of these factors and covariate were significant (all P's > 0.3). The tissue concentration data for tadpoles in experiment 2 raised 2 weeks on diet containing PCBs at 27ºC were near that same regression line, although it appeared that the tadpoles reared at 18ºC fed PCBs 2 weeks were below the line. Indeed, in the tadpoles fed PCB diets for 2 weeks the ratio of PCB in tissue to that in food was significantly lower at 18ºC than 27ºC (respectively, 0.8 ± 0.004 *vs.* 1.25 ± 0.094; $t_6 = 4.8$, $P = 0.003$; Fig. 5B), whereas in tadpoles fed PCB diets for 5 weeks did not differ significantly between 23ºC and 28ºC temperature (respectively, 1.65 ± 0.30 *vs.* 1.18 ± 0.14; $t_{14} = 1.45$, $P =$

0.17). These patterns seem consistent with the idea that steady state ratio (tissue/diet) was approximately 1.42 ± 0.17 (n = 16 for all tadpoles raised 5 weeks) and was reached in all groups except those reared at 18°C.

DISCUSSION

Elimination of PCB and PBDE in *L. pipiens* tadpoles was faster at warmer temperature, as predicted in the hypothetical model in Figure 1A. This is in accord with kinetic principles (Riviere, 2011). A previous study on amphibians showed that sensitivity to toxicants is temperature dependent (Boone & Bridges, 1999), but this is the first demonstration in an amphibian, to our knowledge, of how elimination rates vary with temperature. At first glance, the Q_{10}'s of PCB and PBDE elimination rate constants we measured (2.7 to 4.9 for PCB; 8.5 to 36.5 for PBDE) seem unreliable as they are much higher than the expected range of 2-3 for rate processes in animals (Schmidt-Nielsen, 1990), including amphibians and *L. pipiens* (Clarke, 2006; Moore, 1939). The choice of 18°C as our lower rearing temperature may have resulted in toxicant biotransformation rates too slow to generate an appreciable decline in toxicant tissue concentration over our two-week elimination phase (i.e., k_e was not significantly different than zero). The computation of temperature coefficients, which relies upon the difference in rate constants, could result in an overestimate if based on the unreliable rate constant for tadpoles reared at 18°C. Additionally, it is well known that Q_{10} values are dependent on the temperature range over which rates are measured and are generally higher when computed over lower temperatures (Hochachka & Somero, 2002). For the aforementioned reasons, we used our data to derive elimination rate constant Q_{10} values between temperatures 23 to 27°C. We feel that this temperature range most accurately reflects the temperature increase expected from climate change scenarios for amphibians in midwestern North America (Veloz et al., 2012).

Temperature is known to affect simple physiological rates following the Arrhenius equation which is based on the Boltzmann function of kinetic theory (Hochachka & Somero, 2002). According to the Arrhenius equation:

$$k = Ae^{-E_a/RT} \qquad \text{(eq. 6)}$$

where k is a rate constant, E_a the activation energy of the reaction, R the universal gas constant and T the temperature in Kelvin. Hence, the natural log of rate constants is proportional to the inverse temperature (K), and plotting these two variables will yield a straight line.

Previously, we conducted a very similar elimination experiment at 23°C using the same frog species and the same PBDE mixture (Cary Coyle & Karasov, 2010; Tawnya L. Cary & Karasov, 2013). That rate constant (0.117 ± 0.037) is bracketed by the two rate constants measured in this study, as is expected because we used rearing temperatures that bracket 23°C. We plotted our two PBDE elimination rate constants at 18 and 27°C from the current study along with Cary *et al.*'s elimination rate constant at 23°C into the Arrhenius model (see Methods), and the data points fit a straight line ($R^2 = 0.949$). Hence, the elimination rate constants we measured over the range 18-27 °C were indeed affected by temperature consistent with the Arrhenius equation. Based on this finding, we derived linear equations for both PBDE and PCB elimination rate constants versus temperature. For PBDE:

$$Ln(k_e) = -10471\left(\frac{1}{T}\right) + 33.1\ ;$$

for PCB:

$$Ln(k_e) = -11817\left(\frac{1}{T}\right) + 37.8.$$

This method of deriving elimination rate constants from a larger pool of data (in the case of PBDE) is more robust and an arguably superior approach because it allows the determination of

changes in k_e over a more realistic range of temperatures. Based on these relationships, best estimates for Q_{10} of k_e between 23 and 27°C are 3.3 for PBDE and 3.8 for PCB. These Q_{10} values are closer to the range of 2-3 for Q_{10} in animals generally (Schmidt-Nielsen, 1990). Furthermore, as we will now discuss, these values help explain why frog body toxicant burdens changed very little (or not at all) with increasing rearing temperature.

Our data also indicate that *L. pipiens* reached near steady state concentrations (C_{ss}) that were independent of temperature (Figs. 4 and 5). We predicted this for the case where both elimination and uptake are similarly influenced by temperature (i.e., similar Q_{10}'s; Fig. 1B, C). Considering that C_{ss} corresponds to the quotient of the rates of toxicant uptake to elimination (eq. 2), we can infer that the Q_{10} values for uptake over 18-27 °C are similar to those we measured for k_e. In other words, the data indicate that faster toxicant elimination at warmer temperatures is balanced by faster toxicant uptake, with the effect that exposure is rather independent of temperature. Although the Q_{10} for toxicant uptake was not measured, we can infer that its approximate value is close to that for elimination. Food intake corresponds directly to metabolic rate, because the majority of a tadpoles' energy budget is used for respiration (Pandian & Marian, 1985). We assume that contaminant uptake via food will change with temperature in a fashion similar to respiration. A study done by Parker (Parker, 1967) examining *L. pipiens* metabolic rates at physiologically reasonable temperatures revealed Q_{10} values between 2.6 – 3. Thus, it appears that Q_{10} values for toxicant uptake may be similar to those we estimated for k_e, consistent with our finding that *L. pipiens* reached steady state concentrations (C_{ss}) independent of temperature. Future research should test this hypothesis by determining the temperature dependence of tadpole energetics.

Our kinetics model for toxicant uptake is based heavily on tadpole food intake. We did not

account for toxicant distribution from the diet through the water and then transferred to the tadpoles. There are both theoretical and empirical considerations supporting our assumption. First, the toxicants we used in our study (PCB-70, PCB-126 and PBDEs) have very high K_{OW} and very low water solubility, therefore, the toxicants should bind to the diet and tadpoles with very little distribution to the surrounding water, and an earlier study found that PBDE congeners were not detected in water samples (Cary Coyle & Karasov, 2010). Second, based on past study (Jofré & Karasov, 2008), for *L. pipiens* tadpoles exposed to PCB-70 via water, the bioaccumulation factors (BCFs = PCB concentration in wet tadpole tissue/ PCB nominal concentration in tank water) for PCB-70 ranged from 122 to 194. If we assume, for the sake of argument, that in our experiment all of the PCB-70 in the diet is transferred into the water, and if we use the BCFs from Jofre et al., the predicted tadpole tissue concentration would account for only 2-4% of our actual measured tissue concentration. The theoretical reasoning and the mock calculation for the most extreme assumption (all toxicant added via food dissolves into 12 L of tank water) support that the majority of toxicant uptake for *L. pipiens* in this study comes from food intake and not via water.

There is currently a paucity of studies looking at how temperature affects the kinetic rates of toxicants in animals. This study was designed to provide more data and advance knowledge in this field. Our findings on elimination are consistent with the expectation that warming temperatures, as predicted by climate change models, will increase toxicant elimination rates in ectotherms, perhaps somewhat greater than expected from typical physiological Q_{10} values of 2-3. In addition, the known temperature dependence of animal energetics in ectotherms (physiological rates are higher at higher temperatures) indicate that faster toxicant elimination at warmer temperatures will be approximately balanced out by faster toxicant uptake, which is

supported by our finding that near steady state tissue concentrations were independent of temperature. However, our findings should not be miss-interpreted to mean that there will be no consequences of PCB and PBDE exposure on amphibian populations at increased temperatures. Our study purposefully chose low, but ecologically relevant, concentrations of contaminants in order to study the kinetics of exposure, not acute toxicity. Additional studies are necessary to address target site sensitivity with regard to increased temperatures.

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TABLES AND TABLE LEGENDS

Table 1. Summary of experiment goals, rearing temperatures, toxicant food concentrations (C_{food}) and duration of exposure to toxicants in food.

Exp.	Goal	Rearing Temperatures (°C)	C_{food} (ng/g)[1]	Exposure Duration (d)
1	Measure k_e of PBDEs	18 and 27	753.9	14
2	Measure k_e of PCBs	18 and 27	60	14
3	Measure C_t close to C_{ss} for PBDEs	18 and 27	6.11, 16.81, 39.9, 81.96	85-95[2]
4	Measure C_t close to C_{ss} for PCB 70 and 126	23 and 28	PCB70 – 25, 170 PCB126 – 3.5, 7	37-38 37-38

Notes:

[1]measured sum of all congeners (see Methods)

[2]longer experiment duration at cooler temperature where development was slower

Table 2. Levels[1] of PBDE congeners in tadpoles and their diets in experiment 1.

PBDE Congener	**27 °C Day 14**	**27 °C Day 28**	**18 °C Day 14**	**18 °C Day 28**	**PBDE in diet**
# in pool	4, 5	4, 4	3, 3	3, 2	N/A[2]
Mass (g)	7.43, 8.30	7.77, 7.39	8.59, 7.11	10.2, 7.4	N/A
BDE-28	1, 0.39	ND[3], ND	ND, ND	ND, ND	1.5
BDE-71	16, 8.7	ND, 0.55	7.3, 11	13, 8.4	5.8
BDE-47	450, 310	30, 32	190, 240	130, 170	240
BDE-66	5.9, 4.3	ND, ND	2.7, 3.5	ND, 1.9	7
BDE-100	150, 110	22, 19	64, 90	75, 66	76
BDE-99	640, 440	96, 86	260, 360	240, 280	330
BDE-85	34, 23	2.8, 2.5	15, 20	12, 13	21
BDE-154	46, 33	8.7, 7.6	18, 27	19, 20	29
BDE-153	60, 44	13, 11	24, 35	26, 23	36
BDE-138	8.6, 6.2	1.4, 1.1	3.4, 4.7	3.1, 3.5	6.5
BDE-128	0.25, 0.22	ND, ND	0.11, 0.14	ND, ND	ND
BDE-183	1.4, 1.1	0.34, 0.3	0.54, 0.67	0.65, 0.53	1.1
BDE-190	ND, 0.0067	ND, ND	ND, ND	ND, ND	ND
Total	1413, 980	174, 160	585, 792	518, 586	753.9

Notes:

[1] Levels were measured in pools of tadpoles (n = # in pool) created to have at least 7 grams of wet mass per replicate, the minimum mass required for residue analysis. The mean mass of tadpoles for each pool is provided (“Mass”). All congeners are reported as nanograms of PBDE per gram of wet mass.

[2]N/A = Not applicable

[3]ND = non detectable, levels for this congener were either too low to be detected or were simply non-existent in the sample.

Table 3. PBDE elimination rate constants from experiment 1, and calculated Q_{10} for PBDE elimination.

PBDE Congener	k_e [1]		k_e* [2]		Q_{10} [3]
	18˚C	27˚C	18˚C	27˚C	
BDE-99	0.012 ±0.01	0.126 ±0.01§	0.014 ±0.02	0.152 ±0.02	13.9
BDE-47	0.026 ±0.01	0.178 ±0.01§	0.031 ±0.02	0.214 ±0.02	8.5
BDE-100	0.005 ±0.01	0.131 ±0.01§	0.007 ±0.02	0.158 ±0.01	34.6
BDE-153	0.012 ±0.01	0.104 ±0.01§	0.015 ±0.02	0.125 ±0.02	10.9
BDE-154	0.009 ±0.01	0.112 ±0.01§	0.011 ±0.02	0.135 ±0.02	16.9
BDE-85	0.023 ±0.01	0.168 ±0.01§	0.028 ±0.01	0.203 ±0.02	9.0
BDE-138	0.014±0.01	0.127 ±0.01§	0.017 ±0.01	0.152 ±0.02	11.7
BDE-183	0.002 ±0.01	0.045 ±0.05	0.002 ±0.01	0.054 ±0.06	36.5
Total PBDE	0.015 ±0.01	0.139 ±0.01§	0.022 ±0.02	0.168 ±0.02	11.9

Notes:

[1]calculated using eq. 3

[2]corrected for differences in mass, using eq. 4

[3]Q_{10} calculated using eq. 5

§$P < 0.05$ for slope (i.e., k_e)

Table 4. Levels of PCB congeners in tadpoles and their diets in experiment 2. Levels were measured in pools of tadpoles (n = # in pool) created to have at least 7 grams of wet mass per replicate, the minimum required for residue analysis. The mean mass of tadpoles for each pool is provided ("Mass"). All congeners are reported as nanograms of PCB per gram of wet mass.

PCB Congener	**27 °C Day 14**	**27 °C Day 28**	**18 °C Day 14**	**18 °C Day 28**	**PBDE in diet**
# in pool	4, 4	6, 4	3, 3	3, 2	N/A
Mass (g)	7.13, 7.83	8.03, 8.0	7.84, 8.76	8.73, 7.75	N/A
PCB-70	41, 33	1.7, 2.4	25, 25	12, 13	31
PCB-126	43, 33	4.4, 5.0	23, 23	13, 14	29
Total	84, 66	6.1, 7.4	48, 48	25, 27	60

Notes:
[1]N/A = Not applicable

Table 5. PCB elimination rate constants from experiment 2, and calculated Q_{10} for PCB elimination.

PCB Congener	k_e[1]		k_e^*[2]		Q_{10}[3]	Q_{10} 95% Confidence Interval
	18˚C	27˚C	18˚C	27˚C		
PCB-70	0.05 ±0.003§	0.206 ±0.01§	0.071 ±0.004	0.248 ±0.02	4.01	[3.22, 4.92]
PCB-126	0.035 ±0.003§	0.149 ±0.01§	0.05 ±0.004	0.149 ±0.01	3.39	[2.67, 4.27]
Total PCB	0.042 ±0.003§	0.171 ±0.01§	0.061 ±0.004	0.206 ±0.01	3.89	[3.26, 4.62]

Notes:

[1]calculated using eq. 3

[2]corrected for differences in mass, using eq. 4

[3]Q_{10} calculated using eq. 5

§$P < 0.05$ for slope (i.e., k_e)

FIGURES AND FIGURE LEGENDS

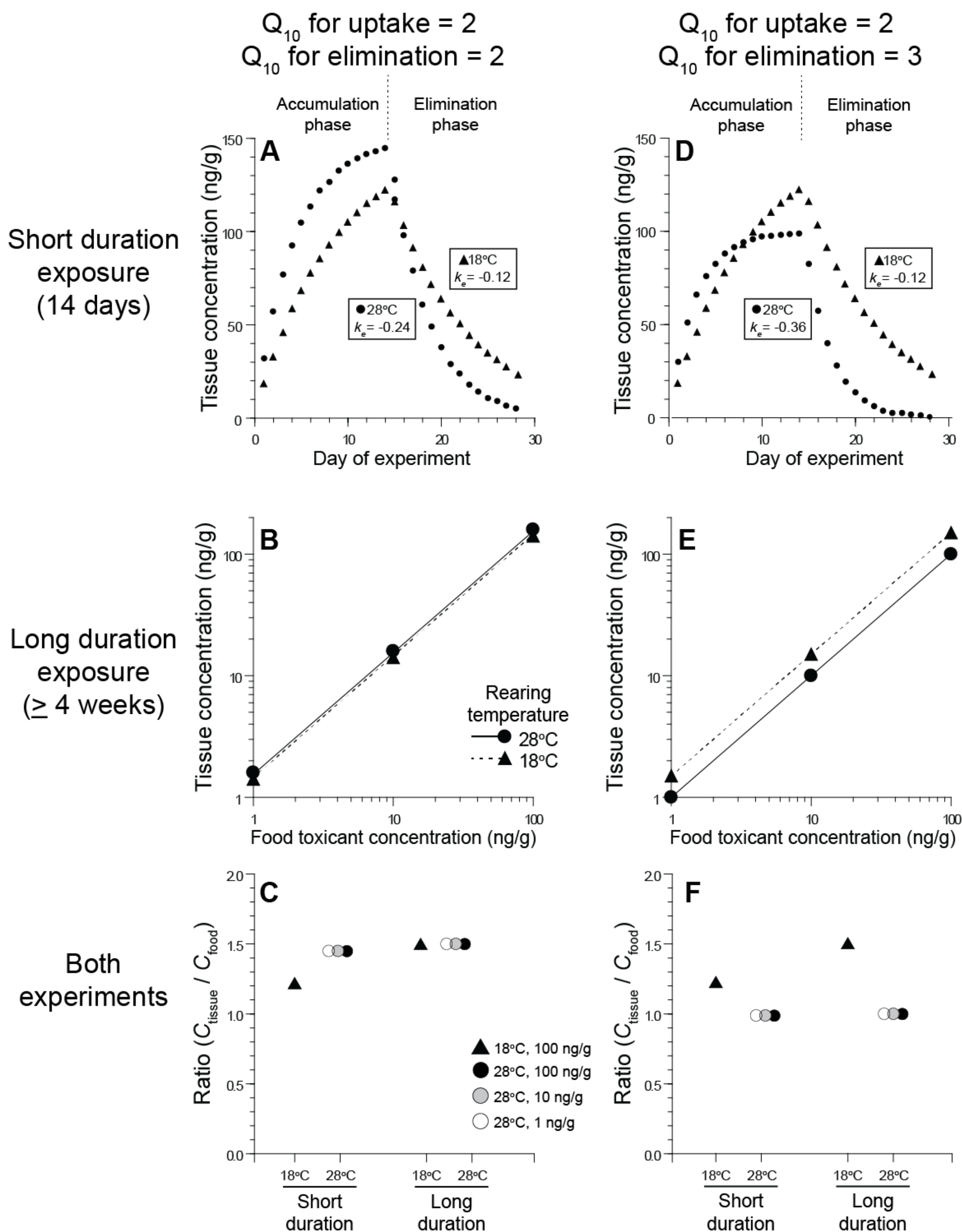

Figure 1. Modeled values (Methods, eq. 1) of tissue toxicant concentration (C_{tissue}, ng/g mass) for tadpoles raised at two temperatures (18 ºC and 28 ºC) fed PBDE-laden diet.

Fig. 1A shows that C_{tissue} of a tadpole consuming toxicant-laden food will quickly rise and approach a steady state level (C_{ss}), which will occur sooner at a warmer rearing temperature. If this accumulation phase is followed by a period when food sans toxicant is consumed (an elimination phase), as in Fig. 1A, C_{tissue} will decline with a rate constant for elimination (k_e) that will be greater (faster) at warmer temperature. Initial parameter values in Fig. 1A were set as follows: daily food intake, I (g wet mass/d) = 20% of body mass/d; tadpole mass, M = 3 g; C_{food} = 100 ng/g, 90% of which is absorbed; rate constant for elimination at lower temperature, k_e = -0.12 d^{-1} (Tawnya L. Cary & Karasov, 2013); temperature coefficient Q_{10} for both intake and elimination = 2.

Fig. 1B shows the asymptotic tissue toxicant concentrations (C_{ss}) for several different values of C_{food}, assuming that toxicant-laden food is consumed for a more prolonged period; all other parameter values are the same as in Fig. 1A. The first order kinetics and the assumption that the temperature coefficient Q_{10} is the same for both intake and elimination leads to linear relationships between C_t and C_{food} that are the same (Fig. 1B), or yield the same ratios of C_{tissue}/C_{food}, which is an alternative way to depict the data when kinetics are first order (Cary and Karasov, 2013; Gibaldi and Perrier, 1982).

Figures 1D, E, and F show analogous plots to A, B, and C, with the only difference being that Q_{10} for intake = 2 whereas that for elimination = 3.

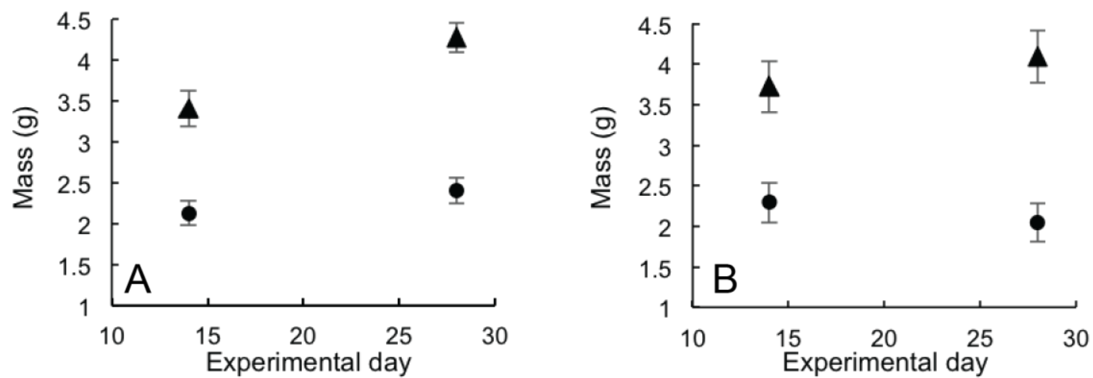

Figure 2. Average tadpole mass (g) for PBDE fed tadpoles (A) and PCB fed tadpoles (B), at the end of exposure (day 14) and elimination (day 28) for animals reared at 18 (triangles) & 27˚C (circles). Each symbol is the average mass of six tadpoles, euthanized, blotted dry, and massed to the nearest milligram; error bars are the standard error of the mean.

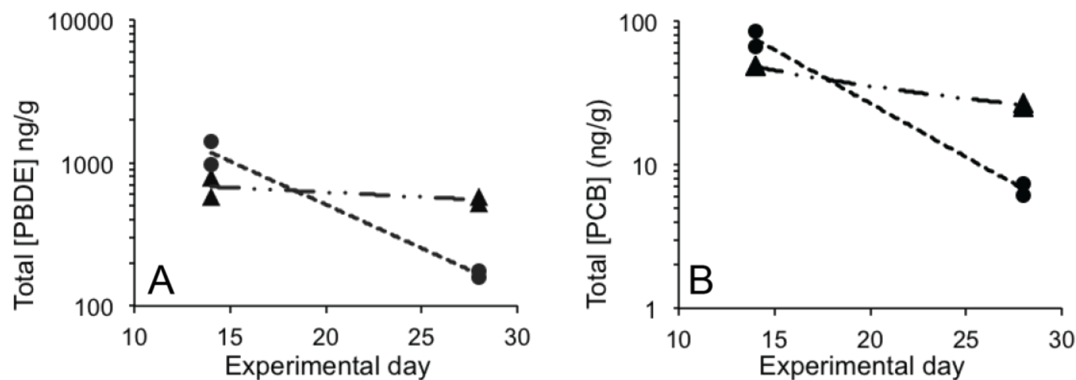

Figure 3. Total PBDE (A) and PCB (B) concentration in nanograms per gram of tadpole tissue at the end of exposure (day 14) and elimination (day 28), for tadpoles reared at 18 (triangles) & 27˚C (circles). Tadpoles fed PBDE are represented by black symbols and PCB by grey symbols. Each symbol represents a group of 2 to 6 tadpoles that were pooled to yield seven grams of tissue for determination of toxicant concentration.

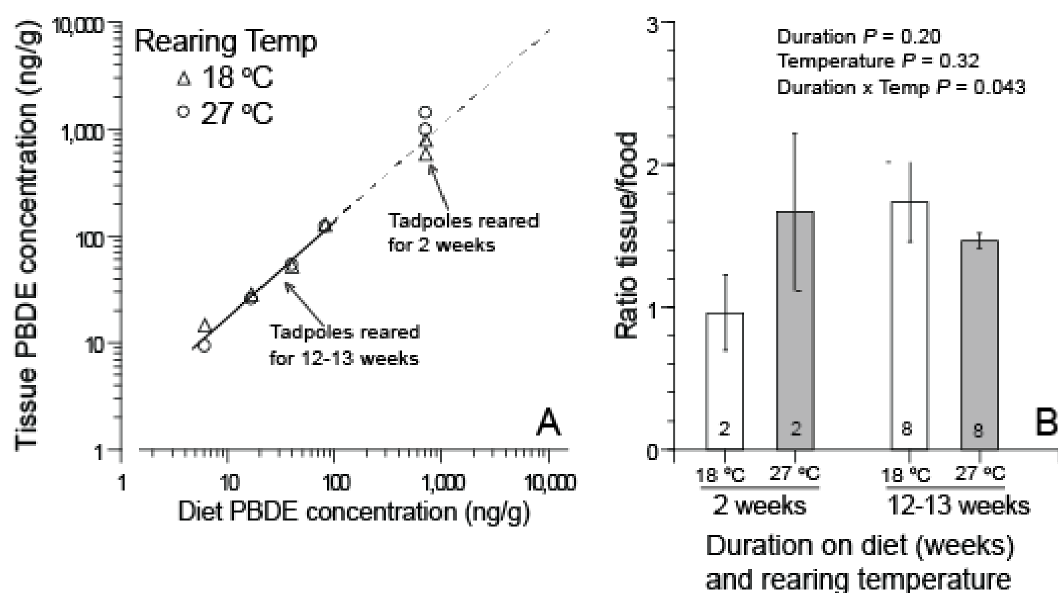

Figure 4. Bioaccumulation of total PBDE in tadpoles as a function of diet total PBDE concentration, rearing temperature, and rearing duration on a log-log plot. (A, left hand figure) In tadpoles reared for 12-13 weeks, rearing temperature had no effect on tissue concentrations, which were linearly related to diet concentrations (see text), as expected for first-order kinetics. The tissue concentration data in tadpoles reared 2 weeks were close to the extrapolated regression (dashed) line. (B, right hand figure) Ratios (= total PBDE in tissue/total PBDE in food) did not differ as a function of either diet or temperature, although there was a significant interaction between the two (see text). The data can be interpreted to mean that steady state (rate of absorption = rate of elimination) was reached after 2 weeks at 27°C but not at 18°C.

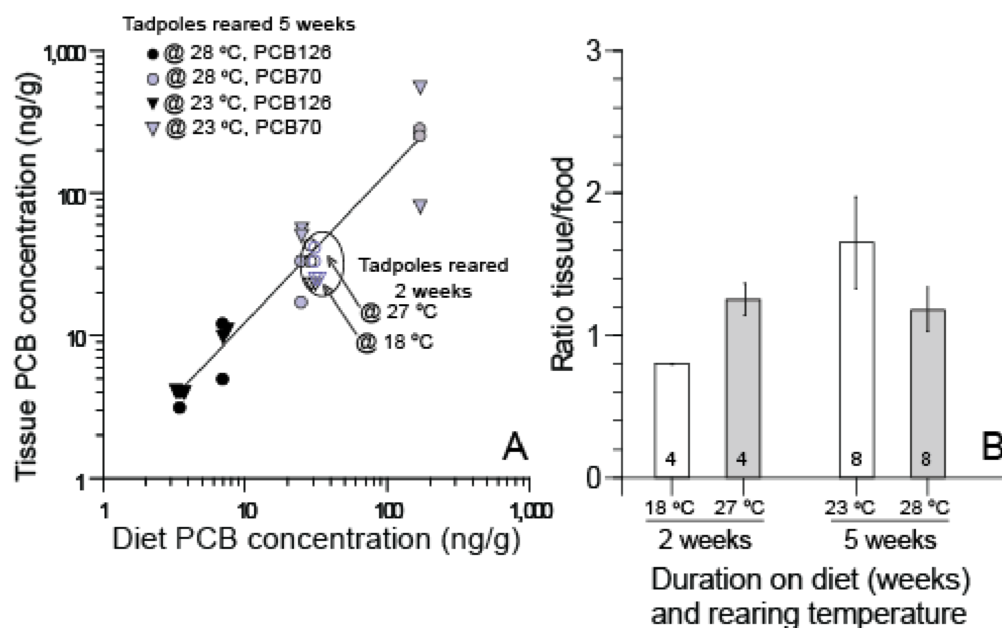

Figure 5. Bioaccumulation of total PCBs in tadpoles as a function of diet concentration, congener, rearing temperature, rearing duration, on a log-log plot. (A, left hand figure) Among tadpoles reared on PCB diet for 5 weeks, tissue concentrations were linearly related to diet concentrations (solid black line), as expected for first order kinetics, but no other factors such as temperature or the particular congener (i.e., PCB 126 *vs.* 70) or any of the interactions of these factors and covariate were significant (see text). The tissue concentration data in tadpoles exposed for 2 weeks (8 values within the ellipse) were close to the regression line, though lowest for those reared at 18ºC. The data can be interpreted to mean that steady state (rate of absorption = rate of elimination) was reached after 2 weeks at 27ºC but not at 18ºC.

CHAPTER II

THE EFFECTS OF REARING TEMPERATURE AND DIETARY PBDE EXPOSURE ON TADPOLE GROWTH, DEVELOPMENT, AND THEIR UNDERLYING PROCESSES

ABSTRACT

Depression of growth rate due to exposure to polybrominated diphenyl ethers (PBDEs) has been documented in birds, mammals, amphibians, and fish at single temperatures. However, the underlying mechanisms for this effect, and how it might change in relation to changing environmental temperature is largely unstudied. Here, we used a simple energy budget to address hypotheses regarding effects of PBDEs on growth in leopard frog tadpoles (*Lithobates pipiens*); namely that reductions in growth could be linked to either increased respiratory costs, reductions in digestive performance, or differences in body composition. We reared tadpoles at 22 & 27 ºC, and from 18 days post hatch (dph) until metamorphosis we exposed tadpoles dietarily to the technical pentabromodiphenyl ether mixture (DE-71) at a nominal concentration of 100 ng DE-71/g diet wet weight. Controls received the same diet without DE-71. After 21 days of exposure, measures of PBDE in tadpoles did not differ for the two temperatures and averaged 148 ± 27.7 ng PBDE/g wet tissue. PBDE exposure resulted in reductions in body length, mass, and development as compared to controls, although there was no interaction with temperature. PBDE in the diet had no significant effect on measures of body composition (no difference in total fat, inorganic or water content) although total fat did increase with age. Measures of dry matter digestibility did not differ between control and PBDE-exposed tadpoles but did decline with Gosner stage and increased with temperature. Rates of mass specific resting oxygen consumption (VO_2; ml g^{-1} h^{-1}) were similarly unaffected by PBDE exposure, but showed a strong dependence on rearing and measurement temperatures. Tadpoles reared and measured at 27 ºC averaged 75%

higher levels of oxygen consumption than those reared and measured at 22 ^{o}C. With all other energetic explanations exhausted we were left to conclude that the difference in growth between PBDE-exposed and control animals must be due to decreased feeding. Future studies on feeding rate and regulation of animals exposed dietarily to PBDEs are warranted.

INTRODUCTION

A leading cause of amphibian declines in Midwestern North America is pollutants, which may act in combination with other stressors such as habitat loss and degradation, pathogens, and climate change (Hof et al. 2011, IUCN 2011). A group of pollutants found in the Great Lakes that are of particular concern are persistent organic pollutants or POPs, which include polybrominated diphenyl ethers (PBDEs). PBDEs increased dramatically from 1980-2000 (Norstrom et al. 2002) and are found in biota of the Great Lakes watershed at levels potentially harmful to wildlife, including amphibians (Cary Coyle and Karasov 2010, Carey et al. 2014). Although industrial and other uses of these compounds has declined as regulatory restrictions have increased, their global distribution and persistence in the environment creates an ongoing need for environmental monitoring of these compounds. Additionally, air temperatures in the Great Lakes watershed are expected to increase by six degrees Celsius by the turn of the century (Veloz et al. 2012), and surface waters are expected to increase by as much as four degrees Celsius (Group 2011). This increase in temperature could act synergistically with other environmental stressors negatively impacting amphibian populations. In a review of 66 species of freshwater animals (Mayer 1986), toxicity of contaminants generally increased as temperature increased. The underlying mechanism(s) are unclear, and understanding the interplay between environmental contaminants such as PBDEs and changing climates on Great Lakes flora and

fauna may be crucial for protecting the integrity of the ecosystem (Noyes et al. 2009; Landis et al. 2014).

Temperature is arguably the most influential environmental factor influencing physiological processes in ectotherms (Clarke 2003; Clarke 2006), generally increasing physiological rates according to a Q_{10} coefficient of 2-3 (the rate change for a 10 ^{o}C change in temperature). Exposure of animals to pollutants is mediated by processes of toxicant uptake, biotransformation, and elimination. These processes link to bioenergetic rates of metabolism, food intake, digestion and elimination, which are known to be temperature dependent in ectothermic vertebrates. For example, respiration (i.e., metabolic) rates of 683 amphibian species had an average Q_{10} of 2.21 (White et al. 2006), and if we assume that food and food-borne toxicant intake scales with metabolism then for every ten degree increase in temperature we would anticipate a two-fold increase in toxicant intake. Depression of growth rate due to PBDE exposure has been documented in birds, mammals, amphibians, and fish at single temperatures (Fernie et al. 2006; Viberg et al. 2008; Cary Coyle and Karasov 2010; Chen et al. 2010). Yet, there are no studies, to our knowledge, of the impacts of PBDE exposure under different temperature regimes in any ectothermic vertebrates, or studies of how PBDE negatively influences growth and development of tadpoles under different temperature regimes.

Reductions in growth of PBDE fed tadpoles could be attributed to differences in features related to energy acquisition and expenditure. Body mass change (Δm, in g/d) can be understood in terms of energy inputs and outputs (in J/d) using a simple balanced equation:

$$\Delta m = ((I \bullet D) - R) / e_{tissue}$$

where, I is daily energy intake, D a digestibility coefficient that determines how much ingested energy is metabolizable, R is respiration (metabolism), and e_{tissue} is the energy density of tissue

(in J/g wet mass) determined mainly by its composition of water, ash, and lipid. (For simplicity we ignore changes in carbohydrate and protein content because their energy densities per gram wet tissue are rather similar to each other, especially in comparison to the much higher energy density per g wet tissue of lipid.) Observed reductions in the growth of PBDE exposed tadpoles directs us to the following hypotheses:

1) PBDE exposure reduces digestibility, *D*, resulting in reduced growth.

2) Slowed mass gain due to PBDE exposure is due to changes in body composition that increase e_{tissue}, such as decreased ash or water content or increased lipid content.

3) PBDE exposure increases metabolic rate, *R,* resulting in reduced growth. Exposure to some other toxicants has increased respiration in frogs (e.g., carbaryl; Marian et al. 1983), perhaps through a direct effect on metabolism or through an increased metabolic cost of toxicant biotransformation and elimination.

To address these hypotheses, we raised tadpoles fed diets without or with PBDE and reared them at temperatures differing by 5°C (22 & 27 °C), which is consistent with the magnitude of temperature change predicted by climate change scenarios during this century (Veloz et al. 2012). In addition to monitoring development and size (length and mass) over time, we tested for changes in body composition (lipid, water and ash content). Digestive performance was measured using a validated inert marker – the comparison of ash content in food samples and ash content in digesta (Gleason et al. 2016b). In order to discern differences in respiration between control and PBDE fed animals at different temperatures, a custom-designed, closed respirometry system was used to measure oxygen consumption. Once a likely component(s) of the energy budget has been implicated in the reduced growth seen in PBDE fed animals, future studies can focus on the likely mechanism(s) underlying this reduction. Our study can improve

our predictive capability for anticipating how environmental change may influence tadpole performance, as well as adding to existing knowledge of the toxic effects of PBDE exposure at environmentally relevant concentrations.

METHODS

Animals, husbandry, and temperature control

Procedures for this study were approved by UW-Madison College of Agricultural and Life Sciences Institutional Animal Care and Use Committee (IACUC) (Protocol number A01336). *Lithobates pipiens* embryos were purchased commercially from Nasco© (Fort Atkinson, WI) on the day fertilization took place (Gosner [GS] stage 1). Immediately upon arrival, embryos were randomly distributed into 36 0.5-L Nalgene containers (approx. 30 embryos/container) containing filtered, dechlorinated municipal water, and placed in temperature-controlled rooms (18 per room) at 22 or 27 °C (± 1 ºC). Water within the containers was changed daily to minimize bacterial and fungal growth; non-viable or dead embryos were removed when found.

Once the embryos developed into free-swimming tadpoles (GS 25; 5-days post hatch [dph] for warmer room, 6 dph for cooler room), they were transferred into 12 (6 per room) 18.9-L glass aquaria (25 tadpoles/tank) with air stones in 12 L of water in temperature-controlled racks. The racks had a system to circulate temperature-controlled water around each aquarium. By flowing temperature-controlled water around the aquaria, as well as maintaining a constant temperature in the animal rooms, we ensured that tadpoles were maintained at their target temperatures, 22 or 27 ºC (± 1 ºC), throughout development. Tank water was changed (> 80% water change) every other day and water quality was monitored weekly for pH, nitrites, ammonia, and dissolved oxygen according to IACUC's standards and was never found to be

outside of the acceptable ranges for any of the aforementioned parameters (pH = 8 ± 0.2; nitrite < 1.0 mg/L; total NH_3 < 1mg/L; dissolved oxygen > 6.0 mg/L). Light/dark cycles were maintained at 14L/10D, via ambient florescent lighting from the ceiling as well as full spectrum light fixtures (Retpi-Sun 5.0 UVB, Zoo Med) in the racks. Tadpoles were fed *ad libitum* a diet that consisted primarily of rabbit chow (Harlan Teklad, catalog 2030) suspended in gelatin/agarose mixture, prepared according to Gleason (Gleason et al. 2016). Wet mass of food provided daily was approximately 20-25% of summed tadpole mass in the tank, and left over food from the previous feeding was syphoned every day prior to feeding.

Tadpole toxicant exposure

Dietary treatment began on day 18 and continued for 21 days (some tadpoles remaining at the end of the experiment were used for measures of resting metabolism (RMR)). DE-71 was incorporated into the food (nominal concentration 100 ng DE-71/g diet wet weight), which was prepared according to a previous study done in our laboratory (Cary Coyle and Karasov 2010). Briefly, stock DE-71 (100 µg/mL in toluene) was dissolved in acetone (equal parts weight/volume) and mixed with ground rabbit chow (250 g/L). Control diet (0 ng DE-71/g) was prepared with the same volumes of toluene and acetone sans PBDE. The mixtures were left on a tray inside a chemical fume hood overnight to allow for acetone and toluene evaporation. The dried rabbit chows (± PBDE) were then mixed with agar (20 g/L), gelatin (14 g/L) and distilled water. The mixtures were brought to boil for 1 min and then cooled to room temperature. Prepared diets were stored refrigerated at -4° C or stored at -20° C for future use.

Measurement of resting metabolism

Tadpoles remained in their respective temperature controlled tanks eating their respective diets for up to 54 dph (approximately 25 tadpoles/tank; 3-4 tanks per treatment; 4 total

treatments, each of which was defined by its particular rearing temperature (22 or 27 ^{o}C) and diet (± PBDE). On 8 separate days spread between 26 – 54dph, 5 -18 individual tadpoles (66 tadpoles in total) were randomly removed from treatment tanks for measurement of resting metabolic rate (RMR), defined as the lowest metabolic rate of nonfasted, resting tadpoles. Between 1000 h and 1700 h we measured the rate of oxygen consumption of tadpoles at either 22° or 27°C in temperature-controlled water-jacketed 106-mL glass respiration chambers (custom built at Miami University [Ohio]) using an oxygen sensor (HIOXY O_2 probe and NEOFOX phase measurement system, Ocean Optics (Dunedin, FL), connected via cable to a data logger (http://oceanoptics.com/product-category/oxygen-sensors/). The chamber contained deionized water and a magnetic stir bar that was rotated by a magnetic stir plate (set at medium) upon which the chamber sat, which ensured homogeneous dissolved oxygen (DO). The sensor, which measured % DO, was calibrated at a specified water temperature by a two-point method recommended by the manufacturer. At the high calibration point, 100% DO, 100% gaseous oxygen was first bubbled into the water in the absence of a tadpole and then stopped, and oxygen concentration was measured until it stabilized. At the low calibration point, 0% DO, granular sodium hydrosulfite was added in (approximately 5-10 grams) until all of the DO was consumed and oxygen level stabilized at 0%.

Tadpoles were randomly netted out of their aquaria, gently blotted to remove excess water and transferred to a dry net, and then weighed (± 0.1 mg) by placing them into a tared 100-mL beaker containing 50 mL clean water used to fill rearing tanks. After developmental staging, the tadpole was placed into the respirometry chamber, which was filled with the same type of water and sealed to ensure no atmospheric oxygen would enter the system. The tadpole was allowed 20 minutes to adjust. During the adjustment period as well as the subsequent

measurement period the tadpole was continuously monitored to ensure that it was not visibly agitated. After DO levels stabilized, data logging commenced for at least 20 min. No sessions lasted more than one hour. Careful attention was paid to the tadpole, noting the behaviors exhibited during data acquisition. Behaviors that could potentially influence measurements, such as frantic swimming, defecation, or if the animal spent time directly adjacent to the sensor, were noted; if these behaviors elicited a noticeable change in the results the data were excluded. Data were downloaded and plotted on a graph in order to determine %DO as a function of time. Slopes, over periods lasting at least 7 min in which tadpoles rested quietly, were used to calculate the rate of oxygen consumption (VO_2), taking into account the DO in saturated water at the specified temperature and the daily atmospheric pressure (Ground-based Atmospheric Monitoring Instrument Suite, University of Wisconsin-Madison; http://metobs.ssec.wisc.edu/aoss/tower/). Rates of oxygen consumption were expressed as mL O_2 /h for tadpoles of measured body mass. After all measurements, tadpoles were returned to their respective treatment tanks and monitored to ensure that they flourished.

Temperature coefficient values for acclimation effect and measurement temperature

The temperature coefficient, or Q_{10} value, is the factor by which a physiological process increases with an increase in ten degrees Celsius. Calculation of a temperature coefficient can be achieved using equation 1, where:

$$Q_{10} = (k_1/k_2)^{(10/(T2-T1))} \qquad \text{(eq. 1)}$$

where k, is the rate value for the reaction and T, is the temperature in Celsius (Hill et al. 2004).

To make clear our comparisons with previous studies done on Q_{10} we make a distinction according to how data are collected. When rates are measured at two temperatures in tadpoles reared (i.e., acclimated) at one particular temperature, we refer to the calculated Q_{10} as an

"acute" Q_{10} coefficient. When rates for tadpoles reared and measured at one temperature are compared to rates for tadpoles reared and measured at a different temperature, we refer to the Q_{10} as an "acclimation" Q_{10} coefficient. The distinction is important because the acute Q_{10}'s can differ from the acclimation Q_{10}'s in frogs (Feder 1985).

Tissue collection and chemical analysis

Approximately weekly, 48 individual tadpoles were randomly removed from treatment tanks (12 tadpoles per diet per rearing temperature) for measurement of GS, body length (snout to tail, ± 1 mm), and body mass (as above). Tadpoles held at 27 °C were collected on 24, 31, and 38 dph and tadpoles held at 22 °C were collected on 25, 32, and 38 dph. For purposes of statistical analysis by ANOVA we assigned ages as 24.5, 31.5, and 38 dph. Tadpoles were euthanized in buffered 1% MS-222 according to IACUC protocol and used for several body composition analyses. In order to remove the contents of the distal 1/6 of the intestine (the rectum), intestines were dissected out, perfused using a small amount of buffered water, and the digesta samples were then dried, weighed, and ashed (Gleason et al. 2016). Diet dry matter digestibility (*DMD*) was calculated based on comparison of ash content in food samples and ash content in digesta in the distal intestine by the inert marker method (Karasov and Martínez del Rio 2007):

$$DMD^* = 1 - (\text{food ash content}/\text{digesta ash content}). \quad \text{(Eq. 2)}$$

If ash were a perfect inert marker (i.e., indigestible, non-absorbable), then its concentration in digesta increases as digestible organic matter is absorbed from the food mass, and the ratio of ash in food/digesta is an index of how much of the food mass is indigestible. We previously validated that this method, using digesta samples from the distal 1/3 of the intestine, provides an estimate of *DMD* similar to that measured by measuring food intake and excreta production

(Gleason et al. 2016). We subsequently found in additional validation trials with 82 other tadpoles that ash content from the distal 1/3 of the intestine did not differ significantly from that in the distal 1/6 ($F_{1,76}$=2.42, P = 0.12), which we therefore used in this study.

To confirm exposure to PBDEs we performed limited chemical analyses on tadpoles at 35 dph, i.e., after 17 days feeding on diets containing PBDE. This length of time exceeds the $t_{1/2}$ for PBDE elimination by 2.9 times at 23 ^{o}C (Cary and Karasov 2013) and by 4 times at 27 ^{o}C (Tsai-Brown, Yahn, and Karasov, unpublished data), so tissue concentrations would be measurable but not necessarily at steady state values. Tissue concentrations of PBDEs were measured in groupings of 3-4 tadpoles per pool in order to insure at least 7 grams of tissue per pool. Diet and tissue samples were sent to ALS Environmental, ALS Group USA (Kelso, WA, USA) for measurement of PBDE concentrations. Chemical analysis (K1413603) was performed according to the laboratory's NELAP-approved quality assurance program (www.alsglobal.com). Minimum detection level was 0.6 ng g^{-1} wet food or wet tadpole.

Constituent analysis

After tadpoles had their G.I. tract removed the remaining carcass was placed in a new vial, capped, and frozen at -20 ^{o}C until analysis of body composition could be performed. To begin the analysis of body composition, a tadpole was thawed and removed from the vial, placed into a mortar, flash frozen with a small amount of liquid nitrogen, and fully homogenized with a pestle. Homogenized tissue was aliquoted into various pools to determine, total lipid content, tissue water content, the proportion of tissue that is dry matter, and the ash content of tissue.

Approximately half of the homogenized tadpole tissue (weights ranged from 0.0365 – 0.7306g) was used to determine total lipid content by methods adapted from Folch (Folch et al. 1957). Briefly, the homogenized tissue was placed in filter paper and the filter paper placed in a

funnel over a massed culture tube. Then a 2:1 chloroform: methanol (CH:MeOH) solution was poured over the tissue and through the filter paper/funnel dissolving all of the lipid and soluble protein in the tissue. The filter paper was then allowed to drain, but not dry, and was then rinsed using a spray bottle containing 0.23% NaCl solution forcing any lipid left in the filter paper into the culture tube. Tubes were then centrifuged at 15G for 20 minutes at room temperature, which separated the lipid and protein phases in the CH:MeOH solution. The protein phase was then drawn off and discarded and the remaining lipid solution was put in an N_2 evaporator at 50 ^oC. The culture tubes containing lipids were weighed following two days in the N_2 evaporator (and then again 24 hours later to ensure that the mass was stable). The final weight minus the culture tube was taken to be the total mass of the lipids in the tissue sample.

For simplicity, we ignore changes in carbohydrate and protein content because they have relatively low effect on tissue energy density compared with that of changes in lipid. This is because the energy density of lipid (approximately 39.5 kJ/g per dry or wet mass) is about eight times higher than those of carbohydrate and protein, which are rather similar to each other (range 18-22 kJ/g dry tissue, or approximately 5 kJ/g wet mass in tissues with 75% water) (Karasov and del Rio 2007).

Other aliquots of homogenized tadpole tissue were used to determine tissue water content and ash content. Tissue was gathered in aluminum weigh boats and weighed to get an initial wet weight and placed in a drying oven at 50 ^oC. Following two days of drying the weigh boats were weighed again to determine the dry weight of the tissue and the water content by difference. Dried samples were then placed in a muffle furnace at 550 ^oC. After being combusted for four hours the samples were weighed a final time to determine the proportion of ash content in tissue dry mass.

Statistical analyses

All statistical tests were run in R version 3.3.0 and R studio, and all reported averages are followed by the standard error of the mean (R Core Team 2016). We fit linear mixed models to test at each age (24.5, 31.5, 38 dph) for the effect of rearing temperature (22 or 27 ºC) and diet (± PBDE) and their interactions on GS, body length, body mass, fat content, tissue water content, ash content, and DMD (each of the aforementioned response variables were fit in separate models), using R's Lme4 package (Bates et al 2015). We used maximum likelihood criteria and fit rearing temperature, dietary PBDE exposure, and their interactions as fixed effects in our models. Rearing tank was included as a random effect to control for repeated measures and non-independence of our samples and to avoid psuedoreplication. To test for the significance of the fixed effects and their interactions likelihood ratio tests were generated for full models vs. models without the fixed effect of interest and compared using a Chi square distribution (Killpack et al. 2013). However, we did not include age as a factor in testing hypotheses about effects of rearing temperature, diet, and their interaction on GS, body length, and body mass because that would most likely obscure our effort, for the following reason. Based on general patterns in ectotherms, we expected and observed that older, more developed tadpoles approach asymptotic size at an earlier age at warmer temperature, which can lead to paradoxical results wherein more slowly developing animals raised in cooler temperature continue to grow and become larger than those in warmer temperature (Karasov and Martinez del Rio 2007). Thus, including age as a factor would surely be complicated with difficult to interpret interaction factors unrelated to our a priori hypotheses. However, we did include age as a factor when assessing whether or not body composition (tissue water content, % lipid, % ash) changed throughout development.

Following our analysis of DMD using the aforementioned linear mixed model at 24.5, 31.5, and 38 dph, we also analyzed DMD as a function of developmental stage. To assess DMD as a function of GS, we used an ANCOVA, and an additional post hoc test, to test for the effect of rearing temperature and dietary PBDE exposure and their interaction on *DMD**, using GS as the covariate as it is more biologically meaningful on physiological processes than age (Kooijman 2000).

To evaluate the effects of temperature and dietary PBDE exposure on respiration rate we first tested for the effect of body mass on respiration rate via ANCOVA. Respiration and wet mass data were log transformed to approximate a more normal distribution and tadpoles were grouped according to the temperature at which they were reared (T_{rear}), diet consumed, and the temperature at which their oxygen consumption rates were measured (T_{ran}). Results from the ANCOVA on all animals in all eight treatment combinations revealed no significant difference in slope for all groups, with a common slope near one (see Results). Thus we normalized respiration rate by dividing by mass raised to the power one and then fit a linear mixed model to test how mass-specific respiration rates were affected by T_{rear}, T_{ran}, and diet (fixed effects) and rearing tank as the random effect (similar to above), and all interactions. Mass-specific respiration rate average and SEM were computed for each group and temperature coefficient values (Q_{10}) were computed.

After each statistical analysis residual plots and Q-Q plots were created to check for constant variables and the assumption of normality respectively, for each dataset. For all tests, the significance level was set at $P < 0.05$, and $0.05 < P < 0.10$ was considered to indicate a trend.

RESULTS

Tissue residues of PBDE

We measured PBDE in tadpole tissues 39 dph, following 21 days of dietary PBDE exposure. Tadpoles reared on control diet at 22 and 27 ºC contained less than 1 ng of total PBDE's per gram of body tissue (Table1) whereas tadpoles fed diet containing PBDE had 148.4 ± 27.7 ng/g total PBDEs ($n = 4$), confirming the difference in exposure to PBDE. There was no significant difference in total PBDEs between tadpoles at the two temperatures ($t_3 = 0.82$, $P > 0.8$), although we had low power to detect a difference considering our small sample size.

Growth and development

At age 18 dph, tadpoles at 22 and 27 ºC did not differ significantly for Gosner stage, body length, or wet mass (Fig. 1 A-C; all *t*-test *P*'s > 0.2; $n = 6$ tadpoles per temperature, each from a different tank). Subsequently, tadpoles at each temperature were fed diets without or with PBDE. Over the next two weeks, measures of both size (length and/or mass) and development (GS) were significantly lower in tadpoles held at cooler temperature and in tadpoles fed diet with PBDE (Fig. 1 A-C; Table 2). Lower GS persisted through 32 dph in tadpoles held at cooler temperature and fed diets with PBDE (Fig. 1 A), but size measures began to converge and to overlap during the fifth week post hatch as increases in tadpole size became asymptotic, more so at the warmer than cooler temperature (Fig. 1 B, C). Notably, the effect of temperature on growth and development at each age group, was not altered by the diet manipulation, as indicated by nonsignificant interactions between temperature and diet at all measurement periods (all *P*'s > 0.11; Table 2).

To characterize the effect of rearing (i.e., acclimation) temperature on rates of growth we calculated Q_{10} values on crude rates of increase over the time interval 18 dph to 31 or 32 dph,

after which time size vs. age became visibly asymptotic (Fig. 1 B, C; Table 3). The rates for increases in length and mass were calculated using the mean values from Table 2 for each of the 4 treatment groups. To better understand rates of development, we calculated for each treatment group the number of days it took to reach GS 35, when starting at the common mean GS at 18 dph (30.3 ± 0.4, n = 12); rate of increase in GS = (35-30.3)/days. Although these calculations yield only two Q_{10} estimates for each parameter, it was apparent that rates of growth in mass and length had Q_{10} values (1.24 ± 0.14, n = 4) considerably lower than Q_{10} for development rate (4.1 ± 1.88, n = 2; $t_4 = 3.44$, $P = 0.026$).

Body composition of tadpoles

Results from the linear mixed model indicate that lipid content increased with tadpole age (Fig. 2A; $P < 0.001$). However, based on comparisons at each age, PBDE in the diet had no significant effect on lipid content, though there were trends for lower lipid content at 24.5 and 31.5 dph. Rearing temperature had no significant effect on lipid except in the youngest tadpoles measured. Neither water content (Fig. 2B) nor ash content (Fig. 2C) varied significantly with age (respectively, $P = 0.055$ and $P > 0.15$), and rearing temperature and PBDE in the diet had no significant effects except for an effect of dietary PBDE significantly lowering the ash content of tadpoles measured at 24.5 dph (Fig. 2C).

Dry matter digestibility

Ash content of food samples, 0.091 ± 0.0022 g ash/g dry food (n = 23), did not differ significantly with type of diet ($F_{1,17} = 1.12$, $P = 0.30$), the measurement period ($F_{2,17} = 0.21$, P = 0.82), or the interaction of the two ($F_{2,17} = 2.71$, $P = 0.10$). Apparent dry matter digestibility was calculated from comparison of ash in digesta with that in food according to equation 2. Results from all linear mixed models revealed no effect of rearing temperature, dietary treatment, or their

interactions on *DMD** at 24.5, 31.5, and 38 dph (all *P's* > 0.2, see Table 4), although DMD* declined with age ($P > 0.001$). Given that there was a trend for an effect of temperature (P = 0.055), and that age and GS are likely correlated, we also performed an ANCOVA with GS as the covariate. That analysis indicated that *DMD** declined with increasing Gosner stage and decreased rearing temperature, with no significant effect of diet (± PBDE) or its interaction with rearing temperature (Fig. 3, Table 5). The decline in *DMD** between 27 ºC and 22 ºC was approximately -0.06, or nearly 10%, and the decline with increasing GS was approximately -0.2 between GS 31 and 42, or about 30%.

Tadpole respiration

Oxygen consumption (VO_2) was monitored for 66 tadpoles, grouped according to the temperature at which they were reared, diet consumed, and the temperature at which their VO_2 was measured (called run temperature). In total there were 8 different groups to which animals were assigned (Fig. 4). Log VO_2 increased with log body mass ($F_{1, 50} = 281.92$; $P < 0.001$), with differences among the 8 groups ($F_{7,50} = 3.59$; $P < 0.001$) but, no difference in slope (i.e., interaction $F_{7,50} = 2.15$; $P > 0.5$; Fig. 4A). The common slope for all 8 groups was 1.15 ± 0.12, so we normalized all rates by dividing by mass raised to the power 1, which we hereon refer to as mass specific resting metabolic rate (RMR) or mass specific VO_2. To confirm our method of mass correction, we plotted mass specific VO_2 against mass or GS and found no significant dependence of mass specific VO_2 on either mass (Fig. 4B) or developmental stage (Fig. 4C; *P's* > 0.05).

Respiration temperature coefficients Q_{10}

Temperature coefficients were computed to understand the rate increase incurred by a ten-degree Celsius increase in temperature. The Q_{10} coefficient for tadpoles reared at 22 ºC,

using the mean VO_2 measured at run temperatures of 22 °C and at 27 °C (termed acute Q_{10}) was 1.63, and the corresponding Q_{10} for tadpoles reared at 27 °C was 1.39 (Fig. 5). The Q_{10} was twice as high using VO_2 of tadpoles reared and measured at 22 °C and those reared and measured at 27 °C (termed acclimation Q_{10} – 3.06; Fig. 5).

DISCUSSION

Tissue residues of PBDE

To test our hypotheses about how PBDE affects growth and development in *L. pipiens* tadpoles we reared tadpoles at two different temperatures (22 & 27 °C), fed diets either not containing or containing a technical PBDE mixture DE-71; a mix of congeners that has been used widely in the Laurentian Great Lakes. Based on a previous study in our laboratory, we chose a dietary concentration of 100 nanograms of total PBDE per gram of diet, a concentration that previously slowed growth and development in leopard frog tadpoles (Cary Coyle and Karasov 2010). After 21 days of dietary exposure tadpoles bioaccumulated total PBDE to a concentration similar to levels measured in their diets, 148.4 ± 27.7 ng/g, and similar to amounts found in Great Lakes salmon (Manchester-Neesvig et al. 2001) as well as other Great Lakes biota (Stapleton and Baker 2003), demonstrating the ecological relevance of our dietary concentration. Interestingly, the average total concentration of PBDE in tissue did not differ between our two temperature treatments, which is a result consistent with our earlier study of PBDE kinetics (chapter 1), but admittedly we had low power to detect such a difference in this study ($n = 4$).

PBDE effects on development and growth

As we expected, development was slowed in tadpoles exposed to PBDE; GS was not significantly different at 24.5 dph but it was significantly lower in PBDE treated tadpoles at 31.5 and 38 dph (See Figure 1. A). It is worth pointing out that in Figure 1A, control animals reared at

27 ^{o}C, did not appear to advance in GS from 31.5-38 dph. However, this is an artifact of our animal husbandry protocol and not a lack of development by this group. Specifically, whenever a tadpole reached GS 42, they were removed from their respective aquarium. Tadpoles held at 27 ^{o}C had the highest developmental rate and after 31.5 dph some of the controls (0 PBDE) had entered metamorphic climax (GS 42) and were removed. This left the less developed tadpoles to be sampled at 38 dph, giving the appearance that animals in this group were not developing. However, this did not affect any of our major conclusions as we focused on the results from 24.5 and 31.5 dph.

The results illustrating PBDE's depression of development are consistent with another study from our laboratory despite a dramatically shorter window of exposure to PBDE, 21 days *vs* 40 days (Cary Coyle and Karasov 2010). In the latter study, tadpoles were fed diets containing concentrations of PBDE ranging from 7, 18, 152, and 277 ng/g, and tadpole development was slowed at all concentrations except the highest. Similar findings in a study done on *X. tropicalis* (West African clawed frog) showed that exposure to BDE-47 & 99 (both of which occur in DE-71) resulted in retarded development relative to control animals (Carlsson et al. 2007). Additionally, when *X. laevis* (African clawed frog) was reared on a diet containing DE-71 the time to reach metamorphosis was delayed by 25% (Balch et al. 2006).

The processes of tadpole development and subsequent metamorphosis is tightly regulated by the thyroid hormones triiodothyronine (T3) and thyroxin (T4) (Wong and Shi 1995). During this process circulating levels of thyroid hormone cause the morphological and physiological changes that characterize tadpole development. In another study by our group, Frietas *et al* found that PBDE-exposed *L. pipiens* tadpoles had lower concentrations of whole-body T3 levels while simultaneously showing significantly higher levels of whole-body corticosterone (Freitas et al.

2016). These hormonal profile changes were consistent with findings that PBDE exposed tadpoles exhibit retarded development. Additionally, studies have shown that certain PBDE congeners structurally resemble receptor binding features of thyroxine and competitively bind with the proteins responsible for their circulation, directly reducing circulating levels of the hormones (Hallgren and Darnerud 2002; Zhou et al. 2002). This effect of competitively inhibiting the binding of thyroxine by PBDE has been shown in rat dams and pups (Zhou et al. 2002), as well as *in vitro* using human transport proteins (Meerts et al. 2000).

Measures of size, both mass and body length, showed a strong influence of dietary PBDE exposure as compared to control tadpoles (see Figures 1 B & C). At 24.5 and 31.5 dph, PBDE exposed tadpoles were significantly smaller in length than were control tadpoles. Body mass showed a similar response, where PBDE exposed tadpoles were significantly smaller at 31.5 dph, but only trending smaller at day 24.5 ($P = 0.06$). These findings are in accordance with findings by Cary Coyle & Karasov, who found PBDE exposed tadpoles were smaller at premetamorphic stages as well as at the end of metamorphic climax (Cary Coyle and Karasov 2010).

Smaller tadpoles are susceptible to a wider variety of predators and delaying development increases the time which tadpoles are exposed to aquatic predation. A 7-year study on *Rana sylvatica* (wood frogs) found that the tadpole was the life stage with the lowest survivorship (*vs.* juveniles or adults) (Berven 1990). Additionally, survival was higher in juveniles that metamorphosed earlier and at larger sizes. These findings are consistent with other studies on amphibians (Semlitsch et al. 1988; Scott 1994) indicating that juvenile and adult fitness are strongly tied to larval size and development rate. Therefore, it is reasonable to assume that the delayed development and retarded growth we saw in PBDE exposed tadpoles could have drastic consequences on wild amphibian populations.

Retarded development in PBDE exposed tadpoles results from inhibiting or antagonizing various hormonal pathways. However, effects of PBDE on growth are less studied and to date no explanatory mechanism has been identified. Studies done on fish, birds, and marine invertebrates, have shown PBDE exposure to negatively affect growth patterns, but have yet to identify the mechanism behind these effect (Chen and Hale 2010; Chen et al. 2010; Han et al. 2015). Therefore, one of the objectives in the present study was to investigate if the effects of PBDE on growth could be explained in terms of energy gains and losses. If differences in digestive efficiency (energy gain), respiration (energy expenditure), or body composition exist then future studies could be directed toward identifying the mechanism underlying these differences.

Testing hypotheses: PBDE effects on body composition

Body composition, in conjunction with mass change, determine energy devoted to production of new tissue. We tested for changes in body composition to see whether proportional changes in mass due to PBDE or temperature correspond to similar proportional changes in production. Overall, the answer seems to be yes, because there were no large changes in body composition due to either PBDE treatment or temperature difference. Curiously, ash content at 24.5 dph, was the only measure of body composition that was significantly lower in PBDE tadpoles (Figures 2 A, B, and C). However, there was a trend for lower lipid content in PBDE-fed tadpoles at 24.5 and 31.5 dph (P = 0.063 & 0.07, respectively) indicating that the proportional decline in production may be greater than the proportional decline in mass in PBDE fed animals, because lipid has about twice the energy content per gram dry mass as protein or carbohydrate. Additionally, there was a marked increase in lipid content with age in tadpoles, however this was somewhat expected because increases in lipid stores is well established during the normal

development of tadpoles to fuel the high costs associated with metamorphosis (Sheridan and Kao 1998). The trend for reduced lipid content in tadpoles fed PBDE (see Figure 2.) could have ecological significance for tadpole development, metamorphosis, and survival. Reduced fat accumulation, which is used to fuel the high costs of metamorphosis, could delay metamorphosis until enough fat is accumulated. Alternatively, if metamorphosis is not delayed but is undergone with insufficient energy stores, tadpoles could starve. Additional studies on reduced body fat content in tadpoles and its' effect on development, metamorphosis, and survival are warranted.

Testing hypotheses: PBDE effects on digestive efficiency

Measures of apparent dry matter digestibility were made to test the hypothesis that reductions in digestive performance could explain the slower growth of PBDE fed tadpoles. However, PBDE fed tadpoles exhibited similar values for *DMD** as their control counterparts. Additionally, to the best of our knowledge, no study to date has demonstrated decreased digestive performance in an animal fed PBDE. Hence, we rejected this hypothesis.

Interestingly, between GS's 31 and 42 *DMD** declined by approximately 0.2 units (approx. 30%), a result which was not found in a previous study done on the digestive performance of tadpoles in our laboratory (Gleason et al. 2016a). However, in that study tadpoles spanned a smaller range of developmental stages 33-41. Additionally, measures of *DMD** in the Gleason study were made by pooling digesta from a pool of three tadpoles from digesta harvested from the distal $1/3^{rd}$ of the GI tract. Here measures were made on individual tadpoles using only the colon (distal $1/6^{th}$), which should afford a more accurate measure, yielding more power to detect a difference. Additional studies of digesta retention time and hydrolysis and absorption rates, both as a function of GS, are needed to understand the underlying mechanistic basis for developmental decline in *DMD**.

Rearing temperature also significantly affected *DMD**, which was approximately 0.06 units (approx. 10%) higher in tadpoles reared at the warmer temperature. A similar pattern was found on *Bufo spinulosus,* where tadpoles reared on low quality diets exhibited higher digestive efficiency when reared at warmer temperatures (Benavides et al. 2005). Increased measures of DMD at higher temperatures could be explained by increased hydrolysis of food and/or increased absorption rate of products of hydrolysis. Alternatively, if tadpoles at 27 ^{o}C increased their food intake to the point where food became limiting they might have engaged in higher amounts of coprophagia, resulting in increased *DMD**.

Testing hypotheses: PBDE effects on respiration

We hypothesized that slower growth in PBDE-exposed tadpoles might be due to higher respiratory energy losses, perhaps due to costs of biotransformation and elimination or direct effects of PBDE on metabolism. Although to date no studies on respiratory rates of amphibians exposed to PBDE (or similar compounds) have been done, an analogous study on *Rana tigrina* tadpoles showed that slower growth in carbaryl exposed tadpoles was associated with increased metabolism (Marian et al. 1983). However, our study revealed no difference in the metabolic rates of control and PBDE fed tadpoles, and so we rejected this hypothesis.

We have confidence in our measures of metabolism based on comparison with literature values of both absolute magnitude and the effect of measurement temperature. For example, Feder (1981) also measured rates of VO_2 in fed tadpoles of a leopard frog - *R. berlandierii* (the Rio Grande leopard frog) (Feder 1981; Frost et al. 2006). In tadpoles acclimated at 25 ^{o}C and measured at 25 ^{o}C, VO_2 was 0.175 mL O_2 g^{-1} h^{-1}. In tadpoles we reared (i.e., acclimated) at 22 ^{o}C, VO_2 was 0.15 mL O_2 g^{-1} h^{-1} at the measurement temperature of 22 ^{o}C and was 0.213 mL O_2 g^{-1} h^{-1} at the slightly higher measurement temperature of 27 ^{o}C, thus bracketing Feder's data. The

calculated Q_{10} for VO_2 of our tadpoles reared at 22 °C (which we call acute Q_{10}) was 1.63, close to Feder's Q_{10} for *R. berlandierii* (Feder 1985; Table 6). Indeed, our acute Q_{10} values for tadpoles reared at either 22 or 27 °C averaged 1.51, a value bracketed by values measured in other studies of leopard frogs (Table 6).

Our data illustrate why acute Q_{10} values are not necessarily appropriate for predicting the impact of a warming climate on tadpole energetics. Measures of VO_2 at both measurement temperatures (22 and 27 °C) averaged 22% higher when the tadpoles were reared at 27 *vs.* 22 °C (Fig. 5). That effect, in combination with the acute Q_{10} effect just discussed, yielded a much higher acclimation Q_{10}, which is the coefficient of rate change for animals acclimated and measured at one temperature (22 °C in our case) as compared with animals acclimated and run at a warmer temperature (27 °C in our case). The acclimation Q_{10} we measured, 3.06 is much higher and is outside the 95% confidence interval of the acute Q_{10} values reviewed in Table 6. We argue that use of this higher Q_{10} yields a better prediction of impact of climate warming on tadpole metabolism than the acute Q_{10} values summarized in Table 6. We recognize the possibility of genetic adaptation to warming temperatures, which might yield a different Q_{10}, but studies to determine this have not been performed. Comparisons of RMR of tadpoles from different populations along a latitudinal gradient do not yield a consistent pattern regarding what Q_{10} applies when comparing tadpoles from different populations that may have evolved at different temperatures (Lindgren and Laurila 2009)

Overview

Our study extends knowledge about how growth and development of frogs may be altered in a warming climate that may or may not have elevated levels of environmental contaminants. Although both temperature and toxicant exposure had significant effects on tadpoles' features

related to growth and development, we found no instances of interaction between these two environmental variables. This could simplify our predictive capability for anticipating how environmental change may influence tadpole performance.

Depression of growth rate due to PBDE exposure has been documented in birds, mammals, amphibians, and fish (Fernie et al. 2006; Viberg et al. 2008; Cary Coyle and Karasov 2010; Chen et al. 2010). We tested whether the reductions in growth of PBDE fed tadpoles could be attributed to differences in features related to energy expenditure or acquisition. Body composition analyses confirmed that the slower growth in length and mass of PBDE exposed tadpoles indeed corresponds to slower rate of energy deposition in new tissue. We rejected the hypotheses that this slower rate is due to a negative effect of PBDE exposure on digestion of food or a higher rate of metabolism in PBDE exposed tadpoles. We are left to conclude that differences in growth rate and size must be attributed to differences in feeding rate. Directly measuring feeding rates is inherently challenging in leopard frog tadpoles (Gleason et al. 2016) and so we did not make those measurements. We know of few studies testing for change in food intake of PBDE-exposed animals, but a study done on benthic invertebrates, reared on PBDE spiked sediment, resulted in smaller individuals and retarded feeding rates for PBDE exposed animals (Leppänen and Kukkonen 2004). Future studies on impacts of PBDE exposure on feeding rate and regulation of feeding are warranted.

Increases are to be expected in many physiological rates of ectotherms as body temperature increases, and the temperature effect is usefully captured in the calculation of the Q_{10} temperature coefficient. Q_{10}'s we calculated for a variety of features related to growth and development ranged from a low of 1.25 for growth rate (Table 3), 1.5 for resting metabolism (Tables 6) to a high of 4.1 for development rate (Table 3). We have also made the distinction

between what we have called acute Q_{10}'s, which are determined in animals acclimated to one particular temperature but measured at two different temperatures, and what we have called acclimation Q_{10}'s, which are determined in animals acclimated to two particular temperatures and in each case measured at the temperature to which they were acclimated. We have suggested that the latter Q_{10} is arguably more useful for predicting the impact of climate warming on physiological rates than the acute Q_{10} values.

Acute and acclimation Q_{10}'s can differ (Feder 1985), as we found in the large difference between the acute Q_{10} for RMR (1.5) and the acclimation Q_{10} (3.06). The distinction is important for predicting exposure to food-borne toxicants in tadpoles in cooler *vs.* warmer environments. Considering that RMR is a major driver of food intake because it typically accounts for the majority of an ectotherm's energy budget (Karasov and Martinez del Rio 2007), one might expect that toxicant intake rate may differ by a Q_{10} near 3 for tadpoles inhabiting warmer *vs*. cooler environments. Elsewhere, we showed that the acclimation Q_{10} for elimination of toxicants PBDE and PCB (polychlorinated biphenyls) ranged 3.3-3.8 for tadpoles over the temperature range 23 to 28 ^{o}C (chapter 1). Considering that exposure to toxicants is a function of the quotient of rates of toxicant uptake and elimination (chapter 1), one might predict that in PBDE-polluted environments faster rates of toxicant intake at warmer temperature may be near balanced by faster rate of toxicant elimination, with the net effect that toxicant exposure, measured as body burden, may not dramatically change as climate warms. However, one would not make this prediction using the (lower) acute Q_{10} for RMR. Future work on the temperature dependence of toxicant uptake and elimination, and how they will interact to influence toxicant exposure, is warranted.

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Tables and Table Legends

Table 1. Levels[1] of PBDE congeners in tadpoles harvested 39 dph and their diets.

PBDE Congener	Diet-PBDE	Diet-Control	27ºC +PBDE (2 replicates)		22ºC +PBDE (2 replicates)		27ºC Control	22ºC Control
# in pool	N/A[2]	N/A	4	4	3	3	4	3
Mass[3] (g)	N/A	N/A	1.52± 0.19	1.58 ± 0.17	2.37 ± 0.04	2.3 ± 0.33	1.73 ± 0.12	2.11 ± 0.02
G.S.[4]	N/A	N/A	40.25	39	38.67	39	40.5	39.33
PBDE 71	1.8	ND[5]	1.3	1.4	0.93	1.4	ND	ND
PBDE 47	33	ND	47	28	42	67	ND	ND
PBDE 100	12	ND	17	12	18	20	0.17	0.18
PBDE 99	48	ND	68	42	62	98	0.78	0.71
PBDE 85	4.2	ND	3.4	2.1	3	5.3	ND	ND
PBDE 154	3.5	ND	5.6	4	5.4	8.3	ND	ND
PBDE 153	8.7	ND	6.5	4.6	6.1	9.8	ND	ND
PBDE 138	ND	ND	0.89	0.59	0.69	1.3	ND	ND
PBDE 183	ND	ND	0.11	ND	ND	ND	ND	ND
∑PBDE [6]	111.2	ND	149.8	94.69	138.12	211.1	0.95	0.89

[1]Levels were measured in pools of tadpoles (*n* = # in pool) where each pool is considered a replicate, created to have at least 7 grams of wet mass per replicate, the minimum required for residue analysis. All congeners are reported as nanograms of PBDE per gram of wet mass.
[2]"Not applicable".
[3]The mean mass ± s.e. of tadpoles for each pool
[4]Gosner Stage ("GS").
[5]"Not detected"
[6]Total concentration of all congeners.

Table 2: Developmental stage, body length, and body mass (means ± s.e.) at three ages in tadpoles reared at two temperatures fed diet with and without PBDEs.

Temp (ºC)[1]	PBDE[2]	*n*	Gosner stage	Length (cm)	Mass (g)
			Age = 24.5 days post hatch		
22 ºC	0	10	33.6 ± 0.22	7.1 ± 0.25 (10)	2.0 ± 0.15
22 ºC	100	11	32.9 ± 0.39	6.4 ± 0.27 (11)	1.7 ± 0.11
27 ºC	0	12	35.4 ± 0.27	7.4 ± 0.14 (12)	2.3 ± 0.13
27 ºC	100	10	35.2 ± 0.31	6.6 ± 0.25 (10)	1.9 ± 0.17
Effect of			**$X^2 = 19.04$**	$X^2 = 0.90$	$X^2 = 1.88$
Temp[3]			**$P < 0.001$**	$P = 0.34$	$P = 0.17$
			$X^2 = 1.87$	**$X^2 = 7.33$**	**$X^2 = 3.46$**
Effect of PBDE			$P = 0.17$	**$P = < 0.001$**	**$P = 0.06$**
			$X^2 = 0.5$	$X^2 = 0.03$	$X^2 = 0.006$
Temp*PBDE			$P = 0.48$	$P = 0.87$	$P = 0.94$
			Age = 31.5 days post hatch		
22 ºC	0	12	37.4 ± 0.35	8.3 ± 0.14 (12)	3.1 ± 0.15
22 ºC	100	11	36.0 ± 0.53	7.9 ± 0.22 (11)	2.8 ± 0.19
27 ºC	0	12	38.2 ± 0.4	8.9 ± 0.16 (12)	3.2 ± 0.12
27 ºC	100	12	37.8 ± 0.36	8.3 ± 0.18 (12)	2.7 ± 0.17
Effect of			**$X^2 = 7.75$**	**$X^2 = 6.76$**	$X^2 = 0.01$
Temp			**$P = 0.005$**	**$p = 0.009$**	$P = 0.91$
Effect of			**$X^2 = 4.17$**	**$X^2 = 7.91$**	**$X^2 = 4.13$**
PBDE			**$P = 0.03$**	**$P = 0.005$**	**$P = 0.04$**
			$X^2 = 2.02$	$X^2 = 0.09$	$X^2 = 0.24$
Temp*PBDE			$P = 0.15$	$P = 0.77$	$P = 0.63$
			Age = 38 days post hatch		
22 ºC	0	12	39.7 ± 0.2	9.3 ± 0.18 (12)	4.2 ± 0.21
22 ºC	100	12	38.2 ± 0.38	8.6 ± 0.32 (12)	3.5 ± 0.32
27 ºC	0	11	41.5 ± 0.52	9.0 ± 0.29 (11)	3.2 ± 1 0.28
27 ºC	100	11	39.7 ± 0.35	9.1 ± 0.13 (11)	3.3 ± 0.2
Effect of			**$X^2 = 17.45$**	$X^2 = 0.45$	**$X^2 = 2.7$**
Temp			**$P < 0.001$**	$p = 0.5$	**$P = 0.1$**
Effect of			**$X^2 = 17.2$**	$X^2 = 2.05$	$X^2 = 1.08$
PBDE			**$P < 0.001$**	$P = 0.15$	$P = 0.3$
			$X^2 = 0.11$	$X^2 = 2.98$	$X^2 = 2.15$
Temp*PBDE			$P = 0.74$	$P = 0.08$	$P = 0.14$

Notes:

[1]Rearing temperature

[2]Total PBDE content of diet, in ng/g wet mass

[3] All p-values are a result of linear mixed model testing the effect of temperature, diet, and their interactions on GS, SVL, and wet mass, with tank as a fixed effect.

Table 3. Calculated acclimation Q_{10} values for crude rates of increase in body length, body mass, and Gosner Stage (GS) over the interval 18 dph to 31 or 32 dph[1].

Diet	Rearing Temp	Rate of increase in body length		Rate of increase in mass		Rate of increase in GS^2	
	(°C)	(mm/d)	Q_{10}	(mg/d)	Q_{10}	(GS/d)	Q_{10}
Control	22	2.7	1.49	122	1.30	0.48	2.78
	27	3.3		139		0.79	
+ PBDE	22	2.7	0.92	96	1.26	0.34	5.44
	27	2.6		108		0.79	

Notes:
[1]Rates calculated from data in Table 1 and Figure 1
[2]calculated at each temperature on each diet from the days to reach a common GS of 35 when starting at the common mean GS at 18 dph (30.3 ± 0.4, n = 12); rate = (35-30.3)/days.

Table 4. Summary results of rearing temperature, diet, and their interactions on dry matter digestibility at each time point and for all ages.

Temp (ºC)[1]	PBDE[2]	*n*	*DMD*[*3]
Age 24.5 (dph)			
22 ºC	0	11	0.61 ± 0.022
22 ºC	100	11	0.60 ± 0.023
27 ºC	0	12	0.62 ± 0.018
27 ºC	100	10	0.61 ± 0.013
Effect of Temp[4]	$X^2 = 0.23$		$P = 0.63$
Effect of PBDE	$X^2 = 0.59$		$P = 0.44$
Temp*PBDE	$X^2 = 0.07$		$P = 0.8$
Age 31.5 (dph)			
22 ºC	0	12	0.58 ± 0.035
22 ºC	100	11	0.57 ± 0.036
27 ºC	0	12	0.59 ± 0.025
27 ºC	100	12	0.56 ± 0.041
Effect of Temp	$X^2 = 0.00$		$P = 0.99$
Effect of PBDE	$X^2 = 0.42$		$P = 0.52$
Temp*PBDE	$X^2 = 0.3$		$P = 0.59$
Age 38 (dph)			
22 ºC	0	12	0.44 ± 0.040
22 ºC	100	12	0.46 ± 0.038
27 ºC	0	7	0.51 ± 0.037
27 ºC	100	11	0.51 ± 0.026
Effect of Temp	$X^2 = 1.65$		$P = 0.20$
Effect of PBDE	$X^2 = 0.08$		$P = 0.77$
Temp*PBDE	$X^2 = 0.025$		$P = 0.87$

Notes:

[1]Rearing temperature

[2]Nominal total PBDE content of diet, in ng/g wet mass

[3]DMD values represent the average for that group followed by the standard error of the mean.

[4]All p-values are a result of linear mixed model testing the effect of temperature, diet, and their interactions on DMD, with tank as a fixed effect.

Table 5. Summary results of ANCOVA for the effects on tadpole dry matter digestibility (*DMD**) on Gosner stage (GS), rearing temperature (Temp, i.e. 22 or 27 ºC), diet (Diet, i.e. ± PBDE), and their interactions.

Effects on DMD*	*D.F.*	*F*	*P*
GS	1,117	27.4	< 0.001
Temp	1,117	9.26	0.003
Diet	1,117	2.52	0.12
Interaction Temp*Diet	1,117	0.0003	> 0.9
Interaction GS*Temp	1,117	1.71	0.19
Interaction GS*Diet	1,117	0.03	> 0.8
Interaction GS*Temp*Diet	1,117	0.06	> 0.8

Table 6. Temperature coefficients (acute Q_{10}'s) of oxygen consumption in tadpoles acclimated and measured at multiple temperatures.

Acclimation Temperature[1]	Measurement Temperature[2]	Average Acute Q_{10}	Source
22, 27	22, 27	1.51[3]	Current Study
16, 25, 33	16, 25, 33	1.83[4]	Feder 85
25	22, 32	1.42[5]	Noland & Ultsch 81

Notes:

[1]Temperatures at which tadpoles were acclimated (T_{rear}), ºC.

[2]Temperatures at which measures of VO_2 were made (T_{ran}), ºC.

[3]The average acute Q_{10} was calculated from averaging the individual acute Q_{10}'s from: the mean VO_2 from tadpoles acclimated to 22 and measured at 22 and 27, and the mean VO_2's for tadpoles acclimated to 27 and measured at 27 and 22 (see Fig. 5).

[4]The Q_{10} was calculated by taking the average of the 3 Q_{10}'s given in the paper, for the acclimation and measurement temperatures listed.

[5]This is the mean of 2 Q_{10}'s measured by the authors, one for younger prometamorphic tadpoles and the other for older premetamorphic tadpoles.

FIGURES AND FIGURE LEGENDS

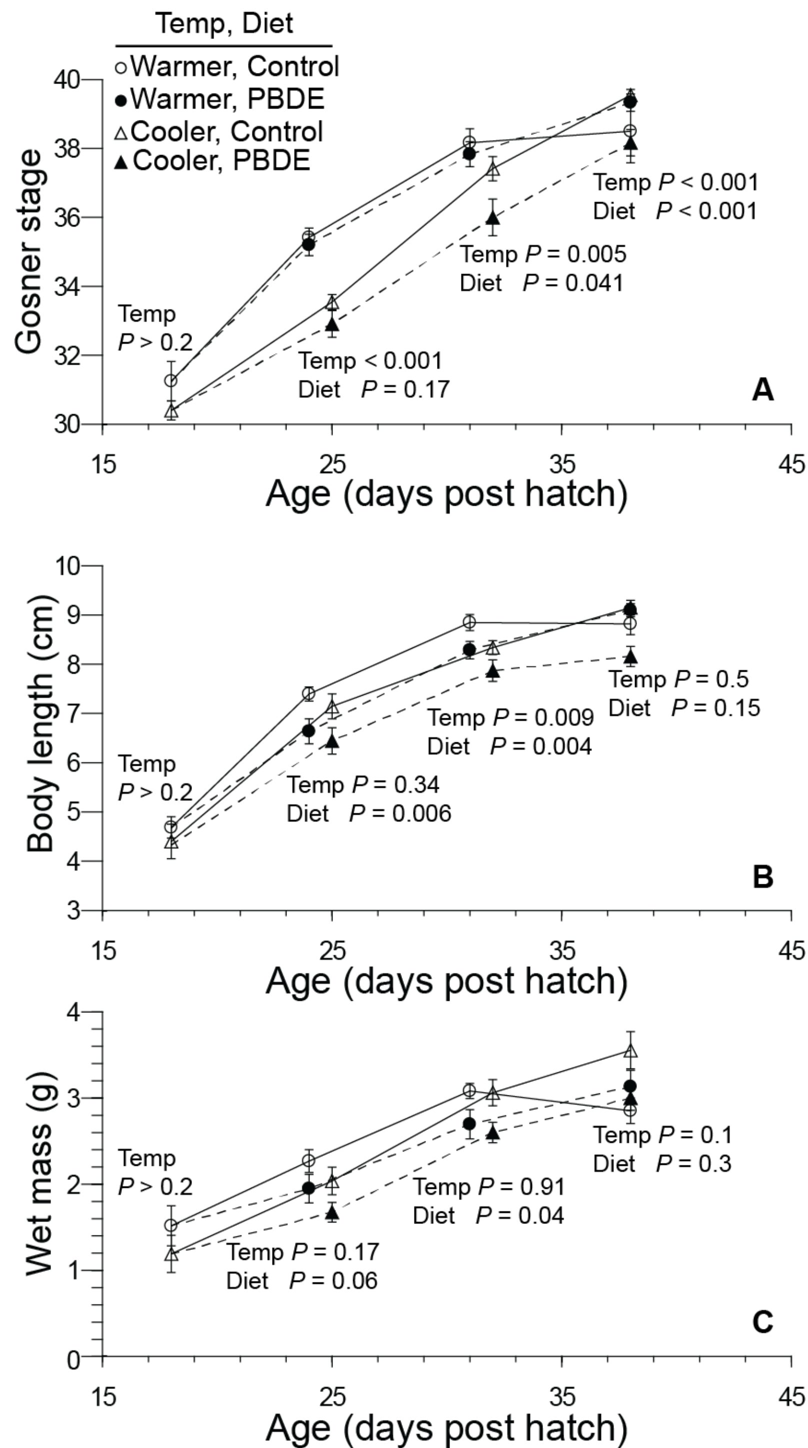

Figure 1. Developmental state (Gosner stage) (A), body length (B), and body mass (C) of tadpoles reared at the two rearing temperatures, 22 (cooler) & 27 (warmer) °C and fed diet with or without PBDE. Data represent the means ± s.e. Statistical results are from analyses summarized in Table 2.

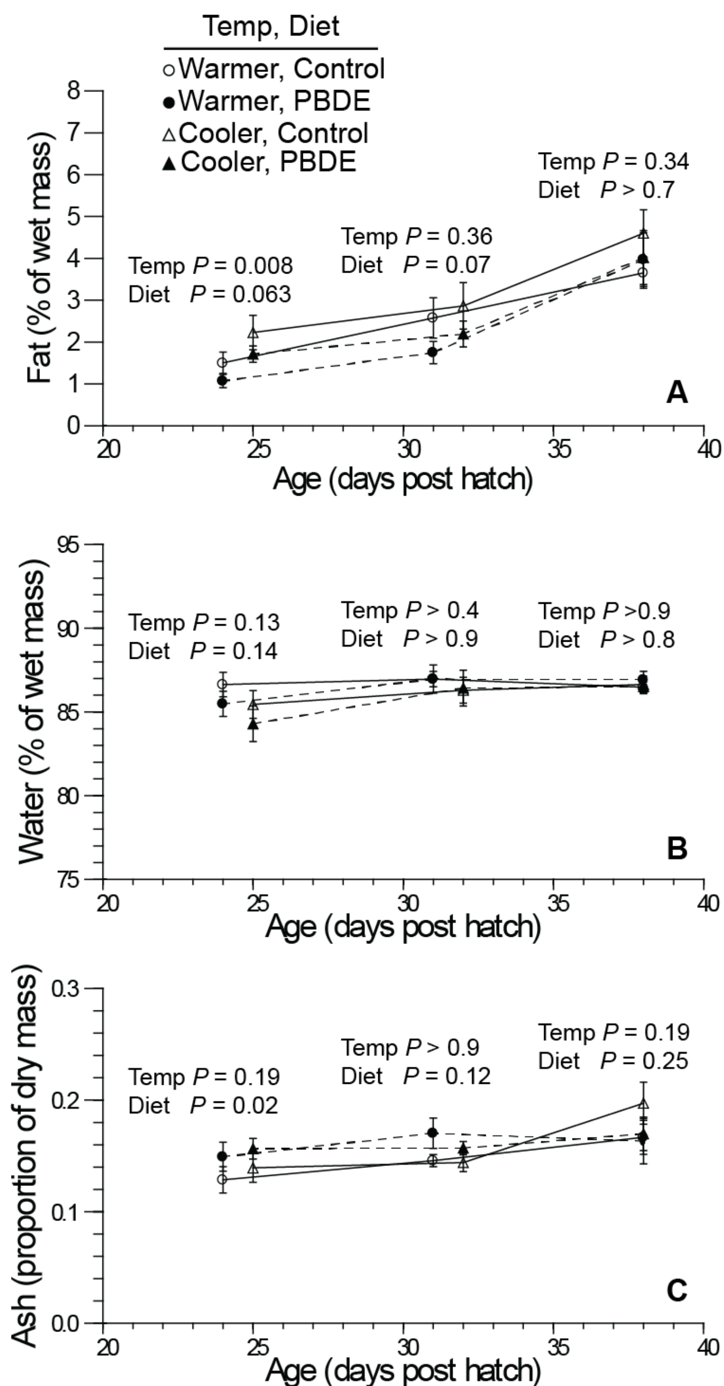

Figure 2 A, B, C. Total lipid content (A) and tissue water content (B) as a percentage of wet mass, and ash content as a proportion of dry mass (C) as a function of age (days post hatch). For all three figures, tadpoles reared at 22 ^{o}C are represented by triangles whereas tadpoles reared at 27 ^{o}C are represented by circles. Dietary PBDE exposure was administered at 18 dph and persisted throughout the rest of the experiment for all tadpoles in that treatment group as indicated by the filled symbols. All p-values are a result of a linear mixed model testing the effect of temperature, diet, and their interactions on lipid, water and ash content of tadpoles, with rearing tank set as a random effect.

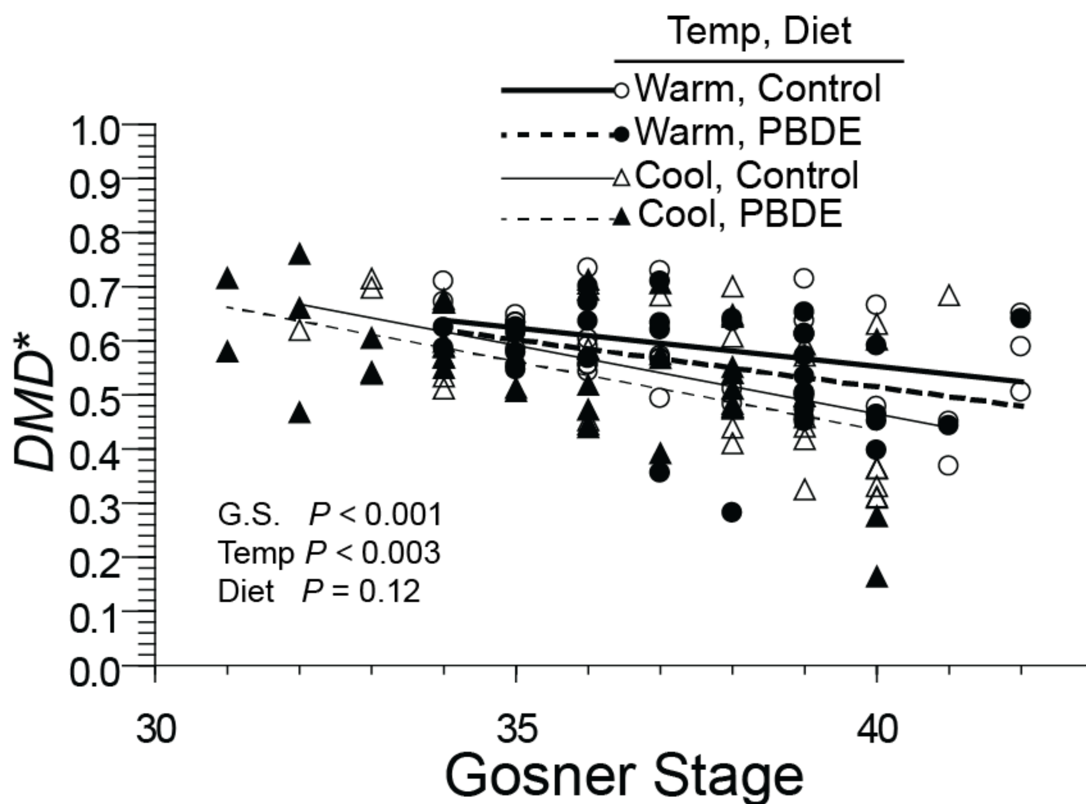

Figure 3. Apparent dry matter digestibility (*DMD**) of tadpoles as a function of developmental (Gosner) stage, rearing temperature (warm = 27ºC, and cool = 22 ºC) and diet (± PBDE). The lines, showing the best fit plots, and the *P*-values are from the ANCOVA (see Table 4). Over the course of development (GS 30-42) DMD* declined about 0.2 units, and averaged about -0.06 units lower at 22 ºC compared with 27ºC.

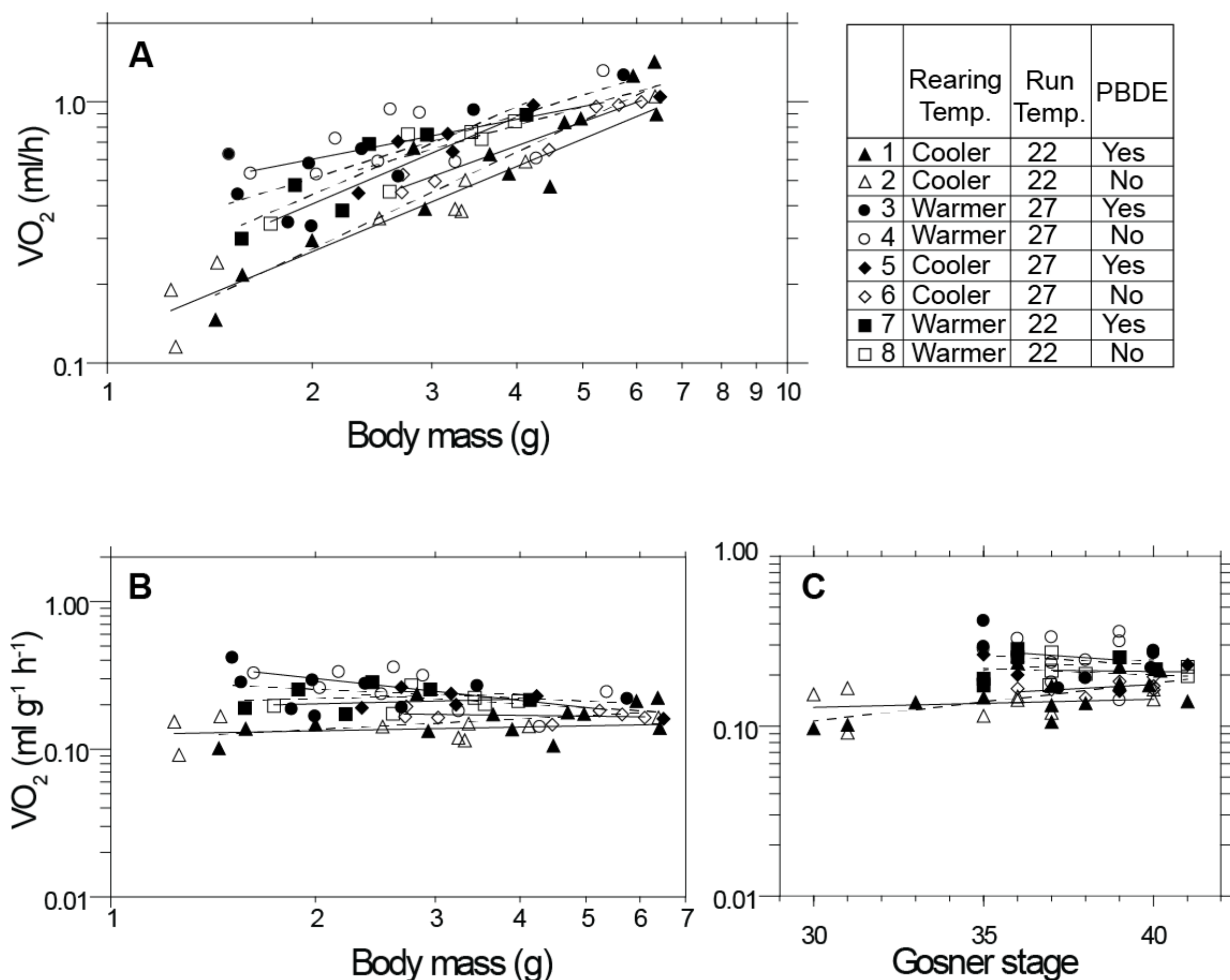

Figure 4 A, B, C. Rates of resting oxygen consumption (VO_2; ml/h) in 66 tadpoles as a function of body mass (g) (A). Each tadpole was a member of one of 8 groups defined by the routine rearing temperature ("Rearing Temp." - Cooler = 22 ºC; Warmer = 27 ºC), the temperature at which VO_2 was measured ("Run Temp." - 22 or 27 ºC), and whether it was raised on diet containing PBDE (Yes=filled symbols; No=unfilled symbols). The 8 lines shown are the linear regressions for the 8 groups (dashed = PBDE-Yes; solid = PBDE-No), which did not differ significantly in slope (common slope = 1.15 ± 0.12; $P > 0.05$). Animals that fed on diet with PBDE did not differ significantly from those that fed on diet without. Mass specific RMR (VO_2) as a function of mass (B) and Gosner stage (C) respectively. Across all masses and Gosner stages, slopes of mass specific VO_2 were nonsignificant, illustrating that the mass specific RMR is independent of Gosner stage and mass. Symbols representing the 8 group assignments are defined in the upper right table and apply to all three figures (A, B, & C). The numbers on regression lines correspond to the group numbers in the table.

In a mixed linear model, mass-specific VO_2 varied significantly with both rearing and run temperature (P's < 0.005), however there was no effect of dietary treatment ($P > 0.5$). Therefore, we removed dietary treatment as a factor from our analyses and tested the effects of rearing temperature and run temperature (Figure 5). Results indicated that both higher rearing temperature and run temperature significantly increased mass specific VO_2 ($P < 0.005$), with no apparent interaction between the two ($P > 0.98$).

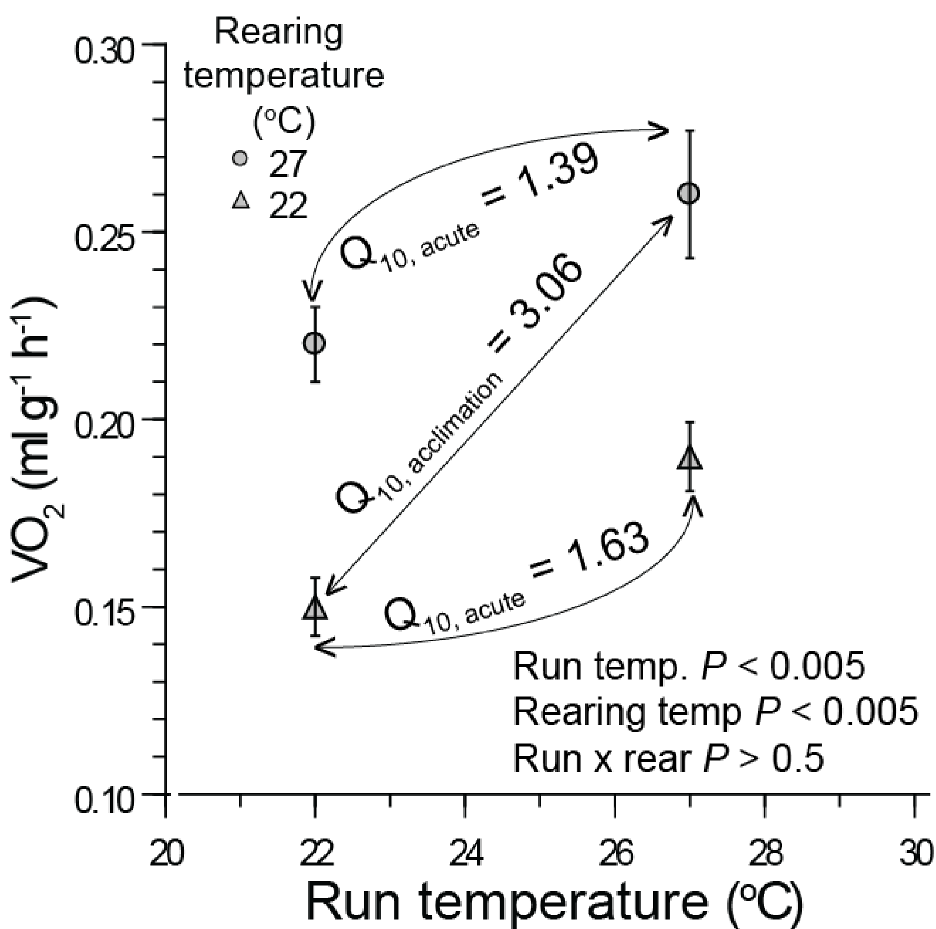

Figure 5. The average mass specific oxygen consumption as a function of run temperature for tadpoles reared at 22 ºC and 27 ºC. Experimental run temperatures were made at either 22 ºC or 27 ºC. VO_2 data are expressed in milliliters oxygen consumed per gram of tadpole wet mass per hour. Q_{10} coefficients were computed using equation 1.

APPENDIX ONE

Digestive Efficiency of Northern Leopard Frog (*Lithobates pipiens*) Tadpoles during Development, Reared on a Laboratory Diet

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Digestive Efficiency of Northern Leopard Frog (Lithobates pipiens) Tadpoles during Development, Reared on a Laboratory Diet

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Abstract: In rasping aquatic feeders, like many tadpoles, it is challenging to measure both the food consumed and excreta produced and thereby calculate digestive efficiency. The goals of this study were to measure the apparent digestive efficiency of Northern Leopard Frog (Lithobates pipiens) tadpoles on an artificial laboratory diet and test the use of putative inert markers to calculate digestibility. The diet used was commercial ground rabbit chow (primary ingredient: alfalfa meal) suspended in agar and gelatin. If dietary ash (8.35% of diet dry mass) were a perfect inert marker, then its concentration in digesta, excreta, or both, should increase as organic matter is absorbed from the food, making the ratio of ash in food to ash in digesta or excreta an index of how much of the food mass is indigestible. Ash content in digesta dissected from the distalmost portion of the tadpoles' intestine was about twice as concentrated as in food, indicating a 50% dry matter digestibility (DMD). This compared well with a DMD value based on our direct measurement of food intake and excreta production (50 6 5%), which did not vary with Gosner stage (Stages 33–41 tested). Excreta collected from the bottom of the holding tank did not yield accurate estimates of DMD. We conclude that total ash of food and digesta can be used to measure DMD when L. pipiens is offered this diet.

Key words: Digestibility; Inert marker; Larval development; Ranidae

Anuran tadpoles include a number of types of feeders such as suspension feeders, macrocarnivores, and rasping tadpoles (Hourdry et al. 1996; Venesky et al. 2011, 2014), the latter of which are thought to consume plants, detritus, and material attached to submerged substrata (variously called periphyton, epilithon, aufwuchs, and biofilm; Altig et al. 2007). Information on energy and nutrient assimilation of rasping tadpoles is sparse, partly because it is challenging to measure their food consumption and excreta production under water. Keratinized labial teeth of rasping tadpoles anchor to a substrate and subsequently lift the foodstuff off of the surface, creating a suspension (Wassersug and Yamashita 2001). Then, as in pelagic or suspension feeders (Jorgensen 1975; Seale and Wassersug 1979), the suspension is subsequently pumped/sucked in a manner that leads it through buccal/pharyngeal structures that separate the water from the suspended solids (Kenny 1969), directing the latter to brachial food traps (specialized tissues that trap the food on a mucosal surface; Wassersug and Hoff 1979). It is thus challenging to measure a tadpole's consumption of food, which is under water, not easily observed, and not taken in discrete, countable units (e.g., number of insects). Further, feces that tadpoles void might lose mass and nutrients to water immediately following discharge. Therefore, as is the case for fish, estimates of diet digestibility are likely subject to some degree of error (De Silva and Anderson 1995). Yet, it is desirable to have this information for a bioenergetic understanding of tadpole growth, development, and metamorphosis (Pandian and Marian 1985).

Northern Leopard Frogs, Lithobates pipiens, the tadpoles of which are rasping feeders, are a commonly used amphibian model in studies of development and

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ecotoxicology. Methods for captive husbandry are available (Nace 1974), and tadpole performance based on a variety of laboratory diets has been compared (Hirschfeld et al. 1970). The digestibility of a laboratory diet has yet to be determined, however, which is an impediment to modeling the tadpoles' feeding energetics during development or toxicant exposure via food in ecotoxicology studies (Cary Coyle and Karasov 2010).

The primary goal of our study was to measure the apparent digestive efficiency of Northern Leopard Frog tadpoles on an artificial laboratory diet and test the use of putative inert markers to calculate digestibility (Karasov and Martı´nez del Rio 2007). Our study focused on tadpoles at Gosner Stages 31–41 (Gosner 1960). Independent feeding starts at Stage 25, but those tadpoles were too small for experiments and we began with older, larger tadpoles of 0.5– 1.0 g in mass. Gosner (1960) considered the period between Stages 30 and 40 as one of "relative stability in key traits," with different stages defined mainly by changes in the toes and hind limbs. Following Stage 40, more dramatic changes of metamorphosis begin, including many changes in the gastrointestinal tract (Hourdry et al. 1996). Consequently, we expected that digestive efficiency would be relatively constant across Gosner stages at least up to Stage 40.

In Experiment 1, we used a direct method for measuring digestive efficiency and compared it to an indirect method based on the change in concentration of marker(s) thought to be indigestible (Karasov and Martı´nez del Rio 2007). We experimented with total ash, which has been used as a marker in tadpoles and fish (Altig and McDearman 1975; De Silva and Anderson 1995; Benavides et al. 2005). Whereas Experiment 1 required the use of isolated individuals in special holding containers and restricted levels of feeding, Experiment 2 collected comparable diet digestibility data on tadpoles housed in the more typical manner of groups in larger tanks and fed ad libitum.

Overall, we predicted that the comparison of ash content in food vs. digesta would yield accurate estimates of diet digestibility in tadpoles housed singly or in groups, and at different levels of food intake. We also anticipated that the apparent digestibility of tadpoles on the laboratory diet would be in the range of that observed in other vertebrates eating a similar kind of diet (0.39–0.68; Karasov et al. 1986; Halver and Hardy 2002). With an accurate estimate of apparent digestibility, the bioenergetics of these tadpoles can be modeled and used to address questions in fields such as habitat suitability, ecotoxicology, and climate change.

MATERIALS AND METHODS

Animal Husbandry

We obtained clutches of Lithobates pipiens embryos on two occasions from Nasco (Fort Atkinson, WI) and raised them following Cary Coyle and Karasov (2010). At Gosner Stage 25, we randomly distributed 23–24 tadpoles to each of 13 tanks containing 15 L of laboratory treated water. The density of tadpoles was low enough to avoid densitydependent inhibition of growth (Gromko et al. 1973). The standard laboratory diet was 88% ground rabbit chow (2030 Harlan Teklad Global Rabbit Diet; primary ingredient 5 alfalfa meal) suspended in 12% agar and gelatin (dry mass basis) in water (3:1 by mass), prepared as described by Hirschfeld et al. (1970) and Cary Coyle and Karasov (2010; Table 1). This diet yields satisfactory growth rates in Northern Leopard Frog tadpoles (Hirschfeld et al. 1970).

We fed tadpoles ad libitum and removed from the aquaria daily any unconsumed food (orts), feces, and any dead tadpoles. We performed an 80% water change for each aquarium every other day with the use of a static-renewal system. We checked water quality weekly (means 6 1 SD, n 5 18: pH 5 8.3 6 0.1, nitrite 5 0.02 6 0.02 mg/L, total ammonia 5 0.08 6 0.03 mg/L). Other environmental conditions included a photoperiod of 14:10 h light:dark, humidity \$ 30%, and water temperature 23 6 1uC.

Experiment 1: Comparing Direct and Indirect Measures of Digestibility

In the first experiment, we placed individual tadpoles \$4 wk in age, (Gosner Stage ,30, mass ,0.75 g) into containers for measurement of food intake and excreta production. The containers were 500-mL polypropylene jars (Thermo Fisher Scientific) equipped with 4 mm mesh suspended 15 mm from the bottom, a design adapted from Peterson and Boulton (1999) to prevent feces from being disturbed. When tadpoles were placed in the

containers simply for collection of excreta they did not defecate, and we found they would do so only when food was also provided and consumed. Consequently, we placed into each container a bottle cap to serve as a feeding dish.

We randomly assigned tadpoles to be fed either the standard diet or the standard diet enriched 1% by dry mass with oxides of either chromium (CrO_2) or yttrium (Y_2O_3). We included the oxides in the diet because they have been used as inert markers in fish (De Silva and Anderson 1995; Ward et al. 2005). The spectrophotometric analyses on some small values for mass were not satisfactory (too variable), however, so we did not use those data on oxides. In a preliminary trial, we provided to 10 tadpoles 37 6 3 mg wet food (equivalent to 3.6% of wet body mass/d); these subjects lost mass at a rate of 3.3 6 0.5% body mass/d. Consequently, the amount of food provided was increased to .80 mg wet mass, and was subsequently adjusted over a 3-d period to an amount of food that could be eaten in a 2-h period without leaving orts. This was followed by a 7-d habituation period in which the daily routine was to remove feces by suction filtration between 1600 and 1800 h, change the water, and then feed each tadpole its respective diet for 2 h, after which the feeding dish was removed. During the 2-h feeding period, any food that fell to the bottom of the container was removed with a pipette and placed back into the food dish. With these procedures, we were confident that we could measure food intake and collect excreta unpolluted by food because food was typically eaten within 2 h, and undisturbed feces could be easily distinguished from any food that might have fallen through the mesh. We cannot rule out, however, that there could be some microbial alteration of the excreta prior to its collection.

Table 1.—Nutritional analysis of the standard diet fed to Lithobates pipiens tadpoles. Sample analysis by University of Wisconsin Soil Forage Analysis Laboratory (n 5 2 measures/sample). Data on dietary ash contents are presented in Fig. 3.

Component	Units	Mean 6 1 SD
Water	Percent of total mass	79.20 6 0.04
Protein	Percent of dry mass	22.60 6 0.13
Neutral detergent fiber	Percent of dry mass	26.60 6 0.31
Nonfiber carbohydrate	Percent of dry mass	34.90 6 0.08
Fat	Percent of dry mass	2.93 6 0.08
P	Percent of dry mass	0.60 6 0.01
Ca	Percent of dry mass	1.02 6 0.01
K	Percent of dry mass	1.44 6 0.04
Mg	Percent of dry mass	0.33 6 0.01

Following the habituation period, we began a 7-d period for measuring food intake and for fecal collection and storage. Any orts remaining after the daily 2-h feeding period were dried and stored for weighing. We collected the feces from each tadpole by suction filtration onto individual filter papers over the 7-d period, so each tadpole's digestive efficiency over 7 d could be calculated. Between the daily collections, we stored the filter papers with feces from each individual at 220uC until the collection period was over (Ward et al. 2005).

After the 7-d fecal collection period (i.e., on Day 8 of the experiment), we fed the tadpoles in each treatment once more for 2 h, and then euthanized them in 1.0% MS-222 solution, buffered to a pH of 7.0. We staged, photographed, and weighed each tadpole (60.1 mg), and then dissected it to remove the gastrointestinal tract. We removed the contents of the distal third of the intestine using an intestinal perfusion of deionized water.

We dried samples of food and two kinds of digesta samples from each individual tadpole (filter papers with 7 d worth of excreta from the bottoms of the containers and intestinal digesta samples) to constant mass at 105uC for at least 24 h and then weighed the samples (60.1 mg; Sartorius ME Series Ultra-Microbalance). Later, we ashed samples at 500uC for at least 6 h in a muffle furnace and weighed the ash residue (60.1 mg). We replicated all sample weighing twice or more until coefficients of variation were ,2%. We found that digesta samples #5 mg were too small for accurate determination of ash content, and so we typically pooled small samples of 2–3 individuals fed the same diet at the same time. Altogether, we ashed 28 samples of food, 17 samples of intestinal digesta, and 22 samples of excreta from cage bottoms.

Experiment 2: Digestibility Estimated in Tadpoles Fed Ad Libitum

In the second experiment, each group of 20 tadpoles, starting at Gosner Stage 25, remained in a 20-L aquarium (four tanks total) that did not have a screen bottom. We fed tadpoles ad libitum each day by placing 3–5 blocks of base diet into the aquaria. We increased the amount of food provided coincident with tadpole growth, but blocks averaged 0.5 g wet mass (range 5 0.25–0.95 g). Prior to water changing and the addition of fresh food, we removed any remaining orts, as well as all feces. We euthanized tadpoles at Gosner Stages 30–41, then weighed and dissected each to remove the gastrointestinal tract. We removed the contents of the distal third of the intestine, perfusing it with deionized water. The digesta samples were then dried, weighed, and ashed as described for Experiment 1.

Calculations and Statistical Analyses

Food consumed in Experiment 1 was calculated as the difference between the daily ration and orts (both on drymass basis). Apparent dry matter digestibility (DMD*) was calculated as

$$DMD = 1 - (Q_e / Q_f), \quad (1)$$

where Q_f is rate of food intake (dry mg/d), and Q_e is rate of excreta production (dry mg/d).

Digestibility was also calculated based on comparison of ash content in food and ash content of digesta in the distal intestine, or in excreta at the bottom of the feeding container by the inert marker method (Karasov and Martı´nez del Rio 2007):

$$DMD_{ash} = 1 - (\text{food ash content} / \text{digesta or excreta ash content}) \quad (2)$$

If ash content was a perfect inert marker (i.e., indigestible, nonabsorbable), then its concentration in digesta and excreta would increase as digestible organic matter is absorbed from the food mass. Thus, the ratio of ash in food/excreta is an index of how much of the food mass was indigestible.

In Experiment 1, we studied 34 tadpoles that were between Gosner Stages 33 and 41 at the end of the study period. The next higher stage, 42, is metamorphic climax, and tadpoles typically do not feed at that stage. Body mass, daily food intake rate, and DMD* (both untransformed and arcsine–square root transformed) were compared as a function of Gosner stage with the use of simple linear regression. Initially, we included the particular diet (base diet or enriched with CrO_2 or Y_2O_3) as a second factor, but diet was never a significant factor in these or other analyses, so data from different diets were typically pooled in the analyses. The single exception was in the analysis of ash content of samples.

Variation in DMD* as a function of rate of food intake was determined with the use of a nonlinear curve fitting to the equation

$$DMD = DMD - E_m / Q_f, \quad (3)$$

where E_m (units dry mg/d) is daily endogenous mass loss (e.g., sloughed cells or microbial matter from the digestive tract), Q_f is rate of food intake (dry mg/d), and DMD is true digestibility, which should reflect the total proportion of food that is digested and absorbed (Karasov 1990). DMD* is considered an apparent coefficient of digestibility (hence, the asterisk) because Q_e includes not only undigested food residue, but also endogenous losses (e.g., sloughed cells of the digestive tract) and residues of gut microbes. The matter and energy in those

losses ultimately come from food, which is why DMD* is arguably the most relevant measure from an energy budget perspective (i.e., it reflects the net matter and energy gained from food).

In Experiment 2, digestibility was calculated based on a comparison of ash content in food and digesta in the intestine. Ash contents as a function Gosner stage were analyzed by simple linear regression.

All values are reported as means 6 1 SE (n 5 number of subjects). In instances for which DMD^*_{ash} was based on the ratio of mean digesta ash content relative to mean food ash content (i.e., Eq. 2), the mean and variance of the ratio was estimated based on the mean and variance for the numerator and denominator (Stuart and Ord 1994). SYSTAT was used for all statistical analyses (Wilkinson 1992), with a significance set at a, 0.05.

RESULTS

Experiment 1: Comparing Direct and Indirect Measures of Digestibility

Among 34 tadpoles, both body mass and daily food intake correlated with increasing Gosner stage (respectively,
$F_{1,32}$ 5 20.90, P, 0.001, Fig. 1A; $F_{1,32}$ 5 26.40, P, 0.001, Fig. 1B), but DMD* did not ($F_{1,32}$ 5 2.35, P 5 0.14; Fig. 1C). Based on rates of intake and excreta production, DMD* averaged 0.50 6 0.05 (n 5 34). ANOVAs were also performed, which included a factor for the particular diet that was fed (standard or enriched with either Cr_2O_3 or Y_2O_3), but diet was never a statistically significant factor in those analyses (P. 0.3).

Some values of DMD* were relatively low and in some cases negative. When daily food intake is low, the relative impact of endogenous mass losses on the calculation of DMD* is large (Eq. 3). We tested for this effect by nonlinear regression of DMD* as a function of daily intake. The fit of the model (r^2 5 0.86, df 5 32, P, 0.001; Fig. 2) indicates this is a plausible explanation for some variation in DMD* in our experiments. The model indicates that endogenous losses were 0.66 6 0.17 mg/d, and that the true DMD, which excludes these endogenous losses, is 0.61 6 0.05.

In order to assess whether digestibility could also be measured indirectly by an inert marker method we measured ash content of the food, the digesta from the distal third of tadpole guts, and the excreta collected at the bottom of tadpole containers. Sometimes, because of very small amounts of dry matter in the distal gut of tadpoles, some samples were pooled for measurement of ash content. The ash contents of these pooled samples represented weighted mean ash content that corresponded to relative intake and excreta production rates of the individuals that contributed to the pools, because the dry matter in the distal guts of tadpoles was correlated with the tadpoles' daily intake rates (t_{32} 5 2.38, P 5 0.02) and daily excretion rates (t_{32} 5 2.28, P 5 0.03).

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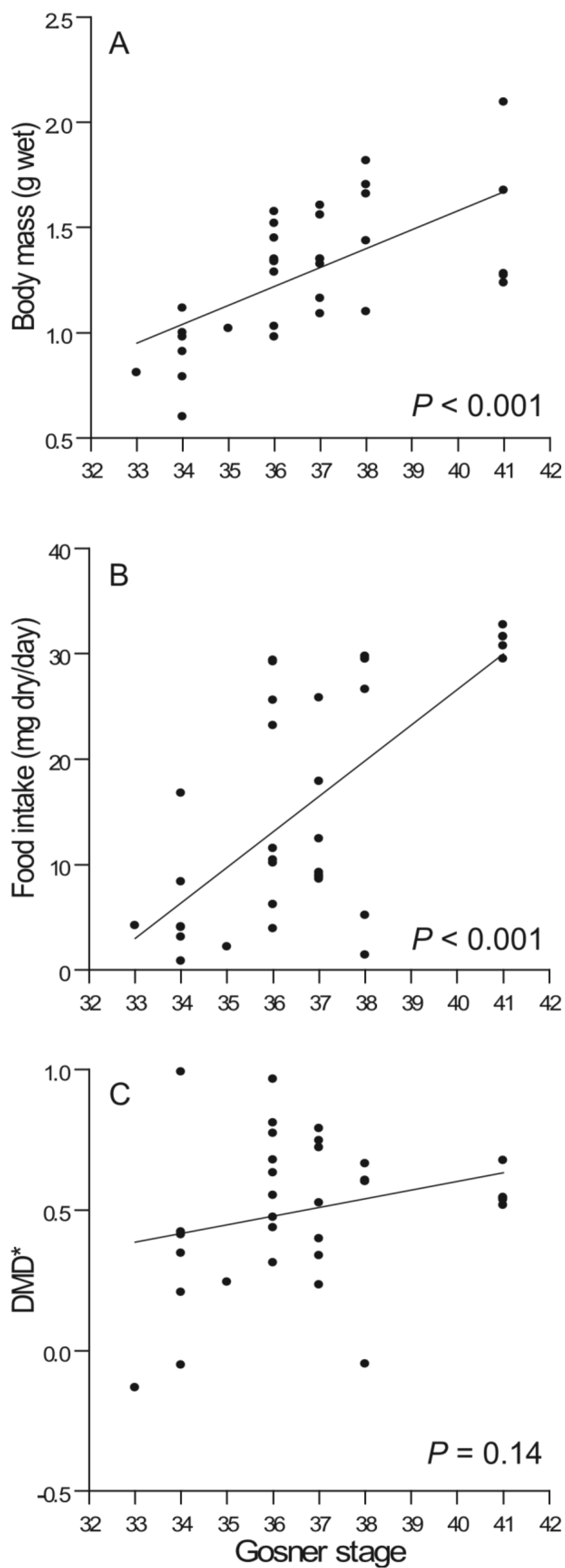

Fig. 1.—Body mass (A), daily food intake (B), and apparent dry matter digestibility, DMD* (C) of tadpoles as a function of Gosner stage in Experiment 1. P values are for significance of linear regressions in each panel, respectively.

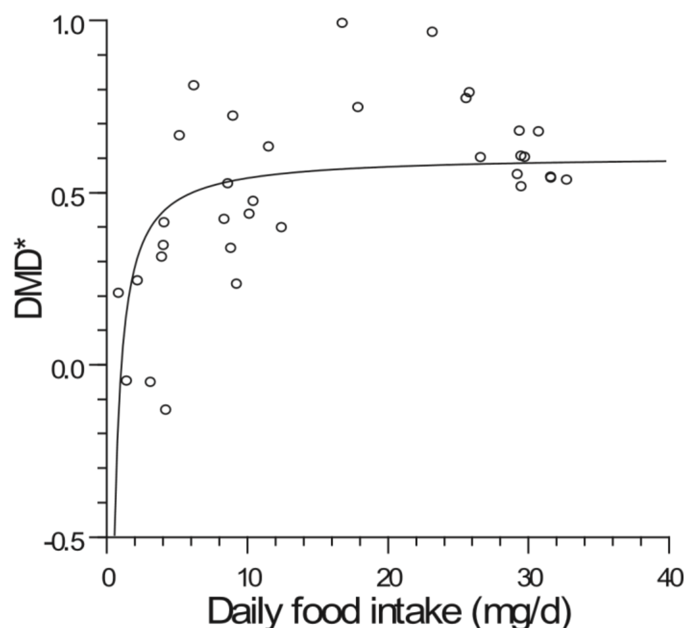

FIG. 2.—Apparent dry matter digestibility (DMD*) as a function of daily food intake of Lithobates pipiens tadpoles in Experiment 1. The line is the least-squares nonlinear fit to Eq. 3 (see Materials and Methods).

Seventeen samples of digesta from the distalmost portion of the intestine were analyzed for ash; 12 of them were pooled from 2–3 tadpoles eating the same diet, but in all but 1 of those pools tadpoles were of different Gosner stages. Although diets (standard or enriched with either Cr_2O_3 or

Y_2O_3) differed in ash content ($F_{2,15}$ 5 9.40, P 5 0.002; Fig. 3), diet had no effect on the ash content of digesta in the distal third of tadpoles' guts ($F_{2,14}$ 5 1.44, P 5 0.27) or of excreta collected from the bottom of the containers

($F_{2,19}$ 5 0.70, P 5 0.51; Fig. 3). The mean ash content of intestinal digesta of tadpoles (0.287 6 0.045, n 5 17) was higher ($F_{2,48}$ 5 20.10, P , 0.001) than the mean ash content of excreta (0.097 6 0.008, n 5 22; P , 0.001) and of food (0.093 6 0.002, n 5 28 all diets pooled; P , 0.001), and the latter values did not differ from each other (P . 0.9). Mean digestibility, calculated using food ash and intestinal digesta ash (i.e., DMD^*_{ash}; Eq. 2) did not differ with diet ($F_{2,13}$ 5 1.05, P 5 0.39) and averaged 0.50 6 0.07 (n 5 17). This value did not differ from the direct measure of DMD*

(0.50 6 0.05, n 5 34; t_{49} 5 0.02, P . 0.9), which was based on food intake and excreta production. We did not analyze DMD^*_{ash} vs. food intake because we could not assign specific feeding rates to correspond with the DMD^*_{ash} estimates (the latter were based on pooled samples).

Experiment 2: Digestibility Estimated in Tadpoles Fed Ad Libitum

We pooled samples of digesta collected from the distal third of the intestine from groups of 2–5 tadpoles maintained in tanks with other tadpoles. The mass of digesta in the distal third of the intestine was positively related to body mass ($F_{1,56}$ 5 22.30, P , 0.001) and was higher when food intake was ad libitum than in Experiment 1 (when food intake was restricted; $F_{1,56}$ 5 119.00, P , 0.001; Fig. 4). The ash content of 17 pools of digesta in Experiment 2 did not correlate with Gosner stage (t_{15} 5 1.40, P 5 0.18; Fig. 5) and averaged 0.164 6 0.003 (n 5 17). We calculated DMD^*_{ash} with the use

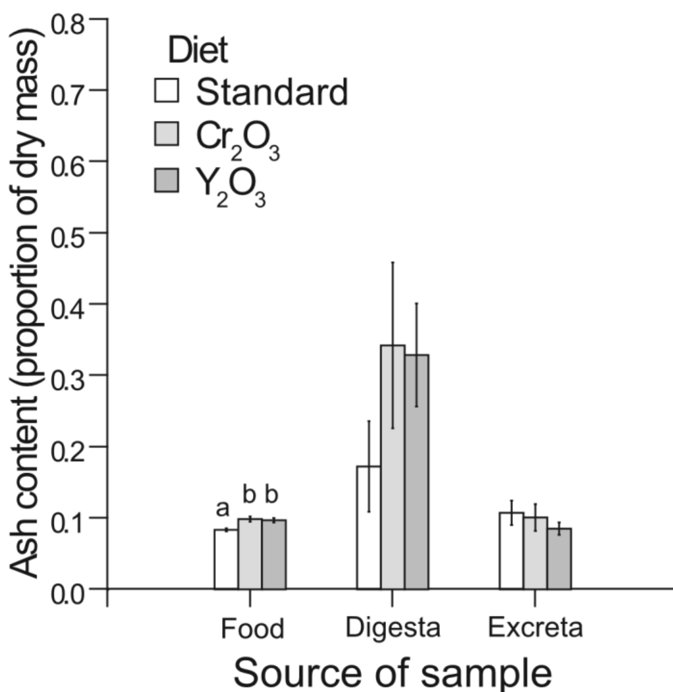

FIG. 3.—Ash content in samples of food, digesta, and excreta of Lithobates pipiens tadpoles, all from Experiment 1. The particular diet that was fed to the tadpoles is indicated with different shading within the bars. Different letters above bars designate different mean values. If no letters are shown, all values within that sample type were similar.

of these ash contents of these samples and those values from the food samples in Experiment 2 (0.0833 6 0.0005; n 5 7 food samples). $DMD*_{ash}$ (0.49 6 0.01) did not correlate with Gosner stage (t_{15} 5 1.31, P 5 0.21; Fig. 5) and was not different from $DMD*_{ash}$ in Experiment 1 when food intake was restricted (0.50 6 0.07, n 5 17; t_{32} 5 0.13, P . 0.9). Using the 17 pooled samples from Experiment 2 as units of replication is arguably pseudoreplication, however, if there were differences among the 4 tanks from which they were drawn (e.g., from competitive or other interactions among tadpoles within each tank that affect feeding, nutrition, and hence $DMD*_{ash}$). Because the tank source of each tadpole was not recorded we could not directly test for tank effects with the use of a nested analysis, but we could determine whether or not $DMD*_{ash}$ differed between Experiments 1 and 2. From the distribution of values for $DMD*_{ash}$ (range 5 0.42–0.55; Fig. 5), we subsampled 14 hypothetical groupings representing 4 tanks (n $ 1 pool analyzed/tank) in ways that would minimize and maximize both the mean and standard error of the mean (ranges 5 0.44–0.53 and 0.002–0.037, respectively). Values of $DMD*_{ash}$ based on the subsamples of Experiment 2 data were all similar to the 17 estimates from Experiment 1 (two-tailed t-tests; P values . 0.2).

DISCUSSION

In rasping aquatic feeders, like the larvae of Northern Leopard Frogs, it is challenging to measure digestive efficiency. A useful finding of Experiment 1 was that the estimate of diet digestibility based on direct measures of food intake and excreta production (Eq. 1) did not differ from the estimate based on comparison of ash content of

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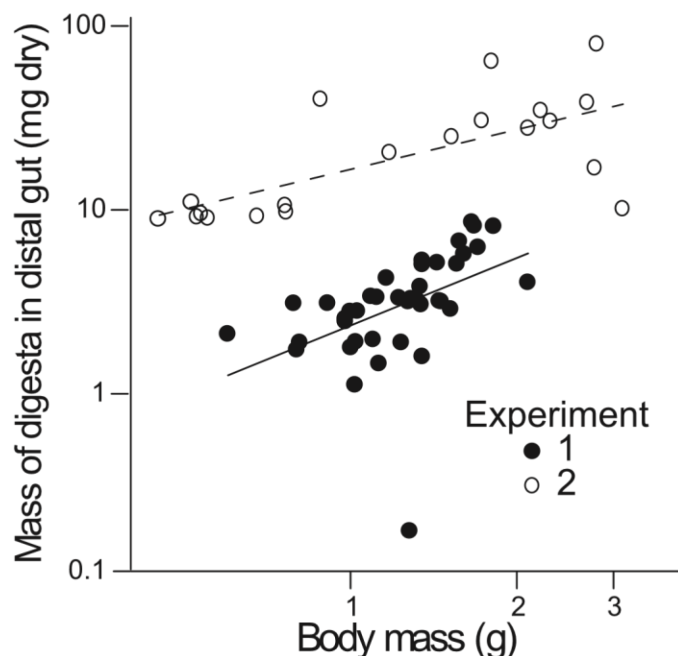

FIG. 4.—Dry mass (milligrams) of the digesta from distal third of the intestine of Lithobates pipiens tadpoles as a function of subject body mass. Food intake was restricted in Experiment 1 and was ad libitum in Experiment 2 but, in both cases, tadpoles were raised at 23uC.

food and digesta (Eq. 2). We could not devise a procedure for Experiment 1 to measure a tadpole's intake under ad libitum feeding conditions directly, and opted instead to feed and measure intake during short intervals when tadpoles were highly motivated to feed because of hunger. One problem with this method occurs when endogenous losses possibly reduce the estimate of apparent digestive efficiency (DMD*; Eq. 3), especially at very low food-intake rates. This effect was apparent in the smallest, youngest tadpoles in Experiment 1 (Figs. 1, 2) and might have confounded our tests for variation in DMD* as a function of housing and feeding conditions (Experiment 1 vs. 2) or Gosner stage (both experiments). This serves to highlight two useful findings in Experiment 2. First, the DMD^*_{ash} (Eq. 2) of tadpoles feeding ad libitum did not differ from the DMD^*_{ash} in Experiment 1 (when food intake was restricted). That finding holds whether we used tadpoles or tanks as the units of replication. Second, results from both Experiment 1 (Fig. 1C) and Experiment 2 (Fig. 5) are consistent with the idea that diet digestibility in both groups was relatively similar across Gosner Stages 33–41. We used tadpoles as units of replication for the analysis in Experiment 2, however, which might be confounded by pseudoreplication. Our study was not designed as a strong test for effect of Gosner stage on diet digestibility, because food intake levels were relatively low, especially at early Gosner stages, and sample sizes were relatively small at the latest Gosner stages.

Overall, measures of DMD* based on tadpoles' dry-matter fluxes and DMD^*_{ash} based on tadpoles' digesta ash as an inert marker indicated that Northern Leopard Frog tadpoles digested about 50% of the laboratory diet dry matter. The estimate based on dry mass fluxes might be an overestimate if some food is lost to the water, but is counted as consumed, or if some excreta is lost to the water. Using ash as an inert marker might yield an underestimate of DMD^*_{ash} because of

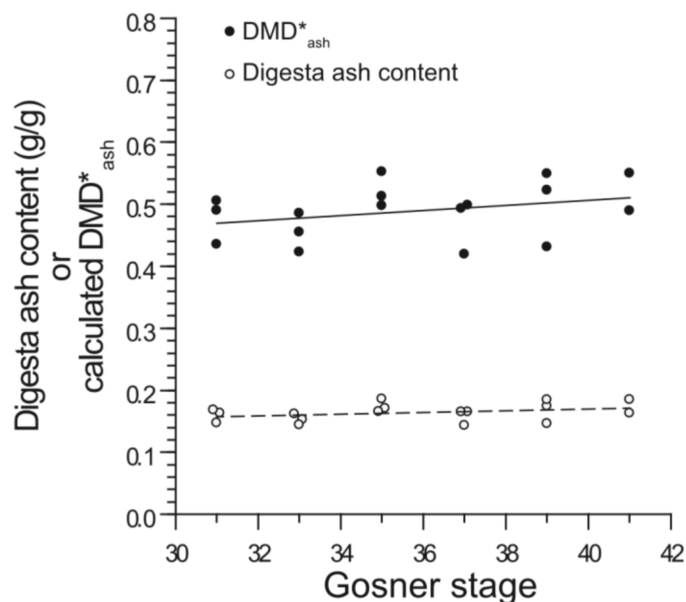

FIG. 5.—Ash content of pooled digesta samples from the distal third of tadpole intestines, and DMD^*_{ash} calculated from those values, as a function of Gosner stage. The lines are least-squares linear regressions, but neither ash content nor DMD^*_{ash} varied with Gosner stage (see text).

some retention of elements (i.e., components of ash) in tissues in the animal. However, the mean estimates of DMD^*_{ash} in Experiments 1 and 2 (two independent experiments with tadpoles reared at 23uC) were similar to each other and to the DMD* based on dry-mass fluxes.

The digestibility values that we measured in tadpoles were within the range we expected based on results with other vertebrates that have been fed similar alfalfa-based rabbit chow diets (cf. Karasov et al. 1986; Halver and Hardy 2002). The variability among animals is likely related to differential fermentative digestion of fiber components, ranging from perhaps negligible in a carnivorous trout, to as much as 20– 30% of fiber in an herbivorous lizard or rodent (Karasov et al. 1986). Tadpoles have fermentative capability (Pryor and Bjorndal 2005), so we expected an apparent digestibility above that of a trout (.0.39), but not as high as in herbivorous rodents (mostly ,0.60; Karasov et al. 1986). Although this still leaves a wide range, it does provide a biologically reasonable prediction for apparent digestibility of the diet. As it turned out, the expected range bracketed our observed values.

For Northern Leopard Frog tadpoles feeding on the laboratory diet, total ash appeared to be a useful marker for digestibility because the calculated value was similar to that measured based on dry-mass fluxes. Note, however, that some procedures we tried were not successful. When we collected excreta from the bottom of holding containers, the ash content was not much higher than that in food. We suspected that material was quickly lost to water, which would invalidate the method. We had more success by measuring ash in digesta from the distal end of the tadpoles' guts (this study), or that procured from the rectum (the last sixth of the gut; J. Yahn, personal observation). With the small quantities of dry matter and ash available, we were not able to measure specific elemental markers such as Cr or Y.

Using total ash as a marker for estimating tadpole digestive efficiency has been examined before, with variable results. Altig and McDearman (1975) fed five anuran species rabbit chow and estimated DMD* using ash content as an internal marker, but their calculated values ranged from 0.078 to 0.857.

This range of values strikes us as biologically unreasonable. Their wild-caught animals were fed the food 2–6 d, which seems not enough time for habituation to a new diet. Also, the equation they used appears incorrect insofar as its numerator includes a difference (food ash content 2 excreta ash content) that would result in a negative number. Benavides et al. (2005) raised Rhinella spinulosa tadpoles at two temperatures, feeding them either boiled lettuce or microalgae spirulina. They measured ash content of food and of excreta samples that were collected continuously over 2-h periods, and used the same calculation method as that employed here (Eq. 3). Their values for DMD* ranged from 0.75 to 0.90 and varied with temperature and diet quality, but there were no comparison data to verify the accuracy of the DMD* values themselves. Along with Benavides et al. (2005), we think that the method of using total ash as an internal marker has utility for studying digestive efficiency of tadpoles, which is inherently challenging to measure.

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APPENDIX TWO

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Effects of environmental temperature on the gut microbial communities of tadpoles

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Summary

Numerous studies have investigated the effects of diet, phylogeny and immune status on the gut microbial communities of animals. Most of these studies are conducted on endotherms, especially mammals, which maintain constant body temperature in the face of environmental temperature variability. However, the majority of animals and vertebrates are ectotherms, which often experience fluctuations in body temperature as a result of their environment. While there have been several studies investigating the gut microbial diversity of ectotherms, we lack an understanding of how environmental temperature affects these communities. Here, we used highthroughput sequencing to inventory the gut microbial communities of tadpoles exposed to cool (188C) or warm (288C) temperature treatments. We found that temperature significantly impacted the community structure and membership of the tadpole gut. Specifically, tadpoles in the warm treatment exhibited higher abundances of the phylum Planctomycetes and the genus Mycobacterium. These results may be due to the direct effects of temperature, or mediated through changes in host physiology. Given that environmental temperatures are expected to increase due to global climate change, understanding the effects of temperature on the diversity and function of gut microbial communities is critical.

Introduction

Recent research has revealed that the communities of microorganisms living within the guts of animals can

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largely affect the physiology, development and fitness of their animal hosts (McFall-Ngai et al., 2013). Specifically, changes in host physiology can be brought about by differences in the presence or absence of microbial taxa or in their relative abundances (Turnbaugh et al., 2006; Kohl et al., 2014; 2015a). Numerous experimental and comparative studies have investigated how factors such as diet, infection status or host phylogeny may impact gut microbial communities (Ley et al., 2008; David et al., 2014; Moeller et al., 2014; McKenney et al., 2015). However, we still lack an understanding of how basic exogenous, environmental factors may impact the gut community structure of animals.

One crucial factor, environmental temperature, has been understudied in relation to gut microbial community structure. To date, most gut microbiome studies have been conducted on mammals, especially laboratory rodents and humans, which maintain consistent body temperatures in the face of environmental variation. While hibernating mammals exhibit changes in gut microbial community structures, these changes seem to be driven by low nutrient availability for microbes rather than by host body temperature (Carey et al., 2013; Stevenson et al., 2014). Conversely, roughly 99% of animal species and 75% of vertebrate species are considered ectotherms (Hammond, 1992), which experience variations in body temperatures in response to their environments. In addition, temperature is known to influence the community structures of environmental microbiomes such as soil or sediment (Yergeau et al., 2012; Sharp et al., 2014). Further, culture-based studies have demonstrated that environmental temperature does alter the gut community structure of ectotherms such as frogs

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(Carr et al., 1976; Gossling et al., 1982) and fish (Lesel and Peringer, 1981; Sugita et al., 1989). However, the effect of temperature on gut microbial communities has never been investigated using high-throughput sequencing techniques.

Amphibians represent a diverse class of animals that is experiencing rapid population declines and species extinctions (Beebee and Griffiths, 2005). Given the large effects that microbial communities have on animal performance and fitness (McFall-Ngai et al., 2013), it is crucial to understand host-microbe interactions in this 1562 K. D. Kohl and J. Yahn

group. The gut microbial ecology of amphibians has only recently been studied using high-throughput sequencing methods demonstrating that the microbiota is largely restructured through metamorphosis (Kohl et al., 2013), and that environmental pollutants can cause persistent alteration of the microbiota (Kohl et al., 2015b). Given the threat that climate change represents to amphibian populations (Beebee and Griffiths, 2005), it is important to study the effects of temperature on the amphibian gut microbiota.

Here, we conducted a laboratory experiment to investigate the effects of environmental temperature on the gut microbial communities of tadpoles of the northern leopard frog (Lithobates pipiens, also known as Rana pipiens). We raised tadpoles under either cool (188C) or warm (288C) temperature treatments. All animals were housed and fed identically, details of which can be found in the supporting information. Gut contents were collected from tadpoles at similar developmental time points (Gosner stage 5 38.3 6 0.31; no difference between treatments: two sample t(30)5 0.20; P 5 0.84). Microbial communities were inventoried by sequencing the 16S rRNA gene from gut contents. Details regarding sequencing and data analysis can be found in the supporting information. We compared measurements of alpha diversity, community membership and structure, and abundances of microbial taxa. We predicted that temperature treatments would significantly influence the gut microbial community composition of tadpoles.

Results and discussion

We obtained an average of 35,5576 1225 sequences per sample. There were no differences in sequencing efforts between treatments or across replicate tanks (Nested ANOVA; temperature: P 5 0.50; Tanks (nested within temperature): P 5 0.16). Measurements of alpha diversity of the gut microbiome (Shannon Index, Faith’s Phylogenetic Diversity, evenness, or number of observed OTUs) were not significantly different between tadpoles reared at cool and warm temperatures (P > 0.1 for all).

Temperature significantly impacted microbial community membership (presence and absence of microbial lineages), and the tank of origin had a near-significant effect (Fig. 1; nonparametric ANOVA: temperature: F 5 3.76, P 5 0.002; Tank [nested within temperature]: F 5 1.12, P 5 0.08). Similarly, we observed significant effects of both temperature and tank on microbial community structure (which takes relative abundance into account; Fig. 1; temperature: F 5 16.46, P 5 0.003; tank [nested within temperature]: F 5 2.22, P 5 0.007). The significant effects of tank may be due to distinct microbial communities in the water of each tank, or perhaps due to slight localized variation in temperature, light, oxygenation, or other variables in each tank. The contributions of varying environmental microbial communities (such as in the water) to the gut microbiota of tadpoles demands further research. Interestingly, the tadpoles from warmer temperatures seemed to exhibit higher interindividual variation, especially in terms of community structure (Fig. 1). These results are consistent with other studies demonstrating that higher temperatures increase heterogeneity of microbial communities among individuals (Erwin et al., 2012; Lokmer and Wegner, 2015).

Comparisons of relative abundances of microbial taxa revealed a number of phyla and genera that were differentially abundant. We identified four microbial phyla and 20 genera that exhibited differential abundances between the two treatments (Fig. 2 and Table 1). Most notably, the phylum Planctomycetes composed almost 30% of the community of tadpoles reared at the warm temperature, but less than 0.5% of the gut microbiome of tadpoles in the cool treatment. The function if this phylum is poorly understood, and usually composes <3% of the gut microbiota of most animals (Rawls et al., 2006; Ley et al., 2008). However, this phylum is dominant in the guts of soil-feeding termites (Kohler€ et al., 2008). Our results are consistent with a previous study demonstrating that elevated environmental temperatures increase the abundance of Planctomycetes in oyster gill tissue (Wegner et al., 2013). Further, a study comparing the stomach microbial community composition Effects of temperature on tadpole gut microbiome
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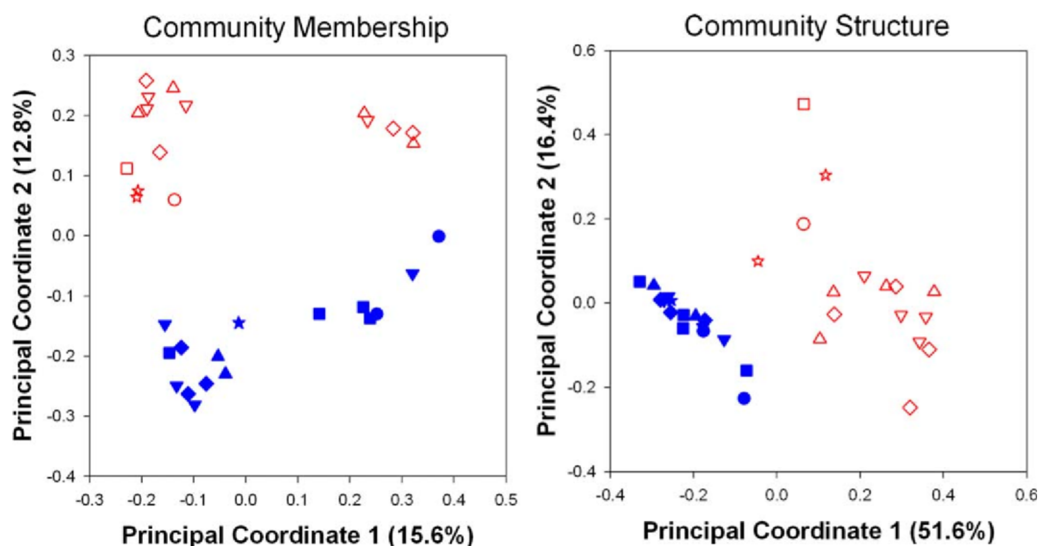

Fig. 1. Principal coordinate analysis of gut communities from tadpoles reared at cool (188C; closed symbols) or warm (288C; open symbols) temperatures (N 5 16 for each temperature treatment). Different shapes of symbols correspond to different replicate tanks. Community membership utilized unweighted Unifrac distances (which investigates the presence and absence of bacterial lineages). Community structure uses weighted Unifrac distances (which takes relative abundances of bacterial lineages into account). See supporting information for details on sequence and data analysis.

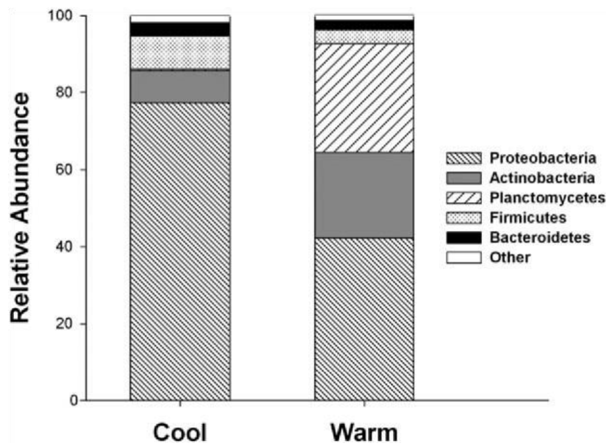

Fig. 2. Relative abundances of bacterial phyla in the guts of tadpoles reared at cool (188C) or warm (288C) temperatures (N 5 16 for each temperature treatment).

Table 1. Relative abundances and statistics for microbial phyla and genera that differed significantly in abundance between tadpoles reared at cool (188C) or warm (288C) temperatures. Higher abundances for each taxonomic group are bolded. Asterisks denote a significant effect of tank (nested within treatment) in the model. N.D. 5 not detected.

	Cool % abundance	Warm % abundance	F	FDR-corrected P
Phyla				
Firmicutes	8.6661.81	3.55 6 1.10	9.53	0.05
Proteobacteria	77.3663.90	42.24 6 4.10	21.55	0.002
Planctomycetes	0.39 6 0.07	28.2262.33	299.04	< 0.001
TM6	0.08 6 0.03	0.1960.03	9.71	0.05
Genera (split by class)				
Alphaproteobacteria				
Aminobacter	0.7760.19	0.05 6 0.02	21.16	0.002
Methylobacterium	0.0560.02	N.D.	9.34	0.04
Devosia	< 0.01	0.0560.01	38.82	< 0.001
Mesorhizobium	< 0.01	0.0260.01	30.60	< 0.001
Pedomicrobium	N.D.	0.0260.01	75.61	< 0.001
Rhodoplanes	0.01 6 0.01	0.6360.07	164.71	< 0.001
Gammaproteobacteria				
Aquicella*	N.D.	0.3060.16	213.69	< 0.001
Rheinheimera*	N.D.	0.0560.04	42.31	< 0.001
Shewanella	N.D.	0.0260.01	49.41	< 0.001
Actinobacteria Frigoribacterium	0.0160.01	N.D.	12.73	0.014
Micrococcus	0.026 0.01	0.01 6 0.01	17.49	0.004
Gordonia	0.01 6 0.01	0.8760.23	28.47	< 0.001
Mycetocola	<0.01	0.0160.01	16.68	0.005
Mycobacterium*	0.21 6 0.11	20.2663.50	99.04	< 0.001
Rathayibacter	N.D.	<0.01	11.24	0.02
Tsukamurella*	N.D.	0.0260.01	28.26	< 0.001
Chlamydiia Parachlamydia	N.D.	0.0360.01	43.34	< 0.001
Clostridia				
Dorea	0.2560.05	0.01 6 0.01	18.83	0.003
Cytophagia				
Runella*	N.D.	0.0160.01	36.12	< 0.001
Planctomycetia Planctomyces	0.07 6 0.04	15.3461.25	313.48	< 0.001

between populations of oysters found high abundances of Planctomycetes in one population (>20%) and low abundance (2%) in another (King et al., 2012). Our results raise the question of whether environmental temperature may have contributed to this population difference.

Additionally, tadpoles reared at the warm temperature exhibited a higher abundance of the genus Mycobacterium in their guts (Table 1). This genus grows optimally at 31–358C and is largely considered a gut pathogen, including for ectothermic hosts (Clark and Shepard, 1963; Clayton, 2005). Interestingly, infection and death as a result of Mycobacterium is more severe in ectotherms held at warmer temperatures compared to those at cooler temperatures (Clark and Shepard, 1963). Although we cannot confirm that the Mycobacterium in our experiment were pathogenic, it would be interesting to further study interactions between environmental temperature and pathogenic gut infections of Mycobacterium in tadpoles.

Tadpoles held at the cool temperature were enriched in much fewer taxa compared to those reared at the warm temperature. Tadpoles reared at the cool temperature harbored gut communities with higher abundances of Firmicutes and Proteobacteria. Additionally, the guts of tadpoles reared in the cool temperature had 153 higher abundances of Aminobacter and 253 higher abundances of Dorea compared to tadpoles reared at the warm temperature.

Overall, temperature had a demonstrable effect on the gut microbial communities of tadpoles. Future studies could elucidate whether these changes are the direct results of contrasting temperatures, or whether they may be mediated through alterations in host physiology. Some of our evidence suggests that temperature may directly be mediating this effect. For example, the warm-adapted genus Mycobacterium exhibited higher abundances in tadpoles reared at warm temperatures.

Conversely, temperature is known to alter many aspects of host physiology in ectotherms, such as immune function (Maniero and Carey, 1997) or gut transit time (van Marken and Lichtenbelt, 1992), both of which can influence microbial community structure (Hooper et al., 2012; Kashyap et al., 2013). To remove host-induced effects, other researchers have conducted in vitro incubations of gut microbial communities exposed to different treatments to look for rapid changes in microbial community structure or function (Maurice et al., 2013). These techniques could be used to disentangle whether temperature and/or host-mediated effects underlie our findings.

Our results are timely given that global temperatures are predicted to increase under models of climate change, which represents a substantial threat to amphibian populations (Beebee and Griffiths, 2005). Gut microbial communities provide a number of functions for their hosts, especially in terms of immunity and nutrition (Hooper et al., 2012; Semova et al., 2012). Understanding the effects of environmental temperatures on the functions provided to ectotherms by their gut microbes may be important for conserving these species.

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