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Environmental Toxicology

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Effects of PBDE and Temperature on Tadpole Growth and Development

The Effects of Dietary PBDE Exposure and Rearing Temperature on Tadpole Growth, Development, and Their Underlying Processes

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Abstract: Depression of growth rate due to polybrominated diphenyl ethers (PBDEs) has been documented in birds, mammals, amphibians, and fish at single temperatures. However, the underlying energetic mechanism for this effect and how it might change in relation to changing environmental temperature remains unstudied. Here, we used a simple energy budget to address hypotheses regarding PBDEs on tadpole (*Lithobates pipiens*) growth; that reductions in growth are linked to increased respiratory costs, reductions in digestive performance, differences in body composition, reductions in food intake, or a combination of these factors. From 18 days post fertilization (dpf) until 42 dpf, tadpoles were exposed dietarily to a pentabromodiphenyl ether mixture (DE-71^{TM}) at

a concentration of 100 ng DE-71/g wet mass under the rearing temperatures of either 22 or 27 ^{o}C. After 20 days of PBDE exposure, total PBDEs in tadpoles averaged 148.4 ng/g wet mass, with no differences by rearing temperature and about 50% higher than in their diet, controls not fed PBDE had levels < 1 ng/g. PBDE exposure resulted in reductions in body length, mass, and development as compared to controls, independent of rearing temperature. PBDE had no effect on measures of body composition, dry matter digestibility, nor oxygen consumption. A simple energy budget using data from this study revealed a ten percent decrease in feeding rate could explain the lower mass gain of tadpoles exposed to PBDE. Growth depression by PBDE could be due to (i) direct inhibition of growth processes by PBDE that indirectly decreases total energy demand and food intake, and (ii) direct inhibition of food intake. Future studies to disentangle these possible pathways of PBDE-effects are warranted.

Keywords: amphibian, climate warming, *Lithobates pipiens*, pollutants, energetics

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INTRODUCTION

Polybrominated diphenyl ethers (PBDEs) have been used extensively as flame-retardants in plastics, textiles, and electronics and are among the chemical pollutants commonly found in aquatic ecosystems worldwide (de Wit 2002, Mikula and Svobodova 2006) at levels potentially harmful to wildlife, including amphibians (Coyle and Karasov 2010, Cary et al. 2014). Although industrial and other uses of these compounds has declined as

regulatory restrictions have increased, their global distribution, and the long environmental half-lives of some congeners (~0.5 yr. (Palm et al. 2002)), create an ongoing need for environmental monitoring of these compounds. Risk assessments for toxic chemicals increasingly consider the possible influence of climate warming (Noyes et al. 2009), and increases in temperature could act synergistically with other environmental stressors to negatively affect amphibian populations. In a review of 66 species of freshwater animals (Mayer and Ellersieck 1986), toxicity of contaminants generally increased as temperature increased. In Leopard frogs (*Lithobates pipiens*), which are common in many parts of North America including the Great Lakes region, where we work with them, hormone profiles during tadpole development were influenced in temperature-dependent fashion by a commercial mixture of PBDEs (DE-71^{TM}) (Freitas et al. 2017). Understanding the interplay between environmental contaminants such as PBDEs and climate warming may be crucial for protecting the integrity of aquatic ecosystems (Noyes et al. 2009, Landis et al. 2014).

Temperature is a major environmental factor influencing physiological processes in ectotherms (Clarke 2003, 2006), generally increasing physiological rates according to a Q_{10} coefficient of 2-3 (the rate change for a 10 ^{o}C change in body temperature). Processes of toxicant uptake, biotransformation, and elimination mediate exposure of animals to pollutants. These processes link to bioenergetic rates of metabolism, food intake, digestion and elimination, which are known to be temperature dependent in ectothermic vertebrates (Karasov 2012). 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, Chen et al. 2010, Coyle and Karasov

2010). Yet, to our knowledge, there are no studies of the impacts of PBDE exposure under different temperature regimes in any ectothermic vertebrates nor studies of how PBDE negatively influences growth and development of tadpoles under different temperature regimes.

The processes of tadpole growth, development and subsequent metamorphosis are tightly regulated by the thyroid hormones triiodothyronine and thyroxine (Wong and Shi 1995). Developmental exposure to PBDEs results in thyroid hormone disruption in vertebrates generally (Zhou et al. 2002), including anuran tadpoles (Freitas et al. 2017). Additionally, reductions in growth of PBDE fed tadpoles could be attributed to changes in features related to energy acquisition and expenditure. Tadpole body mass change (Δm, in mg/d) can be understood in terms of energy inputs and outputs (in J/d) using a simple balanced equation (Pandian and Marian 1985):

$$\Delta m = ((I \bullet D) - R) / e_{tissue} \qquad \text{(eq. 1)}$$

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/mg wet mass) determined mainly by its composition of water, ash, lean tissue, and 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) and heavy metals (Rowe et al. 1998)), perhaps through a direct effect on metabolism or through an increased metabolic cost of toxicant biotransformation and elimination.

4) PBDE exposure depresses food intake, *I*, resulting in slowed mass gain.

To address these hypotheses, we raised leopard frog tadpoles fed diets with or without PBDE and at temperatures differing by 5^{o}C (22 & 27 ^{o}C), which is consistent with the magnitude of temperature change predicted by climate change scenarios during this century in the Great Lakes region of North America (Veloz et al. 2012). In addition to monitoring development (using Gosner stage as the metric (Gosner 1960)) and size (length and mass) as they aged, we tested for changes in body composition (lipid, water, and ash content). Digestive performance was measured using a validated inert marker – ash content in digesta as compared to that in food samples (Gleason et al. 2016). 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. We could not devise a reasonable procedure to quantify food intake under *ad libitum* conditions in rasping aquatic feeders, like Northern leopard frog tadpoles (Gleason et al. 2016). Measuring unconsumed food (orts) is confounded by dissociation of food into the water medium and differentiating the orts from tadpole excreta. We could partly evaluate the fourth hypothesis by rearranging eq. 1 to solve for *I*, based on all the other measurements.

If a component of the energy budget is 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 warming

climate 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

UW-Madison College of Agricultural and Life Sciences Institutional Animal Care and Use Committee (IACUC) approved procedures for this study. *Lithobates pipiens* embryos were purchased commercially from Nasco© (Fort Atkinson, WI) on the day fertilization took place (Gosner [GS] stage 1; (Gosner 1960)), which we refer to as experiment day 0. 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 ^{o}C). Water within the containers was changed daily to minimize bacterial and fungal growth; non-viable or dead embryos were removed when found (checked twice daily).

On day 5, all the embryos in the warmer room and more than half of those in the cooler room became free-swimming tadpoles (GS 25; 5-days post fertilization [dpf]). Tadpoles at GS 25 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 (Fig. 1). Transfer was completed for a minority of the cool room tadpoles on the sixth day when they reached GS 25. The racks had a system to circulate temperature-controlled water around each aquarium. By flowing temperature-controlled water around the tanks, as well as maintaining a constant temperature in the animal rooms, we ensured that tadpoles were maintained at their target temperatures, 22 or 27 ^{o}C (± 1 ^{o}C), throughout development.

Subsequently, 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 acceptable ranges (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 (Repti-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 matrix (Gleason et al. 2016). This diet yields satisfactory growth rates in Northern leopard frog tadpoles (Hirschfeld et al. 1970). Wet mass of food provided daily was approximately 20-25% of summed tadpole mass in the tank and left-over food and excreta from the previous feeding was syphoned every day prior to feeding.

During the first week there was mortality of tadpoles (31% in the warm room, 29% in the cool room), and so at day 14, tadpoles within each room were reallocated among tanks so there were 6 tanks per room each with 17-18 tadpoles. Although the authors are not aware of measures of mortality rate in nature for tadpoles at this stage, similar mortality in control *L. pipiens* (>30%) has been reported in other studies rearing leopard frogs (e.g., (Orton et al. 2006, Coyle and Karasov 2010)). After the reallocation on day 14, there was no more mortality in the experiment. On day 18, one tadpole was randomly removed from each tank for Gosner staging and sizing (length and mass), and then returned to the tank. On that day we began feeding tadpoles in three tanks in each room diet with PBDE, and tadpoles in the other three tanks in each room remained on diet without PBDE (Fig. 1).

Tadpole toxicant exposure

We favor dietary exposure because PBDEs have been shown to bioaccumulate in the sediment of Great Lakes waterways (Samara et al. 2006), where tadpoles tend to feed. Also, PBDE-laden food minimally leaches congeners into the water from the food and thus the primary route of PBDE exposure to tadpoles is dietary (Coyle and Karasov 2010).

The technical pentabromodiphenyl ether mixture DE-71^{TM} was purchased from Wellington Laboratories (product TBDE-71 [Great Lakes DE-71], purity undetermined, dissolved in toluene). This mixture was chosen because the major components are those predominantly measured in biota worldwide including the Great Lakes region (de Wit 2002). Major PBDE congeners, percent by weight, according to the manufacturer were: BDE-47 [32.4%]; BDE-99 [43.9%]; BDE-100 [8.9%]; BDE-153 [3.8%]; BDE-154 [3.3%]. DE-71 was incorporated into the agar-gelatin-matrix food (nominal concentration 100 ng DE-71/g diet wet mass) according to a previous study (Coyle and Karasov 2010). Briefly, DE-71 (100 µg/mL in toluene) was dissolved in acetone (equal parts mass/volume) and mixed with dry, ground rabbit chow (250 g/L). Control diet (0 ng DE-71/g) was prepared with the same volume of acetone (and very small volume of toluene) sans PBDE. The mixtures were left on a tray inside a chemical fume hood overnight to allow for solvent 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 minute and then cooled to room temperature. Ratio of food wet mass/dry mass was 3.8 (Gleason et al. 2016). Prepared diets were stored at -4 ^{o}C for immediate use or at -20 ^{o}C for future use. Dietary treatment began on day 18 and continued for the rest of the study.

Measurement of resting metabolism

Tadpoles in the four treatment groups, each of which was defined by its rearing temperature (22 or 27 ^{o}C) and diet (± PBDE)), remained in their tanks for up to 42 days (Fig. 1). On six separate days spread between experiment days 26 – 42, 11 or 12 individual tadpoles per day were randomly removed from treatment tanks for measurement of resting metabolic rate (RMR), defined as the lowest metabolic rate of nonfasted, resting tadpoles. On any single day we measured tadpoles reared on each diet but at a single temperature. We could not make all the measurements in the study on just a small number of the same days due to a variety of logistical constraints ranging from number of personnel, the concurrent feeding/water changing/tank cleaning schedules, space, time, and equipment requirements of each of the various kinds of measurements. In the case of the six days of respirometry measurements, between 1000 h and 1700 h we measured the rate of oxygen consumption of tadpoles (without food) at either 22 or 27^{o}C in temperature-controlled 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 rotated by a magnetic stir plate (set at medium) upon which the chamber sat, ensuring homogeneous dissolved oxygen (DO) throughout the chamber. 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 100%. At the low calibration point, 0% DO, granular sodium hydrosulfite was added in (approximately 5-10 grams) until all the DO was consumed and oxygen level stabilized at 0%.

Tadpoles were randomly netted out of their tanks, gently blotted to remove excess water and transferred to a dry net, and then massed (± 0.1 mg) by placing them into a tared 100-mL beaker containing 50 mL clean water used to fill rearing tanks. After Gosner 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 at least 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 to determine %DO as a function of time. Slopes, over periods lasting at least seven minutes 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/). VO_2 was expressed as mL O_2/h for tadpoles of measured body mass, and converted to joules using 19.7 J/ml O_2 (Karasov and Martínez

del Rio 2007). After all measurements, tadpoles were returned to their respective treatment tanks and monitored to ensure that they flourished. Consequently, over the entire experiment, a randomly selected but unknown number of tadpoles could have been measured for VO_2 more than once.

The Q_{10} value, which is the factor by which a physiological process such as VO_2 increases with an increase in body temperature of 10 ^{o}C was calculated as follows:

$$Q_{10} = (\frac{k_2}{k_1})^{\left(\frac{10}{(T_2 - T_1)}\right)} \quad \text{(eq. 2)}$$

where *k*, is the rate value for the reaction and *T*, is the temperature in ^{o}C (Hill et al. 2012).

Tissue collection and chemical analysis

Approximately weekly, beginning at day 24, individual tadpoles were randomly removed from treatment tanks for measurement of GS, body length (snout to tail, ± 1 mm), and body mass (as above) (*n*=10-12 tadpoles per diet per rearing temperature; exact sample sizes in Supporting Information Table S1). Tadpoles reared at 27 ^{o}C were collected on experiment days 24, 31, and 38, and tadpoles reared at 22 ^{o}C were collected on days 25, 32, and 38. As explained above, we could not make all the measurements in the study on just a small number of the same days. Tadpoles were euthanized in buffered 1% MS-222 according to IACUC protocol and used for body composition analyses. In order to remove the contents of the distal 1/6 of the intestine (the rectum), intestines were dissected out, and the contents were gently ushered out using a rounded gavage tip, the digesta samples were then dried, massed, 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, which was validated for *L. pipiens* (Gleason et al. 2016):

$DMD = 1 - (\text{food ash content}/\text{digesta ash content})$. (eq. 3)

Body composition analysis

After tadpoles had their gastrointestinal (GI) 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 (masses ranged from 0.0365 – 0.7306 g) was used to determine total lipid content by methods adapted from (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 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 15 g's for 20 minutes at room temperature, which separated the lipid and non-lipid (primarily protein) phases in the CH:MeOH solution. The latter phase was then drawn off and discarded and the remaining lipid solution was put in an N_2 evaporator at 50 ^{o}C. The culture tubes containing lipids were massed following two days in the N_2 evaporator and then again, 24 hours later to ensure that mass was stable. The

final mass minus the empty culture tube mass was taken to be the mass of the lipids in the tissue sample.

Other aliquots of homogenized tadpole tissue (similar to the above, whole animal minus the GI tract) were used to determine tissue water content and ash content. Tissue was gathered in aluminum weigh boats and massed to get an initial wet mass and placed in a drying oven at 50 ^{o}C. Following two days of drying the weigh boats were massed again to determine the dry mass of the tissue and the water content by difference. Dried samples were then placed in a muffle furnace at 550 ^{o}C. After being combusted for four hours the samples were massed a final time to determine the proportion of ash content in tissue dry mass.

Tadpole dry lean mass was calculated as the difference between total mass and measured masses of water, ash, and lipid. To calculate body energy content we used a value of 39.5 kJ/g dry mass for lipid and 20 kJ/g dry mass for lean mass (Karasov and Martínez del Rio 2007).

To confirm exposure to PBDEs we performed duplicated chemical analyses on control and PBDE-fed tadpoles at 38 dpf, i.e., after 20 days feeding on diets containing PBDE. Whole-body tissue concentrations of PBDEs were measured in groupings of 3-4 tadpoles (entire carcass minus the GI) tract per pool in order to insure at least seven grams of tissue per pool (five grams of tissue was the minimum weight needed to measure PBDE tissue concentration). Diet and tissue samples were sent to ALS Environmental, ALS Group USA (Kelso, WA, USA) for measurement of PBDE concentrations. Chemical analyses (K1413603) were 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; any value below this minimum level was categorized as "not detected".

Statistical analyses

Experiment day number was the same as the day post fertilization (dpf), which is hereon reported as age. For data collected on close but different days from tadpoles reared at different temperatures, we assigned the average age (e.g., 24.5 dpf for tadpoles collected on experiment days 24 and 25). We considered this to be a reasonable statistical procedure in response to the fact that we could not make all the measurements in the study on just a small number of the same days (described above), and all our subsequent analyses of how parameters changed with age indicated that this procedure contributed little variation (<2%) in the analyses of main effects due to difference in rearing temperature or diet.

Statistical tests were run in R version 3.3.0 and R studio, and all reported averages are followed by the standard error (*se*). We fit linear mixed models to test at each age for the effect of rearing temperature (22 or 27 ^{o}C) and diet (± PBDE) and their interactions on GS, body length, body mass, lipid content, tissue water content, ash content, *DMD*, and VO_2 (each of the aforementioned response variables were fit in separate models), using R's Lme4 package. 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 pseudoreplication. 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). To characterize how a parameter varied with age, we included it as a covariate in analysis of covariance (ANCOVA). Also, we analyzed $\log_{10}(VO_2)$ as a function of $\log_{10}$(body mass) using ANCOVA.

After each statistical analysis, residual plots and Q-Q plots were created to check for homogeneity of variance and the assumption of normality respectively, for each dataset. For all tests, the significance level was set at $\alpha < 0.05$. Mean values are presented ± *se* (*n* = number tadpoles or tadpole pools). For variables that are the sum or quotient of other variables, the *se* was calculated from the *se*'s of the other variables according to (Harris 2007).

RESULTS

Tissue residues of PBDE

We measured PBDE in tadpole tissues 38 dpf, following 20 days of dietary PBDE exposure. Tadpoles reared on control diet at 22 and 27 ^{o}C contained less than 1 ng of total PBDE's per gram of body tissue (Table 1) whereas tadpoles fed diet containing PBDE had 148.4 ± 27.7 ng/g total PBDEs (*n* = 4 pools of entire tadpole carcass, excluding GI tract), confirming that tadpoles fed PBDE diets took PBDE into their tissues. Levels in those tadpoles averaged about 50% higher than in their diet (Table 1). There was no difference in total PBDEs in tissues between tadpoles at the two temperatures (t_2 = 1.15, P = 0.37), although we had not designed the study to test for this and consequently had low power to detect a difference considering the small sample size.

Growth and development

Tadpoles aged 18 dpf at 22 and 27 ^{o}C did not differ significantly for Gosner stage, body length, or wet mass (Fig. 2 A-C; all *t*-test *P*'s > 0.2*; n* = 6 tadpoles per temperature, each from a different tank; statistical comparisons in Supporting Information Table S1). Power analyses indicated there would be no significant differences even at double the sample size. Subsequently, tadpoles at each temperature were fed diets without or with PBDE. Over the next two weeks, measures of both length and Gosner stage were significantly lower in tadpoles held at cooler temperature and in tadpoles fed diet with PBDE (Fig. 2 A-C; Table S1). Notably, the effect of PBDE on Gosner stage and body size at each age point was not altered by the temperature manipulation, as indicated by no significant interactions between temperature and diet at all measurement periods (all *P*'s > 0.08; Table S1). Size measures began to converge and to overlap during the fifth week post fertilization as increases in tadpole size became asymptotic, and differences in size were no longer significant 38 dpf (Table S1).

Body composition of tadpoles

Based on comparisons at each age, PBDE in the diet had no significant effect on lipid content (Fig. 3A), though the effect approached significance at 24.5 and 31.5 dpf (respectively, *P*=0.06 and *P*=0.07; Supporting Information Table S2). Rearing temperature had no significant effect on lipid content except in the youngest tadpoles measured. PBDE in the diet and rearing temperature had no significant effects on water content (Fig. 3B) nor ash content (Fig. 3C) (Supporting Information Table S2), except for an effect of dietary PBDE significantly increasing the ash content of tadpoles measured at 24.5 dpf (Fig. 3C). Results from ANCOVA indicated that carcass lipid content, overall, increased with tadpole age (Fig. 3A; $F_{1,124} = 65.6$, $P < 0.001$), but no significant increase

with age in either water content (Fig. 3B) or ash content (Fig. 3C) (respectively, $P = 0.06$ and $P = 0.15$). Overall, the main difference observed in body composition was an approximate 2.7 X increase in lipid content (from 1.5 to 4%) between ages 24.5 and 38 dpf, which would increase body energy content about 2% (from 2.4 to 2.5 kJ/g wet mass).

Tadpole respiration

Oxygen consumption (VO_2) was measured for 35 tadpoles aged 26-42 dpf, grouped according to the temperature at which they were reared (same temperature at which they were measured) and diet consumed, and data were analyzed and displayed on both a whole-animal basis (Supporting Information Fig. 1A) and on a mass-specific basis (Fig. 4). At the whole-animal level, $\log_{10} VO_2$ increased with log body mass ($P < 0.01$), with differences among the four groups ($P < 0.01$) but, no difference in slope (i.e., mass x group interaction; $P = 0.07$), which averaged 1.0 ± 0.18 for the four groups (Supporting Information Fig. 1A). Because the slope averaged 1.0, mass specific VO_2 was calculated as whole animal VO_2 divided by body mass$^{1.0}$, which is plotted in Fig. 4. 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 or Gosner Stage (respectively, Supporting Information Fig. 1B and 1C).

Results from linear mixed models revealed that at each age, PBDE in the diet had no significant effect on mass specific VO_2 (Fig. 4; Supporting Information Table S3). Tadpoles that were reared and measured at 27 ^{o}C had 1.9 times higher mass specific VO_2 (0.261 ± 0.017 ml O_2 g^{-1} h^{-1}, body mass 2.77 ± 0.29 g, $n = 18$ tadpoles on both diets) than those at 22 ^{o}C (0.136 ± 0.007, ml O_2 g^{-1} h^{-1}, body mass 2.87 ± 0.14 g, $n = 17$). Results

from ANCOVA indicated that mass specific VO_2 increased slightly (12.8%) between ages 26 and 42 dpf (Fig. 4; $F_{1,30} = 4.18$, $P = 0.05$).

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 ($F_{1,17} = 1.12$, $P = 0.30$), among the three age groups ($F_{2,17} = 0.21$, P = 0.82), or with the interaction of diet and age ($F_{2,17} = 2.71$, $P = 0.10$). Apparent *DMD* was calculated by comparing the ash in digesta with that in food according to equation 3. Results from linear mixed models revealed there was no effect of rearing temperature, dietary treatment, or their interactions on *DMD* (Fig. 5; Supporting Information Table S4). ANCOVA indicated that *DMD* decreased slightly (10%) between ages 24.5 and 38 dpf (Fig. 5; $F_{1,108} = 37.4$, $P < 0.001$).

DISCUSSION

Control tadpoles unexposed to DE-71™ had < 1 ng PBDE/g wet tissue, whereas following 20 days of dietary exposure to DE-71™ leopard frog tadpole carcasses had average PBDE concentration of 148.4 ± 27.7 ng/g wet tissue. This might be considered a near steady-state level, because the 90% elimination time of PBDE at these temperatures is approximately 20 days or less (corresponds to elimination half-time of ~6 d) (Brown et al. 2021). This tissue PBDE concentration is similar to amounts found in Great Lakes salmon (Manchester-Neesvig et al. 2001) as well as other Great Lakes biota (Stapleton and Baker 2003). As expected, based on other studies in which amphibians were exposed to PBDEs (Balch et al. 2006, Carlsson et al. 2007, Coyle and Karasov 2010), development and growth were slowed in the tadpoles (Fig. 2). Rearing at cooler temperature also slowed development and growth, as might be expected from the general

effect of temperature on development and growth in ectotherms (Karasov and Martínez del Rio 2007, Karasov 2012). The dual effects of exposure to PBDE and to warmer temperature were mainly additive because statistical interactions between the two were not significant (Fig. 2 and Table S1). A major goal of our study was to test whether reductions in tadpole growth associated with PBDE exposure could be attributed to differences in features related to energy acquisition and expenditure, which themselves are influenced by temperature. Hence, in the next few paragraphs we consider our specific tests of whether effects of PBDE are mediated through effects on digestive efficiency (energy acquisition), respiration (expenditure), or body composition. Following that, we consider ecological implications including single and interactive effects of temperature, which may be important in considering climate warming scenarios.

PBDE effects on body composition

Body composition, in conjunction with mass change, determines energy devoted to production of new tissue, i.e., in J or J/d. We tested for changes in body composition to see whether proportional changes in mass due to PBDE or temperature would correspond to similar proportional changes in production. Overall, the answer seems to be yes, because there were no large changes in body composition, and hence in J/g, due to either PBDE treatment or temperature difference. Curiously, lower ash content at 24.5 dpf was the only instance in which a measure of body composition differed in PBDE-fed tadpoles (Figures 3 A, B, and C). However, nearly lower lipid content in PBDE-fed tadpoles at 24.5 and 31.5 dpf, although not statistically significant (P = 0.06 & 0.07, respectively), was notable because it supports the interpretation that lower body mass due to PBDE

corresponds to lower production. Indeed, lower lipid content would indicate that the proportional declines in production energy may be somewhat greater than the proportional declines in mass gain in PBDE fed animals, because lipid has nearly an order of magnitude more energy content per gram wet mass as does lean tissue mass. Additionally, there was an increase in lipid content with age in tadpoles, however this was somewhat expected because increase in lipid stores is well established during the normal development of tadpoles in anticipation of fasting during metamorphosis (Sheridan and Kao 1998). Reduced lipid content in tadpoles fed PBDE (see Figure 3) could have ecological significance for tadpole development, metamorphosis, and survival. Reduced accumulation of lipid, which is used to fuel the costs of metamorphosis, could delay metamorphosis until enough lipid accumulates. Alternatively, if metamorphosis is not delayed but is undergone with insufficient energy stores, tadpoles could starve from lack of adequate energy. Additional studies on reduced body lipid content in tadpoles exposed to PBDE and its effect on development, metamorphosis, and survival are warranted.

PBDE effects on digestive efficiency

Measures of apparent dry matter digestibility (*DMD*) 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* to 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 ages 24.5 and 38 dpf *DMD* declined by approximately 10% (Fig. 5), a result that was not found in a previous study done on the digestive performance of tadpoles (Gleason et al. 2016). However, in that study measures of *DMD* were made using 17 pools of digesta (3 tadpoles/pool), each composed of digesta harvested from the distal 1/3rd of the GI tract. In the present study, many more measures were made on 113 individual tadpoles using only the colon (distal 1/6th), which should afford a more accurate measure and yield 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*. Difference in rearing temperature had no effect on *DMD* (Fig. 5, Table S3). This seems consistent with many such tests in aquatic and terrestrial ectothermic vertebrates, as well as invertebrates (Karasov and Martínez del Rio 2007).

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 to 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 resting 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 published values. In making the comparison, we take care to recognize the distinction

between temperature effects that reflect the acute effect of thermodynamics on molecular interactions *vs.* acclimation responses that can occur during chronic differences in rearing temperatures (Havird et al. 2020). Feder (1981) measured mass specific rates of VO_2 in fed tadpoles of the Rio Grande leopard frog (*R. berlandieri*; (Frost et al. 2006)) and found that 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} (Feder 1981). In tadpoles we reared (i.e., acclimated) at 22 ^{o}C, VO_2 was 0.14 mL O_2 g^{-1} h^{-1} at the measurement temperature of 22 ^{o}C and was 0.26 mL O_2 g^{-1} h^{-1} at the higher rearing and measurement temperature of 27 ^{o}C, thus bracketing Feder's data. Our measures yield a calculated "acclimated" Q_{10} for VO_2 of 3.4, higher than the acclimated Q_{10} of 2.1 measured for respiration of fed *R. tigrina* reared between 22 ^{o}C and 32^{o}C (Pandian and Marian 1985), and higher than the average Q_{10} of 2.2 for hundreds of amphibian species (White et al. 2006). Such "inverse compensation", in which acclimation further augments the acute change in metabolic rate, is not uncommon (Havird et al. 2020).

Mechanism(s) of growth reduction

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, Chen et al. 2010, Coyle and Karasov 2010), but researchers have yet to identify completely the mechanism(s) behind these effects (Chen and Hale 2010, Chen et al. 2010, Han et al. 2015). We tested whether the reductions in growth of PBDE-fed tadpoles are 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. However, 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 (all *P*-values >0.05).

Differences in growth rate and size might be attributed to correlated differences in feeding rate. 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 reduced feeding rates for PBDE-exposed animals (Leppänen and Kukkonen 2004). We could not devise a reasonable procedure to quantify food intake under *ad libitum* conditions in leopard frog tadpoles (Gleason et al. 2016), but we used available data to construct simple energy budgets to estimate food intake based on thermodynamic principles in order to make inferences about how PBDE plausibly influenced tadpole energetics. For this calculation, we used data for tadpoles between ages 18 and 32 dpf, the latter a time point when diet had a significant effect on body mass, but temperature did not (Fig. 2C). Based on changes in mean mass over this interval, PBDE depressed daily mass gain and daily production energy (*P*) by ~17% (Table 2). Daily respiration energy (*R*) was not influenced by PBDE in the diet. In a balanced budget, metabolizable energy intake (*MI*) = *P* + *R*. Considering that the *se*'s on the estimates of *MI* are about 10% of the mean values, the predicted differences are not statistically significant, but the exercise suffices to illustrate that a decline in energy intake of ~10% could explain the decline in mass gain of tadpoles exposed to PBDE.

Hence, it is plausible that PBDE exposure reduced food intake about 10%, which was then causal to the observed decline in growth. Alternatively, PBDE might directly inhibit growth processes, which then causes reduction in the tadpole's energy budget

demand for food intake. Additional studies would be necessary to tease apart these two possibilities.

Both of these possibilities might reflect endocrine disruption by PBDE exposure. Certain PBDE congeners structurally resemble receptor binding features of thyroid hormones T3 and T4 and competitively bind with the proteins responsible for their circulation, directly reducing circulating levels of the hormones in rats (Hallgren and Darnerud 2002, Zhou et al. 2002, Zoeller 2010). Consistent with this, PBDE-exposed frogs, including *L. pipiens* tadpoles, had lower concentrations of whole-body T3 levels (Freitas et al. 2017). Because the processes of tadpole growth, development and subsequent metamorphosis are tightly regulated by the thyroid hormones (Wong and Shi 1995), PBDE-associated reduction in T3 plausibly directly reduces growth. Also, local action of T3 in the central nervous system may physiologically regulate appetite (Amin et al. 2011), and PBDE-associated reduction in T3 thus plausibly directly reduces food intake.

Ecological implications

Smaller tadpoles are susceptible to a wider variety of predators, and delaying development increases the time that tadpoles are exposed to aquatic predation. A 7-year field 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 slowed development and growth we

observed in PBDE-exposed tadpoles could have demographic consequences on wild amphibian populations. It is also possible that outcomes from exposure to PBDE might differ under more food-limiting conditions than in our laboratory trial, because other studies revealed that resource limitation can augment toxicity of trace metal to larval anurans (Rowe et al. 2009).

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. Increases are to be expected in many physiological rates of ectotherms living in warmer environments, and data presented here provide some guide for anticipating those singular effects of temperature. Though temperature and toxicant exposure each had significant effects on tadpole size and development (Gosner Stage), our statistical tests for interaction between these two environmental variables were not significant (Fig. 2). This could simplify our predictive capability for anticipating how warming climate might influence tadpole performance even in contaminated environments.

Warmer temperature did increase food intake during the period of rapid tadpole growth (Table 2), as would be expected based on the general effect of higher temperature on physiological rates. In a contaminated environment this would be associated with higher toxicant intake rate. This could lead to higher toxicant body burden, but that effect might be mitigated if toxicant elimination rate were increased at warmer temperature to a similar extent as the rate of toxicant intake. Brown *et al.* found that PBDE elimination rate was increased in *L. pipiens* tadpoles reared at warmer temperature and that there was no effect of rearing temperature on near steady state tissue PBDE levels, which suggests

that faster elimination at warmer temperature was approximately balanced by faster uptake (Brown et al. 2021). However, that finding should not be misinterpreted to mean that there will be no consequences of a warming environment on PBDE toxicology in amphibian populations. Additional studies are necessary to determine whether animals living in a warming environment experience alteration in target site sensitivity to toxicants.

Supporting Information—The Supporting Information are available on the Wiley Online Library at DOI: 10.1002/etc.xxxx.

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Data availability statement—Data, associated metadata, and calculation tools are available from the corresponding author (wkarasov@wisc.edu).

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FIGURE CAPTIONS

Figure 1. Experimental schedule for rearing tadpoles and sampling them for measurements. Numbers along the top of the figure are Experiment days (same as days post fertilization). Rearing temperatures were warm (27^{o}C, orange) and cool (22^{o}C, blue), and diet was with PBDE (+PBDE) or without it (0 PBDE).

Figure 2. Gosner stage (A), body length (B), and body mass (C) of tadpoles reared at the two rearing temperatures, 22 (cooler) & 27 (warmer) ^{o}C and fed diet with PBDE or without PBDE (Control). Data represent the means ± s.e. (sample sizes typically 10-12 per symbol). Statistical results are from analyses summarized in Supporting File Table S1, which includes exact sample sizes.

Figure 3 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) in tadpoles reared at the two rearing temperatures, 22 ^{o}C (cooler) & 27 (warmer) ^{o}C and fed diet with PBDE or without PBDE (Control) as a function of age (days post fertilization). Data represent the means ± s.e., and samples sizes are typically 10-12 per symbol. Statistical results are from analyses summarized in Supporting File Table S2, which also include exact sample sizes.

Figure 4. Rate of oxygen consumption (VO_2) of tadpoles reared at the two rearing temperatures, 22 ^{o}C (cooler) & 27 (warmer) ^{o}C and fed diet with PBDE or without PBDE (Control). Data represent the means ± s.e (n = 3 tadpoles per point, with a single exception of n=2 for the group at 22 ^{o}C with PBDE in diet). Statistical results are from analyses summarized in Supporting File Table S3, which includes exact sample sizes.

Figure 5. Apparent dry matter digestibility (*DMD*) of tadpoles reared at the two rearing temperatures, 22 ^{o}C (cooler) & 27 (warmer) ^{o}C and fed diet with PBDE or without PBDE (Control). Data represent the means ± s.e., and sample sizes are typically 10-12 per symbol. Statistical results are from analyses summarized in Supporting File Table S4, which also includes exact sample sizes.

Table 1. Levels[1] of PBDE congeners in tadpoles harvested 38 dpf, and in their diets.

PBDE Congener	Diet-PBDE	Diet-Control	27^{o}C +PBDE (2 replicates)		22^{o}C +PBDE (2 replicates)		27^{o}C Control	22^{o}C 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	40.3	39	38.7	39	40.5	39.3
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	138.1	211.	0.95	0.89

			2	1	

[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]The mean Gosner Stage ("GS") of tadpoles for each pool

[5]"Not detected"

[6]Total concentration of all congeners.

Table 2. Approximate energy budgets of growing tadpoles between 18 to 32 dpf reared at 22 ^{o}C or 27 ^{o}C while eating diet with PBDE (+PBDE) or without (Control). Values are means ± *se*.

Rearing Temperature (^{o}C)	Diet	Mass gain$^{\alpha}$ (mg $d^{-1}g^{-1}$)	P^{β} (J $d^{-1}g^{-1}$)	R^{λ} (J $d^{-1}g^{-1}$)	MI^{δ} (J $d^{-1}g^{-1}$)
22	Control (*n* = 12)	63.7 ± 9.1	156 ± 22	66 ± 3	222 ± 22
27	Control (*n* = 12)	66.2 ± 8.7	162 ± 21	123 ± 8	285 ± 23
22	+PBDE (*n* = 11)	55.9 ± 9.8	137 ± 24	66 ± 3	203 ± 24
27	+PBDE (*n* = 12)	53.1 ± 9.6	130 ± 24	123 ± 8	253 ± 25

[α]based on mean masses and standard errors from Fig. 2C

[β]*P*, production energy, based on energy density of tissue of 2.45 kJ g^{-1} wet mass (see Results)

[λ]*R*, respiration, based on averages for resting VO_2 at both temperatures (Fig. 4 and Results) and 19.7 J ml^{-1} O_2 (Karasov and Martínez del Rio 2007).

[δ]*MI*, metabolizable energy intake, = P+R

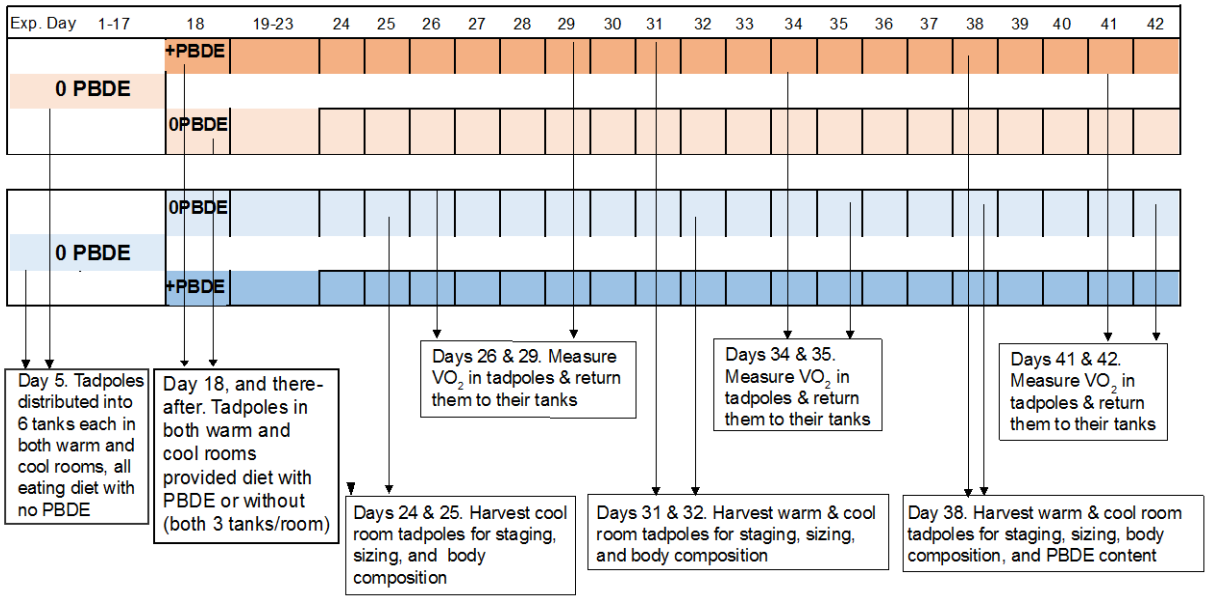

Figure 1. Experimental schedule for rearing tadpoles and sampling them for measurements. Numbers along the top of the figure are Experiment days (same as days post fertilization). Rearing temperatures were warm (27^{o}C, orange) and cool (22^{o}C, blue), and diet was with PBDE (+PBDE) or without it (0 PBDE).

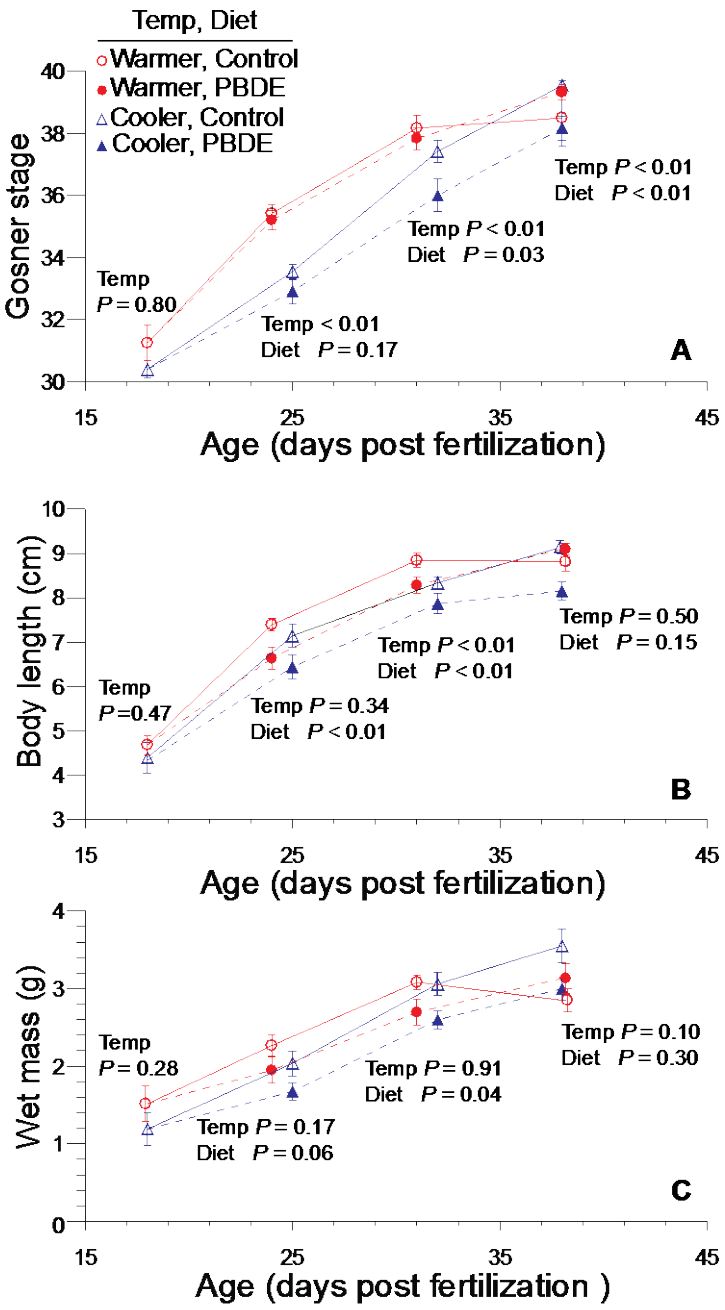

Figure 2. Gosner stage (A), body length (B), and body mass (C) of tadpoles reared at the two rearing temperatures, 22 (cooler) & 27 (warmer) ^{o}C and fed diet with PBDE or without PBDE (Control). Data represent the means ± s.e. (sample sizes typically 10-12 per symbol). Statistical results are from analyses summarized in Supporting File Table S1, which includes exact sample sizes.

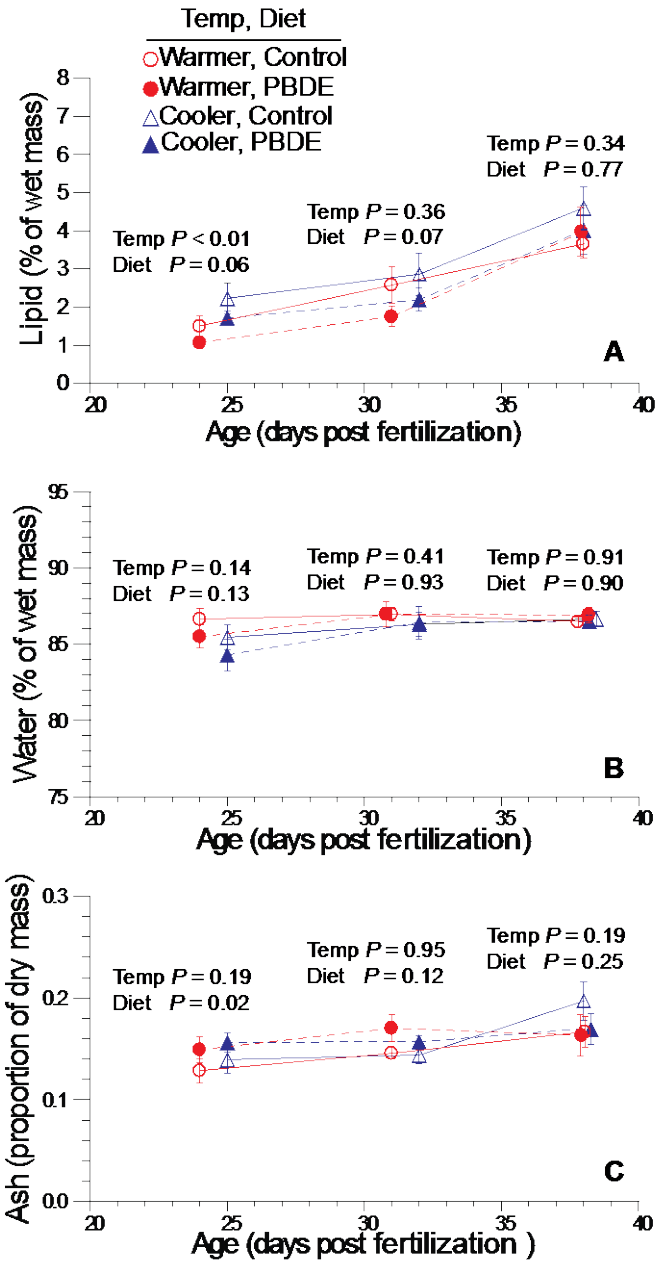

Figure 3 A, B, C. Total lipid content (A) and tissue water content (B) as a percentage of wet mass, and ash content as a proportion of dry mass (C) in tadpoles reared at the two rearing temperatures, 22 ^{o}C (cooler) & 27 (warmer) ^{o}C and fed diet with PBDE or without PBDE (Control) as a function of age (days post fertilization). Data represent the means ± s.e., and samples sizes are typically 10-12 per symbol. Statistical results are from analyses summarized in Supporting File Table S2, which also include exact sample sizes.

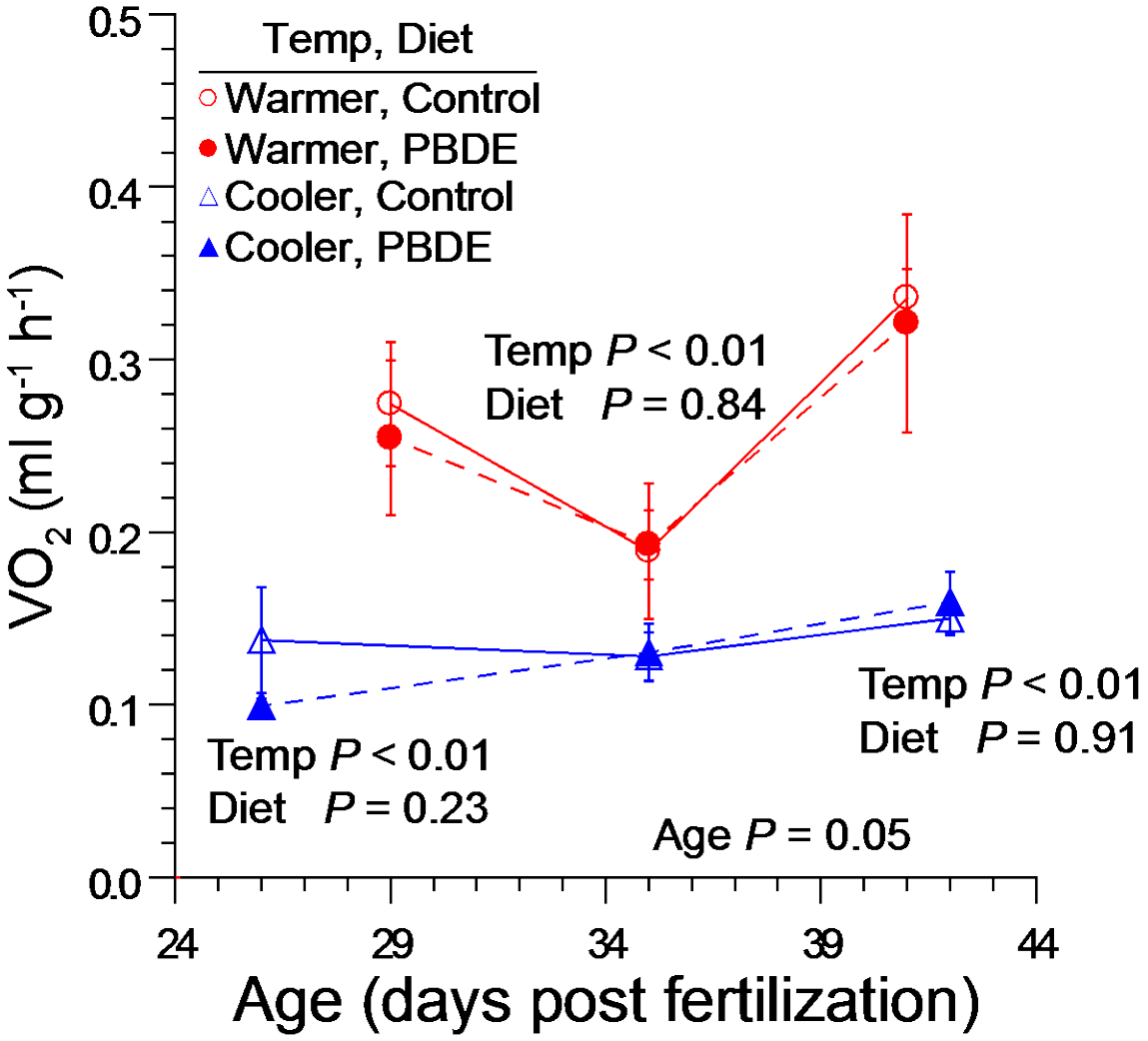

Figure 4. Rate of oxygen consumption (VO_2) of tadpoles reared at the two rearing temperatures, 22 ^{o}C (cooler) & 27 (warmer) ^{o}C and fed diet with PBDE or without PBDE (Control). Data represent the means ± s.e (*n* = 3 tadpoles per point, with a single exception of *n*=2 for the group at 22 ^{o}C with PBDE in diet). Statistical results are from analyses summarized in Supporting File Table S3, which includes exact sample sizes.

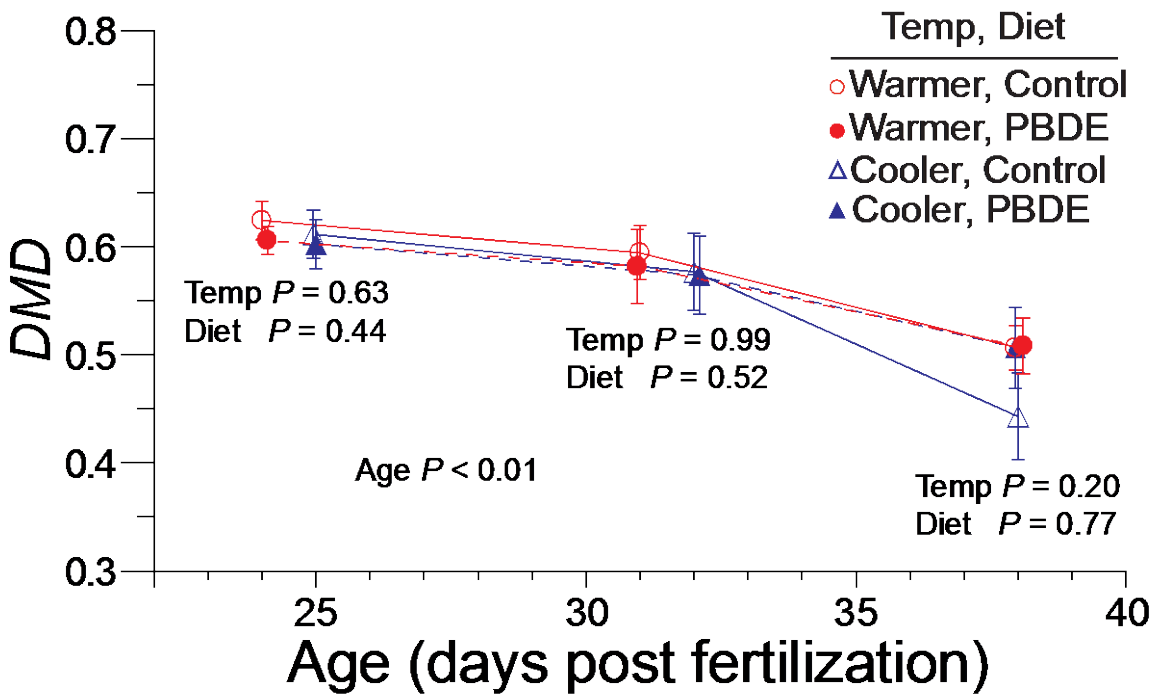

Figure 5. Apparent dry matter digestibility (*DMD*) of tadpoles reared at the two rearing temperatures, 22 ^{o}C (cooler) & 27 (warmer) ^{o}C and fed diet with PBDE or without PBDE (Control). Data represent the means ± s.e., and sample sizes are typically 10-12 per symbol. Statistical results are from analyses summarized in Supporting File Table S4, which also includes exact sample sizes.