Climate change and Ecotoxicology: Examining Persistant Organic Pollutant Effects on Amphibians in Warming Environments

By

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ABSTRACT

The increased temperatures associated with climate change could result in increased impacts of toxicants, like persistent organic pollutants (POPs), on wildlife. However, there is a paucity of knowledge regarding how temperature affects animals chronically exposed to toxicants at environmentally relevant levels. This dissertation research aims to understand whether and how exposure (Chapter 1) and toxicity (Chapter 2 and 3) of environmental contaminants are altered at different temperatures. We studied leopard frogs (Lithobates pipiens) exposed to polychlorinated biphenyls (PCBs) and polybrominated diphenyl ethers (PBDEs) to answer these questions.

In Chapter 1, we focused on the kinetics of PCBs and PBDEs in leopard frog tadpole when reared at two temperatures. Both PCBs and PBDEs 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. Additionally, calculated activation energies for toxicant elimination rates matched those of tadpole metabolic rates, which correspond to diet uptake rates. This suggests that faster uptake to toxicants at warmer temperatures was most likely balanced by faster elimination, and exposure may change very little in tadpoles in a warming climate.

In Chapter 2 we sought to understand how toxicity of PCBs and PBDEs are affected at different temperatures. In particular, we exposed leopard frog tadpoles to environmentally relevant toxicant levels and looked at development and immune function endpoints. In agreement with Chapter 1, there was no temperature effect on tissue toxicant levels after prolonged exposure. Tadpoles reared at warmer temperatures reached metamorphosis faster and displayed a reduction in complement lysis activity compared to tadpoles reared at cooler temperatures. Toxicant treatment did not have significant dose-response effects for any endpoints in both PCB and PBDE studies; however, there were significant

temperature and toxicant interactions in post-metamorphic size and keyhole limpet hemocyanin antibody response in the PCB study. Our data indicate that at warming temperatures, environmental contaminant exposure levels may not change, and amphibian immunity may be lowered, making them more susceptible to pathogens.

In Chapter 3, we looked more closely at how temperature and PBDE exposure would affect development in leopard frogs. Tadpoles were exposed to environmentally relevant PBDE levels at two temperatures and we examined metamorphic hormone profiles. Similar to Chapter 2, tadpoles reared at warmer temperatures were larger and developed faster than tadpoles reared at cooler temperatures. At the warmer temperature in Chapter 3, corticosterone increased earlier compared to tadpoles reared at the cooler temperature, coinciding with the faster development observed. Corticosterone levels were higher in PBDE-exposed tadpoles compared to controls at both temperatures. At both temperatures, PBDE exposure decreased triiodothyronine (T3) and increased corticosterone concentrations, which can potentially impair developing tadpoles. These data suggest both temperature and toxicant exposure effect amphibian development during metamorphosis and more studies are warranted to adequately characterize these combined effects.

DEDICATION

This dissertation is dedicated to my mother, Huei-Yu Chen, who fostered my love for science and provided me with never-ending love and support throughout this journey.

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TABLE OF CONTENTS

CHAPTER I: INTRODUCTION

Amphibian declines have been documented worldwide in the past two and a half decades, with population declines in the Americas, Europe, and Australia. (Drost & Fellers, 1996; Fellers & Drost, 1993; Laurance, McDonald, & Speare, 1996; Reid et al., 2013) There have been many factors that have been associated with amphibian decline, chiefly environmental contaminants, habitat degradation and emerging diseases ("IUCN Amphibian Survival Alliance," n.d.). Most recently, climate change has also been implicated in many deleterious effects on amphibians, including alterations in breeding timing (Blaustein et al., 2001), increased incidence of disease outbreaks (Bosch, Carrascal, Duran, Walker, & Fisher, 2007), and reduced survivorship and reproductive success (Reading, 2007). This dissertation aims to examine the interplay between climate change and environmental contaminants, looking in northern leopard frogs (Lithobates pipiens) at how temperature affects accumulation of polybrominated diphenyl ethers and polychlorinated biphenyl. To provide some general background, here I present a brief introduction on amphibians and our environmental contaminants of concern.

According to the International Union for the Conservation of Nature (IUCN), amphibians are the most threatened taxa. Population declines have been documented throughout the world, with serious declines recorded in the Midwest of Northern America (Adams et al., 2013). Amphibian decline are of great concern due to their important role in many ecosystems. Most amphibians go through two life stages: an aquatic larval stage and a semi-terrestrial or terrestrial adult stage. During these life stages, amphibians act as primary consumers and intermediate predators, as well as prey, contributing largely to the ecological food web. Amphibians also possess substantial biomass (Stebbins, 1995) and contribute to biodiversity with over 4500 species recorded worldwide (McDiarmid & Mitchell, 2000).

Amphibians are also considered useful indicator species to investigate environmental stressors (T. L. Cary & Karasov, 2012), and this is attributed to many of their physiological features. The life stages that occupy different habitats cause the animal to come into contact with many different environmental contaminants. Amphibian species also have very thin integument, especially in comparison with fish or reptile species, and the high skin permeability makes the animal more susceptible to some environmental contaminants. In this dissertation we used northern leopard frogs (Lithobates pipiens, previously known as Rana pipiens) as the animal model. Northern leopard frogs are one of the most widely distributed anuran species in North America (Moore, 1949) and are commonly used in ecotoxicology research (Paetow, Daniel McLaughlin, Cue, Pauli, & Marcogliese, 2012; Relyea & Hoverman, 2006; Thompson, Wojtaszek, Staznik, Chartrand, & Stephenson, 2004; Wojtaszek, Staznik, Chartrand, Stephenson, & Thompson, 2004). Additionally, this species has been used successfully in our laboratory to study the effects of environmental contaminants (T.-H. Chen, Gross, & Karasov, 2009; Gross, Chen, & Karasov, 2007; Jofré & Karasov, 2008a; Karasov, Jung, Vanden Langenberg, & Bergeson, 2005; Michele Laura Rosenshield, Jofré, & Karasov, 1999).

Amphibian declines are associated with multiple stressors, including environmental contaminants. Amphibians have been shown to be sensitive to pesticides and insecticides although effects at sublethal levels are not consistent. For example, research on insecticide carbaryl showed that at sublethal levels swimming ability was impaired, yet carbaryl positively affected mass, time and survival to metamorphosis (Boone, Bridges, & Rothermel, 2001; Boone & Semlitsch, 2001; Bridges, 1997). The aforementioned research on carbaryl also demonstrated that contaminants could cause effects in non-standard laboratory conditions, pointing out the importance of testing under environmentally relevant conditions.

A group of pollutants found in the Great Lakes that are of particular concern are persistent organic pollutants or POPs, which include polychlorinated biphenyls (PCBs) and polybrominated diphenyl ethers (PBDEs). PCBs and PBDEs have been shown to bioaccumulate in the sediment of Great Lakes regional waterways (Samara, Tsai, & Aga, 2006), where tadpoles tend to feed, and both pollutants are routinely found in biota of the Great Lakes watershed and are present at levels potentially harmful to wildlife (Sullivan & Delfino, 1982).

Polychlorinated biphenyls (PCBs) are a class of organochlorines that have been mass-produced and used as flame-retardants, coolants and insulating fluids in the past. Although production and deposition has been banned globally, PCBs chemical properties make them environmentally persistent, and they are still present in the wild at measurable concentrations (Heidtke, Hartig, Zarull, & Yu, 2006). Structurally, PCBs are biphenyl structures with 1 to 10 chlorines substituted on the aromatic rings, resulting in 209 possibile congeners. Many congeners have high octanol-water partition coefficients (log values 3-8), and partition readily into organic matter and bioaccumulate into the food web (Carey, 1998). Uptake of these organochlorines is through the digestive tract, with subsequent transport in the blood in association with lipoproteins. Partitioning into tissues is reported to be proportional to the tissue lipid content and occurs especially in higher lipid tissues such as brain, liver and bone marrow in addition to adipose tissue (Tanabe, Watanabe, Kan, & Tatsukawa, 1988). PCB metabolism relies heavily on certain cytochrome P450 enzymes which are only observed in higher-level vertebrates such as reptiles, birds, and mammals (Carey, 1998). Effects of PCBs reported in wildlife include liver damage, reproductive failure, developmental effects, disruption of estrogen and thyroxine biochemical pathways and impacts on immune function (Jofré & Karasov, 2008b; Kato et al., 1999; Leadley et al., 1998; Leijs et al., 2009). Also, numerous studies have linked PCB exposure to modulation of normal immune response and immunocyte function in fish (Iwanowicz, Blazer, McCormick, VanVeld, & Ottinger, 2009; Regala, Rice,

Schwedler, & Dorociak, 2001; Sures & Knopf, 2004), birds (Grasman, Fox, Scanlon, & Ludwig, 1996; Smits, Fernie, Bortolotti, & Marchant, 2002), and mammals (Sormo, Larsen, Johansen, Skaare, & Jenssen, 2009).

In amphibians, many studies have shown demonstrated PBC accumulation and sublethal toxicity in northern leopard frogs (Y. Huang, Hoffman, & Karasov, 2007; Y. W. Huang, Karasov, Patnode, & Jefcoate, 1999; Y. W. Huang, Stegeman, Woodin, & Karasov, 2001; Jofré & Karasov, 2008a; Michele Laura Rosenshield et al., 1999). At environmentally relevant levels, non-coplanar PCB70 and PCB101 were shown to skew sex ratios and increase the occurrence of intersex gonads (Jofré & Karasov, 2008a). The coplanar congener PCB126 was shown to bind strongly to the aryl hydrocarbon receptor (AhR), which can stimulate a variety of responses (G. S. Chen & Bunce, 2004; Rowlands & Gustafsson, 1997; Schmidt & Bradfield, 1996; Whitlock, 1999). These various responses include activation of cytochrome p450 genes, edema, decreased immune function and reproductive toxicity, which have been observed in previous studies performed in our laboratory (Battershill, 1994; T. L. Cary & Karasov, 2012; Huang, Karasov, Patnode, & Jefcoate, 1999; M. L. Rosenshield, Jofre, & Karasov, 1999; Yao, Panigrahy, & Safe, 1990).

PBDEs, used extensively over the past two decades as flame retardants in various plastics and textiles (Hale, La Guardia, Harvey, & Matt Mainor, 2002), have become an emerging contaminant of concern due to measurable levels in the environment and biota, and potential for environmental persistence. Structurally, PBDEs are diphenyl ethers with 1 to 10 bromines substituted on the aromatic rings, resulting in 209 possibilities. We are mainly concerned with higher substituted PBDEs such as penta-, octa- or deca-congeners as they are used in commercial formulations of flame retardants (EPA, 2010). These PBDEs have high octanol-water partition coefficients (log values 5.5-9.5), and thus can bioaccumulate readily when released into the environment. PBDEs in commercial products are only applied and not

chemically bound, which allows leaching into the environment (Hutzinger, Sundström, & Safe, 1976), and PBDEs have been reported at measurable levels in both abiotic and biotic matrices (Hites, 2004; Law et al., 2006). Summed levels of BDE congeners measured in plankton in Lake Michigan were as high as 720 ng/g lipid (Kuo, Sepulveda, Hua, Ochoa-Acuna, & Sutton, 2010). In leopard frog (Lithobates [Rana] pipiens) tadpoles fed with diet concentrations comparable to these measured plankton levels we reported decreased survival, as well as delayed growth and development (Coyle & Karasov, 2010). Additionally, we found that PBDEs at these levels altered secondary, specific anti-body responses, which we used as a measure of adaptive immune function in juvenile frogs, indicating a potential immunomodulatory effect of PBDEs in amphibians (Tawnya L. Cary, Ortiz-Santaliestra, & Karasov, 2014). Other researchers have shown that the commercial PBDE mix DE-71 in the diet delayed metamorphosis in Xenopus laevis tadpoles (Balch, Velez-Espino, Sweet, Alaee, & Metcalfe, 2006), and kestrels that were fed a similar mix of congeners exhibited altered immune function (Fernie et al., 2005).

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CHAPTER II:

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

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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 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 27oC. Both PCB and PBDE were eliminated faster at the higher temperature, as expected. Using Arrhenius' equation along with published data for tadpoles at 23oC, we calculated that the activation energy for PBDE elimination was 86.9 kJ/mol; a similar analysis for PCB elimination yielded activation energy of 98 kJ/mol. We also 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 and find that the activation energy for toxicant elimination is similar to that likely for toxicant uptake, and thus accumulation of many POPs (indexed by tissue residue levels) will 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 (Hof et al., 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).

The increased temperatures associated with climate change could result in increased impacts of toxicants on wildlife (Noyes et al., 2009). In a review of 66 species of freshwater animals (Mayer & U.S. Fish and Wildlife Service, 1986), acute toxicity of contaminants generally increased with increased temperature. The underlying mechanism(s) are unclear but might involve either or both increased accumulation at warmer temperatures or increased target site sensitivity. Accumulation by biota of contaminants, which operatively can be measured as tissue or whole-body toxicant concentration, 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); 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 toxicokinetics of persistent organic pollutants (POPs) in

any amphibian or aquatic vertebrate with respect to the increased temperatures associated with climate change.

Therefore, the objective of the present study was to establish the temperature dependence of accumulation and elimination of two POPs, polybrominated diphenyl ethers (PBDEs) and polychlorinated biphenyls (PCBs) in *Lithobates pipiens* (Northern leopard frog) tadpoles. PBDE's and PCB's have been shown to bioaccumulate in the sediment of Great Lakes regional waterways (Samara, Tsai, & Aga, 2006), where tadpoles tend to feed, and both pollutants are routinely found in biota of the Great Lakes watershed and are present at levels potentially harmful to wildlife (Sullivan & Delfino, 1982).

There are several ways that the temperature dependence of physiological processes might be described. Although Q_{10} (the change in rate for each 10°C increase) is often used to describe temperature effects, for describing the effect of temperature on key rates we rely instead on the Arrhenius equation, which has a stronger theoretical foundation in thermodynamics. Like Q_{10}, the Arrhenius equation can describe the temperature effect on rates, based on at least two measures of rate at two different temperatures. But, the Q_{10} value is itself a function of, and changes with, temperature (Schmidt-Nielsen, 1990), whereas the Arrhenius equation yields a process' fixed characteristic activation energy (E_a, in kJ/mol), which along with the gas constant (R), is not dependent on temperature:

$$k = Ae^{(-E_a/RT)} \quad \text{(eq. 1)}$$

where k is a rate constant, E_a the activation energy of the reaction, A is a constant specific for each chemical process, R the universal gas constant and T the temperature in Kelvin.

Our study of temperature impacts on bioaccumulation and elimination was guided by two main predictions from kinetic modeling, illustrated in Fig. 1. In Fig. 1A, tissue toxicant concentration ($C_{tiss,t}$)

rises with increasing days eating toxicant-laden food up to an asymptotic steady state concentration (C_{ss}), where rate of toxicant uptake, the product of an uptake rate constant (k_u) multiplied by food toxicant concentration (C_f), is balanced out by the rate of toxicant elimination, which is the product of elimination rate constant (k_e) and C_{ss}:

$$k_u C_f = k_e C_{ss} \quad \text{(eq. 2)}$$

Rearranging eq. 2 illustrates that C_{ss} could be fairly independent of rearing temperature if warmer temperature increases k_u to the same extent as it increases k_e (i.e., if they have the same apparent activation energies [E_a*]; Fig. 1A). The plot in Fig. 1A was in fact generated assuming that rates of uptake and elimination both double for a 10 ^oC increase in rearing temperature (Q_{10} = 2; E_a* = 50.5 kJ/mol).

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 (e.g., Q_{10} = 2 *vs.* 1.2, respectively; E_a* = 50.5 *vs* 13.3 kJ/mol respectively) (Fig. 1B). Our null hypothesis was that the effect of warmer temperature will be similar on both rates of toxicant uptake and elimination, resulting in no change in C_{ss} with rearing temperature. Hence, our Prediction #1 was that even after tadpoles were raised at different rearing temperatures on diets containing POPs for prolonged periods of time there would be no significant difference in their toxicant tissue concentrations.

We would like to address here the two common types of activation energy used in scientific literature. One corresponds to the more traditional definition of activation energy, describing the difference in Gibb's free energy between the initial state and the transitional state in a chemical reaction (Tro, 2013). The second is what we call "apparent" activation energy, stemming from the field of metabolic ecology, and which describes an observed phenomenon, not one particular reaction (Clarke, 2006; Gillooly, Brown,

West, Savage, & Charnov, 2001). Both follow the Arrhenius principal, and since we are measuring and comparing the same processes in tadpoles reared at different temperatures, we feel comfortable using apparent activation energy as the endpoint.

Because considerably more is known about the temperature dependence of energy expenditure and intake in vertebrates (Gillooly et al. 2001, Karasov 2012) than about toxicant elimination, we also sought to measure the temperature dependence of the rate constant for toxicant elimination (k_e), for which there are established measurement methods for frogs and tadpoles (e.g., (Leney et al. 2006, Cary and Karasov 2013). Based on the likely involvement of biotransformation enzymes in elimination of POPs (Kania-Korwel & Lehmler, 2016; Kannan, Reusch, Schulz-Bull, Petrick, & Duinker, 1995; Stapleton, Kelly, Pei, Letcher, & Gunsch, 2009), and our null hypothesis (above), our Prediction #2 was that k_e would increase with rearing temperature consistent with enzyme-mediated processes (e.g., range of values of E_a 48-76 kJ/mol, corresponding to Q_{10} range 2-3), and not consistent with elimination by diffusion ((e.g., E_a =13 kJ/mol, corresponding to Q_{10} = 1.2; (Thomson & Dietschy, 1980). Hence, results from this study will help shed light on the temperature dependence of toxicant accumulation kinetics that could result from the increasing temperatures projected to occur in climate change scenarios.

MATERIALS AND 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 two congeners: 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 the PBDE mixture and both PCB congeners could exhibit a range of elimination rates (Leney et al., 2006).

Our highest target test temperature (27^{o}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^{o}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, Hartman, Hay, Sauer, & Dhuey, 1998). However, based on results from our first experiments we decided that key rates at 18 ^{o}C were slower than desired and that the study's findings would be improved if subsequent experiments used 23 ^{o}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 at steady state (C_{ss}, ng/g mass) is modeled using an open, single-compartment model with first-order toxicant elimination (Ritschel, 1998):

$$C_{ss} = (I \bullet C_{food} \bullet a)/(M^{*}k_{e}) \qquad \text{(eq. 3)}$$

where 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 PBDEs and PCBs by tadpoles is best described by a single compartment and first order elimination kinetics (Tawnya L. Cary & Karasov, 2013; Leney et al., 2006). PBDEs and PCBs 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).

If an animal at or near steady state is switched to a toxicant-free food, then tadpole tissue concentration (C_t) declines exponentially daily with e^{-kt}. The temperature dependence of eq. 3 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, corresponding to E_a* = 50.5 kJ/mol).

In the following sections two types of experiments are described (Table 1). In experiments 1 & 2, we reared tadpoles at different temperatures and fed them for prolonged periods of time (> 5 weeks) diets with environmentally relevant levels of POPs to yield tissue concentrations that are equal to or at least close to C_{ss} (Fig. 1). Toxicant elimination rate constants (k_e) were measured in experiments 3 & 4, 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).

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 (Gosner, 1960)). 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 days 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 rearing 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 (GS42) tadpoles were removed from tanks and excluded from the study.

To create diets containing contaminants, specified concentrations of DE-71™ , PCB-70, and PCB-126 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 jello-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 C_t close to C_{ss}

Experiment 1 using PBDE was done concurrently with experiments 3 and 4 whereas experiment 2 using PCBs was performed 12 months later. In experiment 1, tadpoles were fed diets containing different concentrations of PBDE (6.11, 16.81, 39.9, or 81.96 ng/g DE-71^{TM}; a commercial mixture of PBDE congeners; Wellington Laboratories Inc., Ontario, Canada) and reared at either 18 or 27 ^{o}C. In experiment 2 tadpoles were reared at 23 or 28 ^{o}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; AccuStandard®, New Haven, CT). 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 (GS25) until they reached metamorphic climax (GS42).

Experiments 3 and 4 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 et al., 2006). We also designed it to ensure that 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 either PCB mixture (31 ng/g PCB-70 + 29 ng/g PCB-126) or PBDE mixture (753.9 ng/g DE-71^{TM}). 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 without toxicant for two weeks to allow for toxicant elimination (Fig. 1). Measures of tissue toxicant concentrations and elimination rates are described in subsequent sections.

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 et al., 2006), which allows us to use only two time points to determine the elimination rate constant (Klaassen, 2007).

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

$$\ln C_t = k_e\, t + \ln C_0 \qquad \text{(eq. 4)}$$

where C_t is the tissue residue levels, t is the time when the measurements are made (days), and C_0 is the residue in tissue at $t = 0$, which varies with amount of toxicant in the diet. The elimination rate constants were corrected for body size differences using the equation:

$$k^*_e = \frac{k_e}{M^{-1/4}} \qquad \text{(eq. 5)}$$

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 apparent activation energy (E_a*) was obtained by rearranging the Arrhenius equation (eq. 1) to the Arrhenius plot:

$$ln(k_e) = -\frac{E_a^*}{R} \cdot \left(\frac{1}{T}\right) + ln(A) \qquad \text{(eq. 6)}$$

See eq. 1 for definitions of the equation terms.

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, analysis of covariance (ANCOVA) was used to compare the linear relationships of $\ln C_t$ *vs*. $\ln C_{food}$ at two different rearing temperatures. 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.

In experiments 3 and 4, 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. 6. Multiple linear regression with an additional variable designed to qualitatively distinguish data points was used to determine whether elimination rate constants at different temperatures were significantly different from one another (Draper, 1981).

RESULTS

Tadpole mortality across all temperature and toxicant treatments was less than 1% during the exposure and elimination phases (data not shown).

Experiments 1 and 2: measure C_t close to C_{ss}

In experiment 1, tadpoles reared at 18 and 27 ^oC for 12-13 weeks on diets containing different levels of PBDE had $\log_{10}$ tissue concentrations that 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. 2). Likewise in experiment 2, tadpoles reared at 23 and 28^oC for 5 weeks on diets containing PCB congeners 70 or 126 had $\log_{10}$ tissue concentrations that were linearly

related to $\log_{10}$ diet concentrations (data not shown; Multi-way ANOVA PCB-70: $F_{2,6} = 5.18$, $P = 0.04$; PCB-126: $F_{2,6} = 20.5$, $P = 0.002$) as expected for first order kinetics (Gibaldi & Perrier, 1982), but no other factors such as temperature (PCB-70: $F_{1,6} = 0.14$, $P = 0.72$; PCB-126: $F_{1,6} = 0.46$, $P = 0.52$), or the interactions of these factors and covariate were significant (all P's > 0.7).

Considering the apparent first-order kinetics over the entire concentration range, we normalized the total PBDE in tissues to that in the diet by calculating their quotient (ratio tissue/diet) (Gibaldi & Perrier, 1982). PBDE ratios in tadpoles reared for 12-13 weeks at either 27 ^{o}C (1.74 ± 0.2, $n = 4$) or 18^{o}C (1.47 ± 0.04, $n = 4$) did not differ (ANOVA $F_{1,6} = 1.3$; $P = 0.29$; Fig. 3), and their mean value (1.60 ± 0.12, $n = 8$) is arguably close to the steady-state concentration. In tadpoles fed PCB diets for 5 weeks the ratio of PCB-70 in tissue to that in food did not differ significantly between 23^{o}C and 28^{o}C temperature (respectively, 1.99 ± 1.14, $n = 4$ *vs.* 1.25 ± 0.39, $n = 4$; $F_{1,6} = 1.49$, $P = 0.27$), and similarly in tadpoles fed PCB-126 (respectively, 1.32 ± 0.20, $n = 4$ *vs.* 1.11 ± 0.44, $n = 4$; $F_{1,6} = 0.75$, $P = 0.42$).

Experiments 3 and 4: measure elimination rate constants (k_e^*)

A goal in experiments 3 and 4 was to minimize growth associated dilution of tissue toxicant concentrations and then measure the temperature dependence of the rate of toxicant decline in tissues. 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 4 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 metamorphosing ectotherms tend to be larger when reared at cooler temperatures (Karasov & Martínez del Rio, 2007).

In Experiment 3, 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. 5B, Table 2). After two weeks of elimination, tadpoles reared at 27˚C had significantly lower mean tissue PCB levels (6.75 ± 0.7 ng/g) 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 3), 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 3).

In Experiment 4, 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 5A; Table 4). Notably, the tissue concentration data for tadpoles reared only 2 weeks on PBDE diet fell near the extrapolated regression line in Fig. 2, despite the much shorter rearing time on toxicant-containing diet. 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) (Fig. 5A; $F_{1,2} = 125$, $P < 0.01$). 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. 4A). 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 ($F_{1,2} = 1.6$, $P = 0.34$, but the calculated k_e

for 18˚C is clearly lower than that for 27˚C. The elimination rate constant for total PBDE (k_e; eq. 4) was 9.3 times higher at 27˚C than at 18˚C (Table 5), though once the differences in body size were considered (eq. 5), k_e^* for PBDE was 7.6 times higher at 27˚C than at 18˚C (Table 5).

Experiment 3 and 4: Determine apparent activation energies (E_a*) for PCB and PBDE

For PCBs, we used the Arrhenius plot (eq. 6). Based on the slope, the calculated E_a* value for total PCB was 98.3 ± 15 kJ/mol.

For PBDEs, 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) from just our experimental data. Therefore, in order to increase confidence in the estimation of E_a* for PBDEs, we also included a k_e value we previously measured in 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 d^{-1}) is bracketed by the two rate constants measured in this study, as is expected because we used rearing temperatures that bracket 23°C. We used our PBDE elimination rate constants at 27°C from the current study along with Cary *et al.*'s elimination rate constant at 23°C in the Arrhenius model (see Methods). Based on these two elimination rate constants, we estimate the E_a* value for total PBDE elimination is 86.9 ± 60 kJ/mol.

DISCUSSION

Elimination of PBDE and PCB in L. pipiens tadpoles was faster at warmer temperature, as predicted in the hypothetical model in Figure 1. 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. 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., ke 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 only on the unreliable rate constant for tadpoles reared at 18°C. We think that the E_a* value computed with inclusion of our previous data on k_e at 23°C is a more valid estimate.

Our data indicate that *L. pipiens* reached near steady state concentrations (C_{ss}) that were independent of temperature (Figs. 2, 3). We predicted this for the case where both elimination and uptake are similarly influenced by temperature (i.e., similar Q_{10}'s; Fig. 1A). Considering that C_{ss} corresponds to the quotient of the rates of toxicant uptake to elimination (eq. 2), we can infer that the E_a* values for uptake (k_u) over 18-27 ^oC 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 net effect that accumulation at steady state is rather independent of temperature. Although the k_u for toxicant uptake was not measured, we can infer that its approximate value is close to that for elimination. Food intake corresponds to metabolic rate, because the majority of a tadpoles' energy budget is used for respiration (Pandian & Marian, 1985). We first assume that contaminant uptake via food will change with temperature in a fashion similar to respiration. A study done by Yahn (Yahn, 2016) examining *L. pipiens* metabolic rates at 22 and 27 ^oC temperatures revealed E_a* to be 82.4 kJ/mol. These E_a* values for

metabolic rates are similar to the E_a* values for eliminations rates from our experiment (E_a PBDE: 86.9kJ/mol; PCB: 98.3 kJ/mol). Thus, data corroborate that E_a* values for toxicant uptake are mainly influenced by the temperature-dependence of respiration rates, as we found that *L. pipiens* reached steady state concentrations (C_{ss}) independent of temperature.

In our study, we predicted that the steady state concentrations would be independent of temperature mainly due to the fact that PBDE and PCB elimination E_a* values are matched with similar E_a* values for *L. pipiens* metabolic rates. It is important to note that elimination E_a* values vary from compound to compound. Therefore, we also predict that compounds with lower elimination activation energy, yet that still have their uptake activation energy mainly influenced by metabolic rates, will have lower temperature dependence for elimination than that for uptake, which will lead to the alternative situation in Fig. 1B, showing different steady state concentrations at different temperatures.

We were interested to compare our measures of the temperature dependence of toxicant elimination with other published data. We identified 16 studies of toxicant elimination for 13 compounds eliminated from 16 species of aquatic ectotherms that had measured k_e at two temperatures (Table 6). We plotted all k_e data on an Arrhenius plot and computed the E_a values for all the species and compounds in these studies. Our E_a values in tadpoles eliminating PCBs and PBDEs were consistent with those in the literature for other species and toxicants, which showed a wide range of values averaging around 61.5 ± 26.9 kJ/mol.

Based on this comparative analysis, we suggest a general hypothesis for aquatic ectotherms in warming environment. We predict that for most aquatic ectotherms toxicant elimination activation energy would match with metabolic (toxicant uptake) activation energy and overall toxicant accumulation may not increase in a warming climate. However, for aquatic ectotherms that rely heavily on diffusive

processes for elimination (E_a lower or close to = 23.5 kJ/mol, [Thomson & Dietschy, 1980]) and still have toxicant uptake activation energies close to metabolic activation energy, we expect accumulation to increase in warming environments. Future research should test these predictions by testing accumulation at different temperatures for animals that showed low toxicant elimination activation energy and high metabolic activation energy.

Our kinetics model for toxicant uptake assumes exposure mainly through food intake. We did not account for any toxicant distribution from the diet through the water followed by uptake into tadpoles across integument. 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 rates of toxicant uptake and elimination in animals. This study was designed to provide more data and advance knowledge in this field. Our findings on elimination of two POPs are consistent with the expectation that warming

temperatures, as predicted by climate change models, will increase toxicant elimination rates in ectotherms. This increase in elimination is also expected to be more pronounced for POPs, because their elimination tend to involve biotransformation enzymes, and therefore have higher apparent activation energies when compared to compounds that rely on diffusion for elimination. In addition, the known temperature dependence of animal energetics in ectotherms (rates of energy intake and expenditure 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 a warming environment on PCB and PBDE toxicology in amphibian populations. Our study focused on kinetics that influence tissue toxicant concentrations, yet other studies are necessary to determine whether animals living in a warming environment experience alterations in target site sensitivity to toxicants.

TABLES AND FIGURES

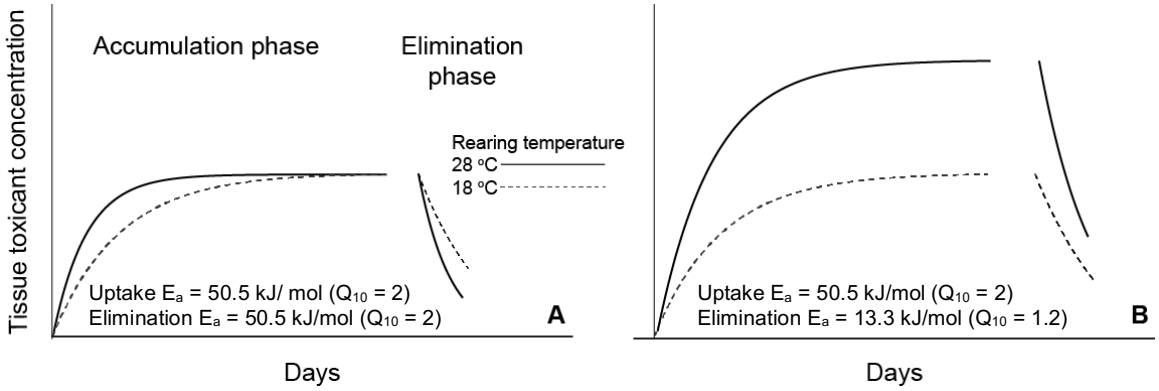

Figure 1. Theoretical values of tissue toxicant concentration ($C_{tiss,t}$) for tadpoles reared at two temperatures (18 ºC and 28 ºC) fed toxicant-laden diet. **Fig. 1A** shows that $C_{tiss,t}$ of a tadpole consuming toxicant-laden food will rise and approach a steady state level (C_{ss}), which will occur sooner at the warmer rearing temperature. If this accumulation phase is followed by a period when food without toxicant is consumed (an elimination phase), as in Fig. 1A, $C_{tiss,t}$ will decline exponentially with a rate constant for elimination (k_e) that will be greater (faster) at warmer temperature. **Figure 1B** shows an analogous plot to 1A, with the only difference being that the apparent activation energies (E_a*), i.e. the effects of temperature on rate constants, differ for toxicant elimination and uptake.

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 C_t close to C_{ss} for PBDEs	18 and 27	6.11, 16.81, 39.9, 81.96	85-95[2]
2	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
3	Measure k_e of PCBs	18 and 27	60	14
4	Measure k_e of PBDEs	18 and 27	753.9	14

Notes:

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

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

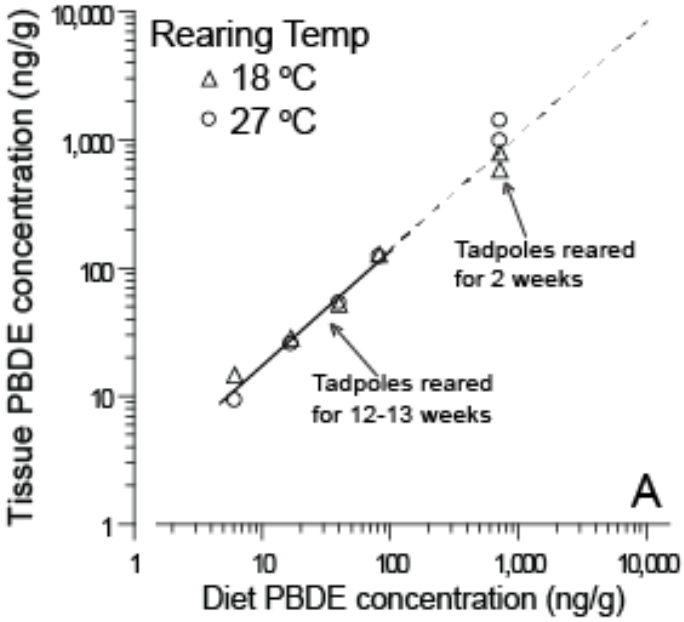

Figure 2. Bioaccumulation of total PBDE in tadpoles as a function of diet total PBDE concentration, rearing temperature, and rearing duration on a log-log plot. In tadpoles reared for 12-13 weeks, rearing temperature had no effect on tissue concentrations, which were linearly related to diet concentrations (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. The data can be interpreted to mean that steady state (rate of absorption = rate of elimination) was nearly reached after 2 weeks.

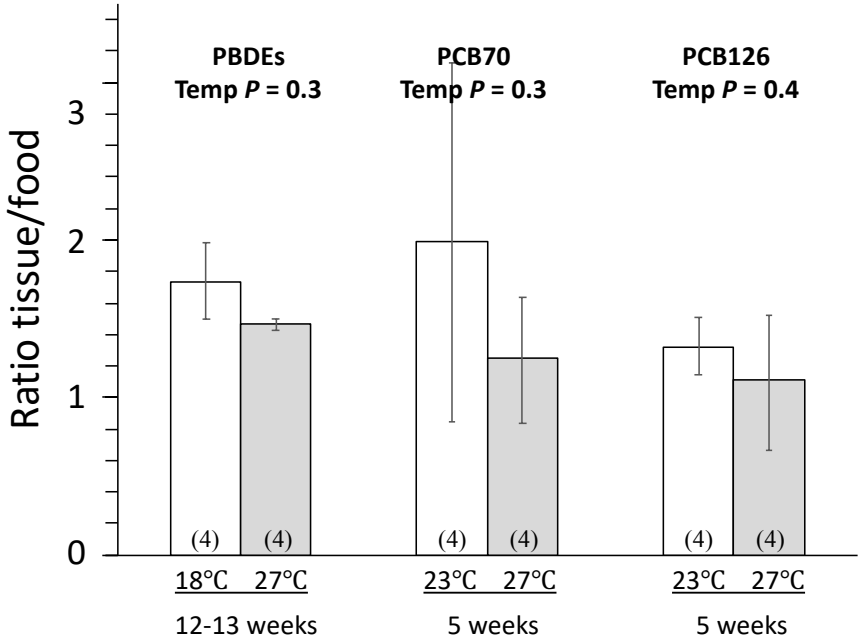

Figure 3. PBDE and PCB Ratios (= total toxicant in tissue/total toxicant in food) did not differ as a function of either diet or temperature (p-value in figure). For each pair of bars, the filled bar corresponds to the warmer rearing temperature. The number in parentheses at the base of each bar is the number of tissue pools measured for toxicant level. The data can be interpreted to mean that steady state (rate of absorption = rate of elimination) was reached at the same toxicant level in both cooler and warmer rooms.

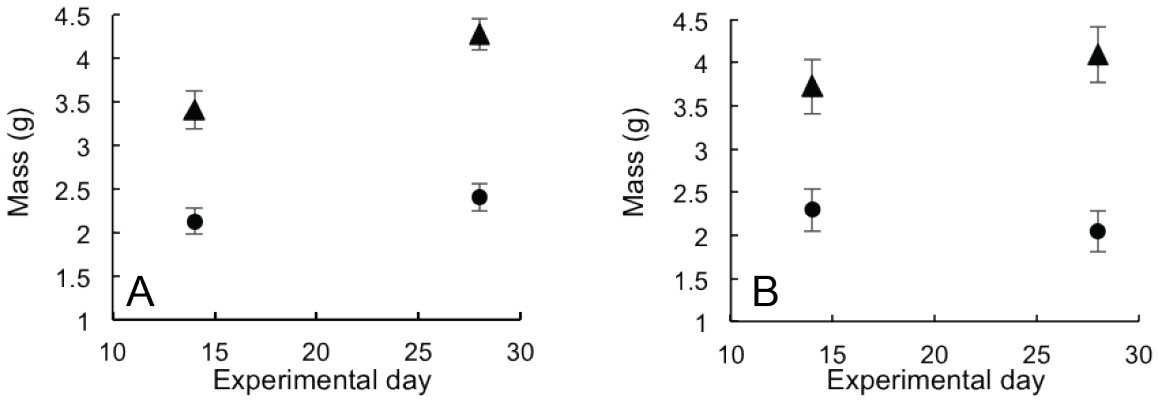

Figure 4. Average tadpole mass (g) for PBDE fed tadpoles (**A**) and PCB fed tadpoles (**B**), at the end of exposure (day 14) and elimination (day 28) for animals reared at 18 (triangles) & 27˚C (circles). Each symbol is the average mass of six tadpoles, euthanized, blotted dry, and massed to the nearest milligram; error bars are the standard error of the mean.

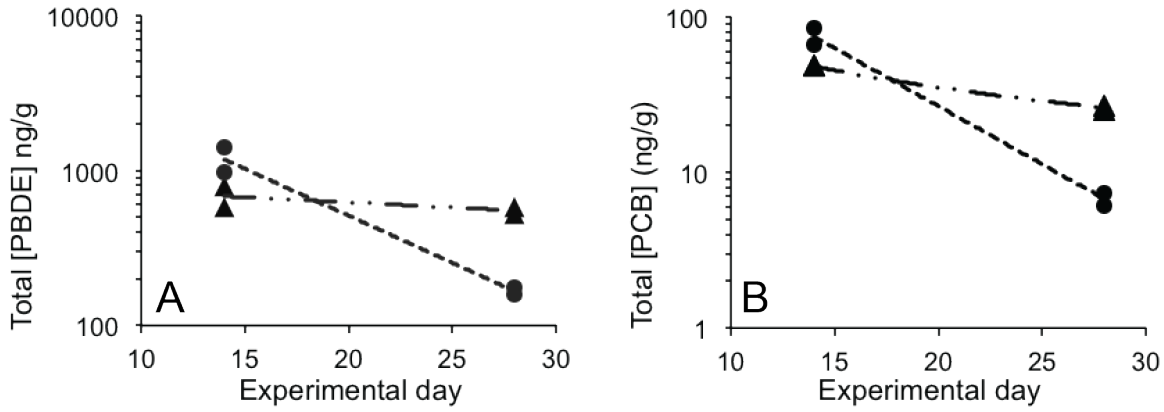

Figure 5. Total PBDE (**A**) and PCB (**B**) concentration in nanograms per gram of tadpole tissue at the end of exposure (day 14) and elimination (day 28), for tadpoles reared at 18 (triangles) & 27˚C (circles). Each symbol represents a group of 2 to 6 tadpoles that were pooled to yield seven grams of tissue for determination of toxicant concentration.

Table 2. Levels of PCB congeners in tadpoles and their diets in experiment 3. 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. Two numbers separated by a comma represent two replicates.

PCB Congener	27 ºC Day 14	27 ºC Day 28	18 ºC Day 14	18 ºC 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 3. PCB elimination rate constants from experiment 3, and calculated E_a* for PCB elimination.

PCB Congener	k_e[1] (d^{-1})		k_e*[2] (d^{-1})		E_a*[3] (kJ/mol)
	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	101
PCB-126	0.035 ±0.003[§]	0.149 ±0.01[§]	0.05 ±0.004	0.149 ±0.01	88
Total PCB	0.042 ±0.003[§]	0.171 ±0.01[§]	0.061 ±0.004	0.206 ±0.01	98

Notes:

[1]calculated using eq. 4

[2]corrected for differences in mass, using eq. 5

[3] E_a* calculated using eq. 6

[§] $P < 0.05$ for slope (i.e., k_e)

Table 4. Levels[1] of PBDE congeners in tadpoles and their diets in experiment 4.

PBDE Congener	27 ºC Day 14	27 ºC Day 28	18 ºC Day 14	18 ºC 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 PBDE	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. Two numbers separated by a comma represent two replicates.

[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 5. PBDE elimination rate constants from experiment 4, and calculated E_a for PBDE elimination.

PBDE Congener	k_e [1] (d^{-1})		k_e [2] (d^{-1})		E_a*[3] (kJ/mol)
	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	39.1
BDE-47	0.026 ±0.01	0.178 ±0.01§	0.031 ±0.02	0.214 ±0.02	111.6
BDE-100	0.005 ±0.01	0.131 ±0.01§	0.007 ±0.02	0.158 ±0.01	
BDE-153	0.012 ±0.01	0.104 ±0.01§	0.015 ±0.02	0.125 ±0.02	
BDE-154	0.009 ±0.01	0.112 ±0.01§	0.011 ±0.02	0.135 ±0.02	
BDE-85	0.023 ±0.01	0.168 ±0.01§	0.028 ±0.01	0.203 ±0.02	
BDE-138	0.014±0.01	0.127 ±0.01§	0.017 ±0.01	0.152 ±0.02	
BDE-183	0.002 ±0.01	0.045 ±0.05	0.002 ±0.01	0.054 ±0.06	
Total PBDE	0.015 ±0.01	0.139 ±0.01§	0.022 ±0.02	0.168 ±0.02	86.9

Notes:

[1]calculated using eq. 4

[2]corrected for differences in mass, using eq. 5

[3] E_a calculated using eq. 6 and incorporating TL Cary data

§$P < 0.05$ for slope (i.e., k_e)

Table 6. Literature review of ectotherm elimination rate constants at different temperatures and the calculated activation energy (E_a^*). All cited literature had measured elimination rate constant (k_1, k_2) at 2 temperatures (respectively T_1, T_2) reported in degrees Celcius. E_a^* was calculated using the Arrhenius plot (eq. 6) and using 8.314 J/mol K as the gas constant. E_a^* is reported in kJ/mol.

Species	Compound	T_1	k_1	T_2	k_2	E_a^*	Citation
Rainbow trout (*Oncorhynchus mykiss*)	radiocesium	8	0.009	16	0.014	37.3	Cocchio, Rodgers, & Beamish, 1995
Arctic charr (*Salvelinus alpinus*)	radiocesium	6.3	0.00339	15.8	0.00467	22.6	Forseth, Ugedal, Næsje, & Jonsson, 1998
Bream (*Abramis brama*)	radiocesium	7	0.00085	15.9	0.0017	52.4	
Atlantic salmon (*Salmo salar*)	radiocesium	6	0.00297	15.9	0.00672	55.3	
Brown trout (*Salmo trutta*)	radiocesium	6	0.00217	15.6	0.00501	58.4	
Atlantic salmon (*Salmo salar*)	radiocesium	6	0.00192	15.9	0.00592	76.3	
Arctic charr (*Salvelinus alpinus*)	radiocesium	6.3	0.00109	15.9	0.00508	107.7	
Rainbow trout (*Oncorhynchus mykiss*)	propofol	12	0.467	17	0.615	37.9	(Gomułka, Fornal, Berecka, Szmagara, & Ziomek, 2015)
Channel Catfish (*Ictalurus punctatus*)	oxolinic acid	14	0.010	24	0.017	37.4	(Kleinow, Jarboe, Shoemaker, & Greenless, 1994)
Nile tilapia (*Oreochromis niloticus*)	florfenicol	25	0.33	30	0.36	15.0	(Kosoff et al., 2009)
Walleye (*Sander vitreus*)	florfenicol	20	0.29	25	0.43	58.9	
Hybrid triped bass (*Morone chrysops* x	florfenicol	20	0.63	25	1.39	114.6	

Morone saxatilis)							
Turbot (*Scophthalmus maximus*)	enrofloxacin	10	0.01	16	0.02	78.6	(Liang, Li, Zhao, Liu, & Chang, 2012)
Gibel carp (*Carassius gibelio*)	moroxydine hydrochloride	15	0.08	25	0.26	84.2	(Liu et al., 2016)
Mummichog (*Fundulus heteroclitus*)	toxaphene-d	15	0.0503	25	0.0983	47.9	(Maruya, Smalling, & Vetter, 2005)
	toxaphene-l	15	0.0256	25	0.0532	52.2	
Rainbow trout (*Oncorhynchus mykiss*)	silver	4	0.188	16	0.43	45.9	(Nichols & Playle, 2004)
Largemouth bass (*Micropterus salmoides*)	radiocesium	15	0.002	20	0.003	49.2	(Peters & Newman, 1999)
Channel Catfish (*Ictalurus punctatus*)	radiorubidium	20	0.042	27.5	0.044	3.6	(Peters, Schultz, & Newman, 1999)
	radiocesium	20	0.0045	27.5	0.0082	58.5	
Sea bass (*Dicentrarchus labrax*)	oxolinic acid	13	0.0102	22	0.046	117.5	(Rigos, Alexis, Andriopoulou, & Nengas, 2002)
European eel (*Anguilla anguilla*)	geosmin	15	0.2	25	0.26	18.7	(Schram, Schrama, Kusters, Kwadijk, & Palstra, 2016)
Rainbow trout (*Oncorhynchus mykiss*)	flumequine	3	0.0012	13	0.0051	93.6	(Sohlberg, Aulie, & Soli, 1994)
Goldfish (*Carassius auratus*)	marbofloxacin	15	0.02	25	0.06	78.5	(Zhu et al., 2009)

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CHAPTER III:

THE EFFECTS OF TEMPERATURE AND PERSISTENT ORGANIC POLLUTANT - POLYBROMINATED DIPHENYL ETHER AND POLYCHLORINATED BIPHENYL - ON DEVELOPMENTAL RATE AND IMMUNE FUNCTION IN NORTHERN LEOPARD FROGS (LITHOBATES PIPIENS)

Cherry T Brown and William H Karasov

ABSTRACT

The predicted increasing temperatures associated with climate change could affect how persistent organic pollutants (POPs) impact wildlife, but no study has been done that focuses on the effects of environmentally relevant POP levels on amphibians at different temperatures. We exposed Lithobates pipiens tadpoles to varying levels of persistent organic pollutants, polybrominated diphenyl ether (PBDE) and polychlorinated biphenyl (PCB), at cooler and warmer temperatures and compared the effects on development and immune responses. Animals were exposed as soon as they became free swimming until they reached metamorphic climax (Gosner Stage 42). For the PBDE study, tadpoles were exposed orally to the commercial PBDE mixture DE-71 at 0, 10, 25, 50, 100 ng/g wet weight diets at 18 and 27°C. For the PCB study, tadpoles were exposed orally to PCB-126 at 0, 10, 25 ng/g wet weight diets at 23 and 27°C. After at least 12 weeks of exposure, tissue concentrations of PBDE and PCB were significantly positively correlated with diet toxicant concentrations, and there was no effect of rearing temperature on tissue toxicant level in tadpoles. Tadpoles reared at warmer temperatures reached metamorphosis faster than tadpoles reared at cooler temperature in both PBDE and PCB studies, there were no significant effects of PBDE on rate of development, and development was faster with increasing PCB level only at the warmer rearing temperature. When assessing immune function, we found complement lysis activity was significantly depressed at warmer temperatures in both PBDE and PCB studies, and after the animals were immunized with keyhole limpet hemocyanin (KLH), KLH-specific antibody response significantly increased in the warmer temperature treatment in the PBDE study. For the PCB study, KLH-specific antibody response was depressed in animals exposed to PCB at the warmer temperature. Toxicant treatment did not have significant dose-response effects for all endpoints in both PCB and PBDE studies. In the PCB study, there were temperature and toxicant interactions on size immediately post-metamorphosis and KLH-specific antibody response. Our data indicate that at warming temperatures, amphibian accumulation of these POPs may not change, and amphibian immunity may be lowered and

make them more susceptible to pathogens. In order to increase our predictive ability for the future, more studies are needed on temperature and environmentally relevant toxicant exposure in amphibians.

INTRODUCTION

Declining amphibian populations have stimulated research to identify the 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 climate on amphibians is an area greatly in need of research (Landis et al., 2014; Noyes et al., 2009).

It has been proposed that the increased temperatures associated with climate change could result in increased impacts of toxicants on wildlife (Noyes et al., 2009). We tested for direct and interactive effects of altered rearing temperature and toxicant exposure on developmental rate and immune function of tadpoles and young frogs. Depressed growth and development can lead to lowered long-term viability (Cabrera-Guzmán, Crossland, Brown, & Shine, 2013), and depressed immune function can raise the animal's susceptibility to environmental pathogens (Carey, Cohen, & Rollins-Smith, 1999).

As in most ectotherms, amphibian performance is greatly influenced by environmental temperature, and physiological rates usually increase as temperature increases. In many cases, ectotherms reared at warmer temperature reach developmental maturity at a faster rate and a smaller size (Karasov & Martínez del Rio, 2007). In *L. pipiens*, previous studies have shown that exposure to environmentally relevant levels of DE-71^{TM} slowed developmental rates (Cary Coyle & Karasov, 2010), whereas exposure to

environmentally relevant levels of PCB-126 did not (Cary, 2013). Studies have shown that many stressors can alter amphibians' physiological responses, and change in one physiological response can alter another, creating a positive feedback and exacerbate the animal's decline in physiological condition (Linder, Krest, Sparling, & AMPHIB, 2003). It is plausible that as developmental rate is accelerated by higher temperatures, the ability to mitigate effects of toxicants is lowered, resulting in a more pronounced toxicant exposure effect. However, such interactive effects have rarely been tested for in frogs.

In addition to growth and development, we are also interested in how amphibian immune functions are affected when frogs are concurrently exposed to toxicants and reared at different temperatures. Studies have shown that temperature does affect amphibian immune functions, although the direction of change and the changes in different immune branches are not consistent (Raffel, Rohr, Kiesecker, & Hudson, 2006; Ruben, Edwards, & Rising, 1977; Terrell et al., 2013). Maintaining an immune system is purportedly energetically costly, and some animal species exhibit apparent trade-offs in allocation between immune function(s) and growth (van der Most, de Jong, Parmentier, & Verhulst, 2011). Studies have shown that exposure to environmental pollutants increases energy expenditure (Zachariassen et al., 1991) and it widely known that exposure to warmer temperature increases resting energy expenditure in amphibians (Feder, 1992). As the demand for energy increases to cope with environmental pollutants and temperature changes, *L. pipiens* might trade-off energy allocations in the immune aspect. Previous studies in our laboratory have shown that at environmentally relevant levels, both the commercial PBDE mixture DE-71^{TM} and PCB-126 lessened the adaptive immune response in *L. pipiens* (Cary, Ortiz-Santaliestra, & Karasov, 2014).

Current literature leads us to hypothesize that developmental rate and immune responses to toxicant exposure will be impacted when *L. pipiens* are exposed at higher temperatures. However, there are no studies (to our knowledge) that address the interaction between temperature and environmental toxicant

exposure in amphibians. In order to address this issue, we exposed *L. pipiens* to varying levels of POPs, polybrominated diphenyl ether and polychlorinated biphenyl, at cooler and warmer temperatures and compared the effects on growth, development and immune responses. Results from this study can provide insight into how amphibians will respond to environmental contaminants as climate change ensues.

MATERIALS AND METHODS

The PBDE study and PCB study were carried out consecutively. The first study on PBDE used 18°C and 27°C for the two temperature treatments, but relatively high mortality during metamorphosis in the 18°C room led us to reconsider our selection of temperatures and so in the following PCB study we used 23°C as the lower temperature. Other than the cooler room temperature and the particular toxicants used for exposure, all other aspects of the two studies were the same. We will refer to the two temperature rooms as the "cooler" and "warmer" room in the following descriptions, where the "cooler" room is 18°C for the PBDE study, and 23°C for the PCB study; the "warmer" room was 27°C for both studies.

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 (Protocol number A01336). *Lithobates pipiens* embryos were purchased commercially from Nasco© (Fort Atkinson, WI) on the day fertilization took place (Gosner stage [GS] 1;(Gosner, 1960)). Immediately upon arrival, embryos were randomly aliquoted into 92 0.5L Nalgene containers (40 embryos/container) containing filtered, dechlorinated municipal water, and placed in temperature-controlled cooler or warmer rooms. Water within the containers was changed daily to ensure proper dissolved oxygen content as well as 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; approximately 6-8 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 (+/- 1˚C), throughout development. Relative humidity was kept > 30%, with static renewal of the aquaria water (> 80% water change) every other day, to provide satisfactory water quality (pH = 8 ± 0.2; nitrite < 1.0 mg/L; total NH3 < 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) on the racks.

Tadpole toxicant exposure.

Tadpoles were fed control diet or diets containing a mixture of PBDEs (10, 25, 50, 100ng/g DE-71^{TM}; a commercial mixture of PBDE congeners; Wellington Laboratories Inc., Ontario, Canada) or PCB-126 (10, 25ng/g PCB-126; Accu Standard®,New Haven, CT). Exposure levels were chosen to yield ecologically relevant concentrations based on Great Lakes sample data (Karasov, Jung, Vanden Langenberg, & Bergeson, 2005). The diets containing PBDE or PCB were made according to previous studies (Cary Coyle & Karasov, 2010). Briefly, targeted concentrations of PCB-126, and DE-71^{TM} in acetone were mixed with ground rabbit chow and stirred for 15 minutes to ensure proper adsorption of each toxicant into the chow. Rabbit chow for control diet was also mixed in the same fashion with only acetone. 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

jello-like consistency, similar to that of the control diet. The final diet contained: 19.5% rabbit chow, 1.5% agar, 1% gelatin and 78% water. Wet mass of food provided daily was 5-10% of summed tadpole mass in the tank, and left over food from the previous feeding was syphoned every day prior to feeding.

Tadpoles were fed *ad libitum* until they reached metamorphic climax (GS 42). Then they were removed from the tanks and housed individually in 500 ml polypropylene jars (Nalgene) with 50mL filtered, dechlorinated municipal water, and tilted to provide both a wet and dry surface. We conducted full water changes until the animals completed metamorphous (GS 46), indicated by complete tail reabsorption. *Lithobates pipiens* do not consume any food during metamorphosis.

After the metamorphic phase, animals were switched to a diet of live food (crickets and mealworms) without any PCB or PBDE until they reached 16 weeks post metamorphosis. This is to mimic the expected low contaminant residue levels in terrestrial insects that are the primary food source for wild *L. pipiens* (Paine, McKee, & Ryan, 1993).

Data collection

Developmental Rate

Days to reach metamorphic climax (the amount of time to reach Gosner Stage GS46) and days to complete tail reabsorption were used to assess developmental speed. Snout-vent length (SVL) and body mass were measured immediately upon completion of metamorphosis (GS46) to assess effects on animal size.

Assessment of immune functions

In order to assess both innate and adaptive immune functions of the animals, the following procedure was performed on all animals(Cary, 2013). At 10 weeks post metamorphosis, animals were

intraparitoneally injected with 25µL keyhole limpet hemocyanin (KLH) (1mg/g in PBS; EMD Chemicals, Inc. San Diego, CA) in combination with 25% Titermax™ adjuvant (Sigma-Aldrich, St. Louis, MO). At 14 weeks post metamorphosis, animals were injected with 25µL keyhole limpet hemocyanin (1mg/g in PBS) without the adjuvant. At 16 weeks post metamorphosis, animals were euthanized via exsanguination, and the plasma was collected for complement lysis assay and ELISA for KLH antibodies.

Complement Lysis Assay

Innate immune response was assessed by the complement protein activity towards lysing foreign cells. Frog plasma was incubated with rabbit red blood cells and we measured hemolytic ability. Based on previously established protocol (Cary, 2013), 15µL frog plasma samples were diluted with 60µL PBS and then incubated with 75µL 2% rabbit red blood cell solution in a 37°C water bath for 30 minutes in 600µL microcentrifuge tubes. The tubes were then centrifuged for 5 minutes at 2500 RPM, forming a pellet of any unlysed red blood cells. The supernatant was then plated to a 96-well plate and the absorbance was read at 540nm using a plate reader. Heat-activated plasma (pooled plasma incubated at 54°C for 30 minutes) was used as negative controls, and 1µL Triton-X-100 was added to the 2% rabbit red blood cell solution resulting in complete lysing of the cells was used as a positive control. Lysis activity is reported as a percentage, using the positive control absorbance as 100%.

$$Lysis\ activity = \frac{Sample\ Absorbance}{Positive\ control\ Absorbance} \times 100\% \qquad \text{(eq. 1)}$$

ELISA of KLH-specific antibodies

Adaptive humoral immune function was assessed by measuring the animal's KLH-specific antibody concentration. This method uses *Xenopus* antibodies with the procedures optimized for *L. pipiens* by Cary et al (Cary, 2013). High-binding 96-well plates were coated with 50µL 1mg/mL KLH antigen in coating

buffer and incubated at 4°C overnight. The plates were washed (wash protocol: 3 times with 0.1% Tween in phosphate buffered saline [PBS]), and then blocked with 300µL blocking buffer (5% dry milk) for 1 hour at room temperature. Plates were then washed and 100µL diluted frog sample plasma (1:100 in PBS) was added in triplicates. Plates were then incubated for 2 hours at room temperature. Plates were then washed and 100µL of primary antibody (mouse-anti-frog IgY) was added and incubated for 1 hour at 33°C. Afterwards, the plates were washed and 100µL of secondary antibody (horseradish peroxidase-conjugated rabbit anti-mouse IgG) was added and incubated for 1 hour at 33°C. Plates were then washed twice with wash protocol and 150µL of ABTS substrate was added and incubated for 40 minutes at room temperature. The reaction was then stopped with 50µL 1% SDS and the absorbance was read at 405nm using a plate reader. Frog plasma, from frogs that have not been injected with KLH and zebra finch plasma, were used as negative controls. Frog plasma from previous studies that are known to have a high KLH-specific antibody concentration was used positive controls. The data are reported as corrected absorbance, using the negative control samples average absorbance as the baseline.

Chemical Analysis.

Whole body tissue samples sans digestive tract were collected at 12 weeks post hatch and analyzed for toxicant levels to confirm exposure. The tadpoles' digestive tract along with any remnant diet was removed as to not confound the chemical analysis of tadpole tissues. Tadpoles were euthanized by buffered 1% MS-222, eviscerated and pooled to yield at least 7 grams of tissue per sample to warrant proper analysis. Diet and tissue samples were sent to ALS Environmental, ALS Group USA (Kelso, WA, USA) for PBDE and PCB detection and quantification. Chemical analysis (K1413603) was performed according to the laboratory's NELAP-approved quality assurance program (www.alsglobal.com). The method reporting limit (MRL) for PBDE is 0.21 ng/g wet tissue mass, and the MRL for PCB is 1.9 ng/g wet tissue mass.

Statistical Analysis.

All statistical analyses were performed using R version 3.1.2 (R Foundation for Statistical Computing). Analysis of covariance was used to compare the linear relationships of measured tissue toxicant concentration versus diet toxicant concentration at two different rearing temperatures (Figure 1). Survival, developmental rate (the amount of time to reach GS46), size post metamorphosis, complement lysis activity and KLH-specific antibody levels (absorbance) for each combination of temperature and diet treatment were compared using two-way ANOVA with interaction, followed by post-hoc Tukey HSD comparisons. The complement lysis assay data, reported as a percentage, were normalized using arcsine square-root transformation.

RESULTS

Tissue Residue Analysis.

We measured PBDE and PCB in animal tissues at 12 weeks post hatch. Animals reared on control diets had non-detectable amounts of toxicants. Animals fed diets containing PBDE and PCB had varying amounts of toxicant in their tissue corresponding to each diet treatment, confirming the difference in exposure to PBDE and PCB among treatment groups. Tissue concentrations of PBDE and PCB were significantly positively correlated with diet toxicant concentrations (p-value < 0.001), and analysis of covariance showed no effect of rearing temperature on tissue toxicant level (PBDE: p-value = 0.71; PCB: p-value = 0.47; Figure 1).

Survival.

For the PBDE study, survival during metamorphosis was lower in animals raised at 18°C (48 ± 19%, n = 20) than in those raised at 27°C (85 ± 15%, n = 20) (F = 44.08, p-value < 0.001). This led us to adjust the cooler room temperature in the subsequent PCB study to 23°C instead of 18°C. Survival during

metamorphosis was slightly lower in animals raised at 23°C (93 ± 8%, n = 9) than in those raised at 27°C (100 ± 0%, n = 9 tanks) ($F = 10.12$, p-value < 0.01). Across both studies, there was no significant difference in survival between dietary toxicant levels nor were there significant temperature and dietary toxicant interactions (PBDE treatment p-value = 0.95, temperature x PBDE p-value = 0.27; PCB treatment p-value = 0.10, temperature x PCB p-value = 0.10).

Development rate.

For both PBDE and PCB trials, animals in the warmer room took significantly less time to reach metamorphosis than in the cooler room (PBDE: $F_{1,161} = 234$, p-value < 0.001; PCB: $F_{1,162} = 14.27$, p-value < 0.001; Figure 2). For animals exposed to PCB, time to reach metamorphosis was inversely related to dietary PCB level ($F_{2,162} = 8.71$, p-value < 0.001; Figure 2).

Size at 0 days Post-metamorphosis

For animals exposed to PBDE, both temperature and dietary treatment affected size when it was measured at 0 days post metamorphosis (dpm) (Figure 3A, Figure 3B). Animals reared at 27°C were significantly smaller in both SVL and body mass than animals reared at 18°C (SVL: $F_{1,161} = 275.27$, p-value < 0.001, Figure 3A; body mass: $F_{1,161} = 6.02$, p-value < 0.001, Figure 3B). Animals exposed to the highest dietary PBDE concentration (100 ng/g wet mass) at both rearing temperatures were significantly larger in body mass than animals exposed to other treatments ($F_{4,161} = 2.03$, p-value < 0.001).

For animals exposed to PCB, there was a significant temperature and dietary treatment effect in both SVL and mass at 0 dpm (Figure 3C, Figure 3D). Animals reared at 27°C were significantly larger in both

SVL and body mass than animals reared at 23°C at 0 dpm (SVL: $F_{1,162} = 84.39$, p-value < 0.001, Figure 3C; body mass: $F_{1,162} = 13.6$, p-value < 0.001, Figure 3D). There was also a significant temperature and dietary treatment interaction for mass ($F_{2,162}= 4.531$, p-value <0.05). However, post-hoc Tukey test indicates there was no difference between control groups from both temperature treatments (SVL: p-value = 0.1; mass: p-value = 0.999).

Complement Lysis Assay

For animals exposed to PBDE, there was a significant temperature effect on lysis activity (Figure 4A). Animals reared at 18°C showed higher lysis activity than animals reared at 27°C ($F_{1,136} = 30.8$, p-value < 0.001). There was a significant dietary effect: animals exposed to 25ng/g PBDE showed higher lysis activity than other dose levels ($F_{4,136} = 2.79$, p-value <0.05), however post-hoc Tukey comparisons showed that it did not differ from control animals (p-value = 0.305). This pattern held true at both rearing temperatures, and there was no significant temperature and dietary treatment interaction ($F_{4,136}= 0.597$, p-value = 0.0665).

For animals exposed to PCB, there was a significant temperature effect on lysis activity (Figure 4B). Animals reared at 23°C showed higher lysis activity than animals reared at 27°C ($F_{1,161} = 14.5$, p-value < 0.001). There was no significant dietary effect ($F_{2,161} = 0.31$, p-value = 0.735) nor significant temperature and dietary treatment interaction (F = 1.70, p-value = 0.185).

KLH-specific antibodies

For animals exposed to PBDE, there was a significant temperature effect on KLH-specific antibody levels. Animals reared at 18°C showed lower levels of KLH-specific antibody than animals reared at 27°C ($F_{1,146} = 4.63$, p-value < 0.05; Figure 5A). There was no significant dietary effect ($F_{4,146} = 1.00$, p-

value = 0.409) nor significant temperature and dietary treatment interaction ($F_{4,146}$ = 0.909, p-value = 0.460).

For animals exposed to PCB, there was no significant temperature effect ($F_{1,161}$ = 0.063, p-value = 0.802) or dietary effect ($F_{2,161}$ = 0.536, p-value = 0.586) on KLH-specific antibody levels. However, there was a significant temperature and dietary treatment interaction ($F_{2,161}$ = 3.16, p-value < 0.05; Figure 5B). Although post-hoc Tukey with all treatments did not distinguish which treatments differ, testing for all combinations of grouping showed that for animals in the warmer room, KLH-specific antibody levels were significantly lowered in all exposed animals than control animals (F = 6.76, p-value <0.05), whereas there was no depression effect observed in animals in the cooler room.

DISCUSSION

Overview

The animals in this study were exposed to different environmentally relevant toxicant levels at two different rearing temperatures. The aim was to see how amphibians might respond to environmental contaminants in a warming climate. Past studies have shown that some of the endpoints we tested were impacted individually by either increased temperature or by exposure to contaminants, but possible interactions among these two factors have not been studied. Thus, in the following paragraphs as we discuss in more detail the various responses to temperature and toxicant exposure we will highlight especially these interactions, which were signified by significant statistical interactions between temperature and toxicants. Additionally, it is worth noting that rearing tadpoles at different temperatures did not change toxicant accumulation (Fig. 1). Therefore, in instances where interactions between temperature and toxicant were apparent, it seems quite plausible that they result from temperature-related

differences in sensitivity of target site(s) to the respective toxicant rather than simply to temperature-related differences in toxicant accumulation.

Temperature and toxicant effects on developmental rate and size post-metamorphosis

As expected from previous studies, animals reared at warmer temperatures developed faster, taking fewer days to reach metamorphosis (Alvarez & Nicieza, 2002; Rumschlag, Boone, & Fellers, 2014). This was most evident in the study involving PBDEs (Fig. 2A), where the differences in rearing temperature were larger than in the study involving PCB-126. In many ectotherms, faster development at warmer temperature leading to earlier metamorphosis is also associated with smaller size post-metamorphosis (Karasov and Martinez del Rio 2007), which was an effect also most evident in the study involving PBDEs (Fig. 3A, 3B).

Our results also indicated that although exposure to PCB-126 also led to faster development (shorter time to metamorphosis; Fig. 2B), in that case the faster development was associated with larger size especially in the animals reared at warmer temperature (Fig. 3C, 3D; no temperature-related size differences in controls). This seemed to be an interactive result of being reared at a warmer temperature while also exposed to PCB-126.

There was one other possible interaction between warmer rearing temperature and toxicant on development or growth. Other studies in which leopard frog tadpoles were exposed to similar dietary concentration of DE-71, but reared at 23 ºC, found that DE-71 exposure was associated with slower development (Cary and Karasov 2010; Cary et al. 2014)), and this effect was not apparent in our study (Fig. 2A). The effect of DE-71 on development in those other studies was much smaller than the effect of temperature that we recorded here. This discrepancy might reflect another subtle effect of an interaction between temperature and toxicant exposure, which could be tested for in further studies. Also, this kind of

discrepancy could be due to different methods in assessing development. Some past studies have chosen a specific time-point and sampled all the animals' Gosner stage to quantify development. While Gosner staging is a well-established process, this numbering system does not consider how different stages take up different amount of times. For instance, animals can go from Gosner stage 20 to 25 in a few days, whereas animals also typically take a few days to progress from Gosner stage 36 to 37(Gosner, 1960). Due to this complication, we feel that measuring the time it takes from hatch to metamorphosis is a less confounding end-point and might explain some discrepancies between this study and previous studies.

Temperature and toxicant effects on immune functions

Lysis activity was significantly reduced in animals that were reared at warmer temperature (Fig. 4). Interestingly, DE-71 only at a midrange exposure level increased lysis activity at both rearing temperatures (Fig. 4A), an effect of a PBDE previously unreported. PCB-126 had no significant effect on lysis activity.

Other studies, however, have shown that warmer temperature increased aspects of innate immune function in amphibians (Terrell et al., 2013; Jozkowicz & Plytycz, 1998; Maniero & Carey, 1997), however, upon closer inspection, these studies were comparing temperature ranges (5 - 22°C) well below our temperature ranges (18/23/27°C). In our study, animals held at 18 and 27°C (9°C difference) showed a larger depression in lysis activity than animals held at 23 and 27°C (4°C difference). It is plausible that for our study in *L. pipiens*, the optimal temperature for complement protein production is closer to 18°C.

KLH-specific antibody production increased with temperature for frogs exposed at 18 and 27°C (Fig. 5A) yet did not show an increase with temperature for frogs exposed at 23 and 27°C (Fig. 5B). It is possible that the smaller difference in temperature was not sufficient to elicit a significant difference for this endpoint. There was no overall effect of exposure to DE-71 on KLH-specific antibody production

(Fig. 5A), but exposure to PCB-126 depressed antibody production under warmer rearing conditions only (Fig. 5B). A depressing effect of exposure to PCB-126 on antibody production has not been previously reported.

There was no dose response effect with exposure to PBDE for both complement lysis and KLH-antibody production in animals reared at 18 and 27°C, and this could indicate that the environmentally relevant levels that we used for our study were below the LOEC for PBDE in *L. pipiens*.

Prospects for growth, development and immune function in frogs in a warming environment

There is good evidence in this study (Fig. 2), and others, that development time of frogs will be faster in a warmer environment. Exposure to toxicants has also been shown in some instances to influence development time(Boone & Bridges, 2003; Carey & Bryant, 1995), but our results with both PCB-126 (Fig. 2B) exposure and PBDE (DE-71) exposure indicate that these toxicant effects in leopard frog tadpoles depend on rearing temperature. Through apparently interactive effects between warming temperature and toxicant exposure, the common association of smaller froglet size and faster metamorphosis (Karasov and Martinez del Rio 2007) seems reversed in tadpoles raised at warmer temperature that are also exposed to environmentally relevant levels of PCB-126 (Fig. 3C, 3D).

There is also good evidence in this study that lysis activity may be reduced in leopard frogs growing at the warmer temperatures that are predicted in climate change scenarios for temperate North America (Fig. 4). The finding of a general reduction in lysis activity with increased rearing temperature is new, and suggests a possibly increased risk of infection in frogs in a warming environment. However, disease outcomes from infectious agents can also be influenced by adaptive immunity. Our study showed that froglets reared at 27°C had more KLH-specific antibodies when they were challenged with KLH injections, and Greenspan et al. showed that tree frogs (*Litoria spenceri*) exposed to repeated heat pulses

from 18 to 29°C were more resistant to chytrid infection than animals held at a constant 18°C (Greenspan et al., 2017). These results shed light on how increased environmental temperatures alter immune function in amphibians. Studies looking at how temperature affects chytrid infection in amphibians have shown trends that there may be an interaction between innate and adaptive immunology branches where they respond inversely to an immune challenge, and similar trade-off effects are observed in reptile and humans as well (McDade, Georgiev, & Kuzawa, 2016; Ribas et al., 2009; Sandmeier, Tracy, Dupré, & Hunter, 2012). With this in mind, it is reasonable that our frogs exposed to PBDE at 27°C showed higher KLH-specific antibody production and lower complement lysis ability compared to frogs reared at 18°C. However, exposure to a different toxicant, PCB-126 in frogs reared at expected warmer temperature, resulted in declines in both innate immunity (lysis activity; Fig. 4B) and adaptive immunity (specific antibody production; Fig. 5B). Fairly analogous to our findings with PCB-126, (Rumschlag et al., 2014) found that Pacific tree frogs were more susceptible to chytrid fungus when exposed at 25°C, compared to animals exposed at 15 or 20°C. Overall, interactive effects between warming temperature and toxicant exposure were most apparent for PCB-126.

Considering that actual tissue levels of PCB-126 were similar in leopard frogs raised at both cooler and warmer temperature (Fig.1), it seemed that growing at warmer temperature increased frog's sensitivity to PCB with regards to both growth/development and immune response. Especially for the two immune responses we studied, PCB was more toxic when frogs were exposed in a warmer environment. The depression in KLH-antibody production and complement lysis activity (above) at warmer temperature shows us amphibians may be more susceptible to disease outbreaks as climate change ensues and global temperature increases. However, in order to increase our predictive ability for the future, more studies need to be completed that look at environmentally relevant toxicant levels simultaneously with the predicted increases in climatic temperature.

TABLES AND FIGURES

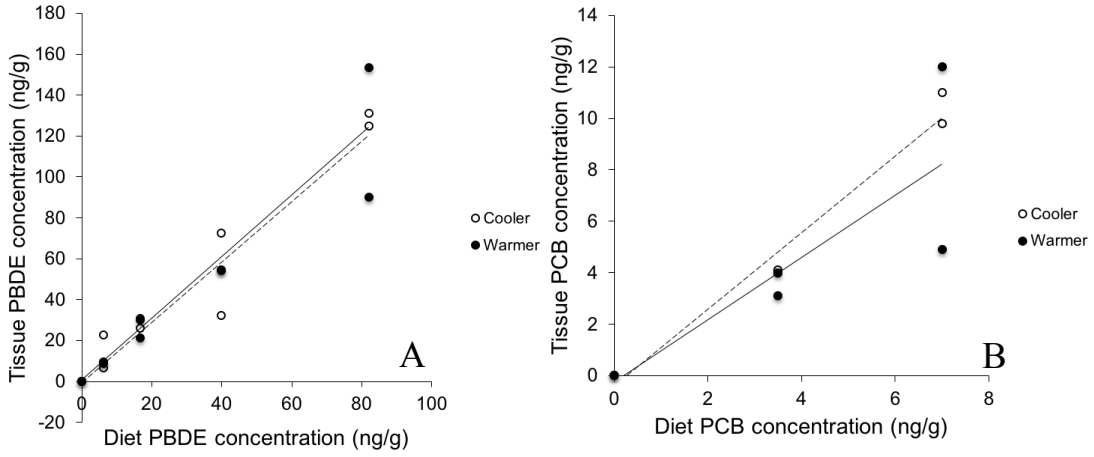

Figure 1. Tissue toxicant concentration in tadpoles as a function of diet toxicant concentration and rearing temperature. Tissue concentrations of PBDE (**A**) and PCB-126 (**B**) were significantly positively correlated with diet toxicant concentrations (p-value < 0.001), and analysis of covariance showed no temperature effect on tissue toxicant level (PBDE: p-value = 0.71; PCB: p-value = 0.47). Tissue pools of tadpoles raised at the warmer temperature, 27 ^{o}C are represented by filled circles and solid line, and tissue pools from tadpoles raised at the cooler temperature are represented by unfilled circles and dashed lines.

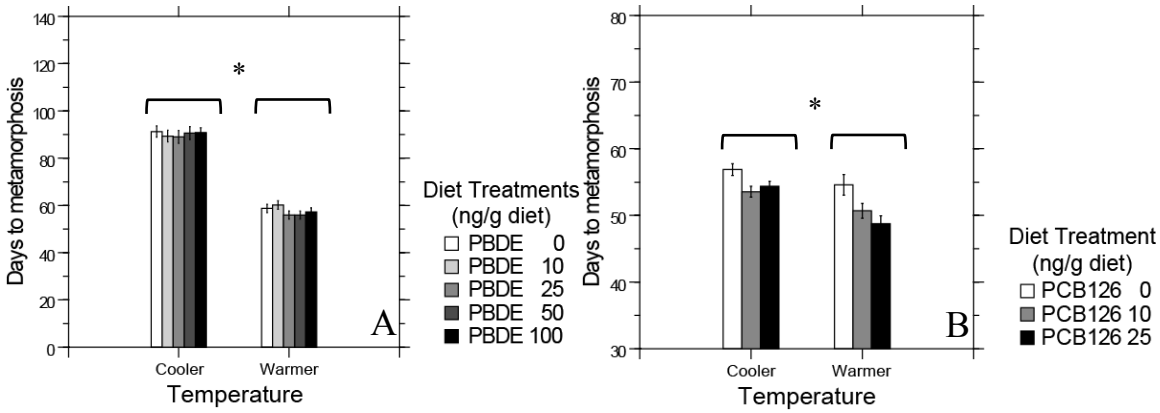

Figure 2: Days to metamorphosis in tadpoles reared at two temperatures. For both PBDE (**A**) and PCB (**B**), animals reared at warmer temperature took significantly less time to reach metamorphosis than those reared at cooler temperature. (PBDE: $F = 234$, p-value < 0.001; PCB: $F = 14.27$, p-value < 0.001. For animals exposed to PCB, time to reach metamorphosis was inversely related to dietary PCB level ($F = 8.71$, p-value < 0.001). In both panels, the asterisks indicate there is significant difference between the bracketed groups.

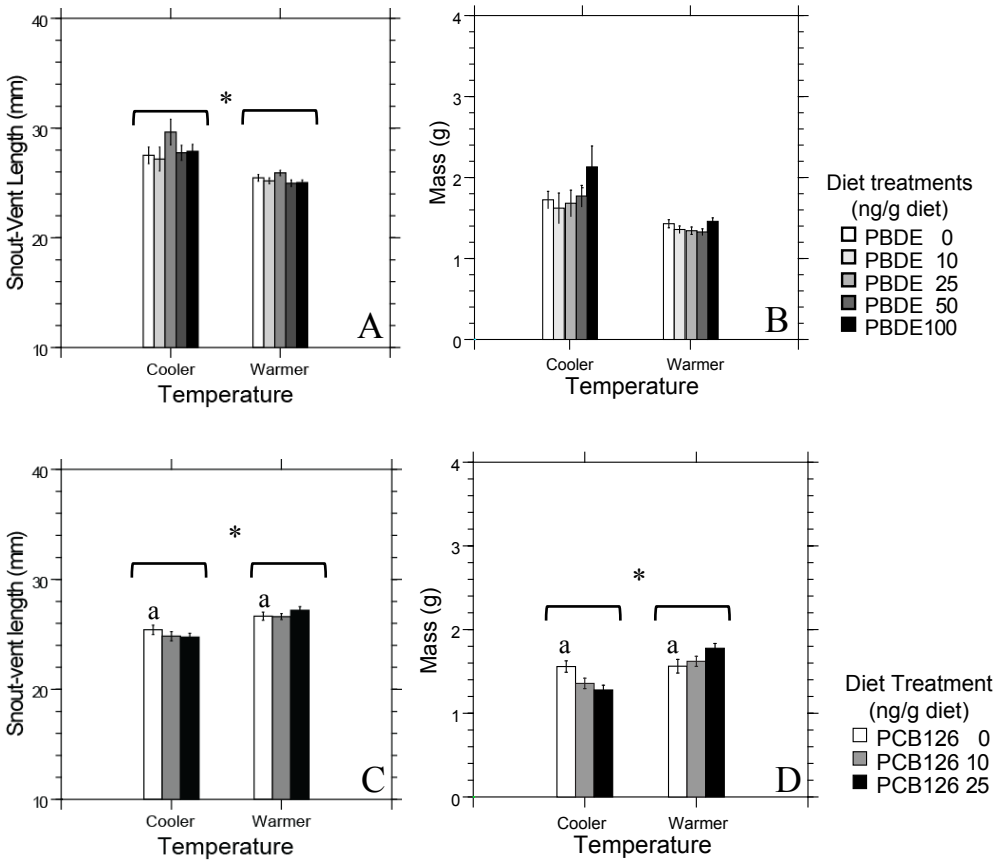

Figure 3: Size measure of frogs reared at two temperatures at 0 days post-metamorphosis. For PBDE (top figures), animals reared at 27°C were significantly smaller than animals reared at 18°C for both SVL (**A**; $F = 275.27$, p-value < 0.001) and body mass (**B**; $F_{1,161} = 6.02$, p-value < 0.001). For PCB (lower figures), animals reared at 27°C were significantly larger than animals reared at 23°C for both SVL (**C**; $F = 84.39$, p-value < 0.001) and for body mass (**D**; $F_{1,162} = 13.6$, p-value < 0.001). However, post-hoc Tukey tests indicated there was no difference between control groups from both temperature treatments for either SVL (p-value = 0.1) or body mass (p-value = 0.999), labeled as "a" in panels C and D. In all panels, the asterisks indicate there is significant difference between the bracketed groups.

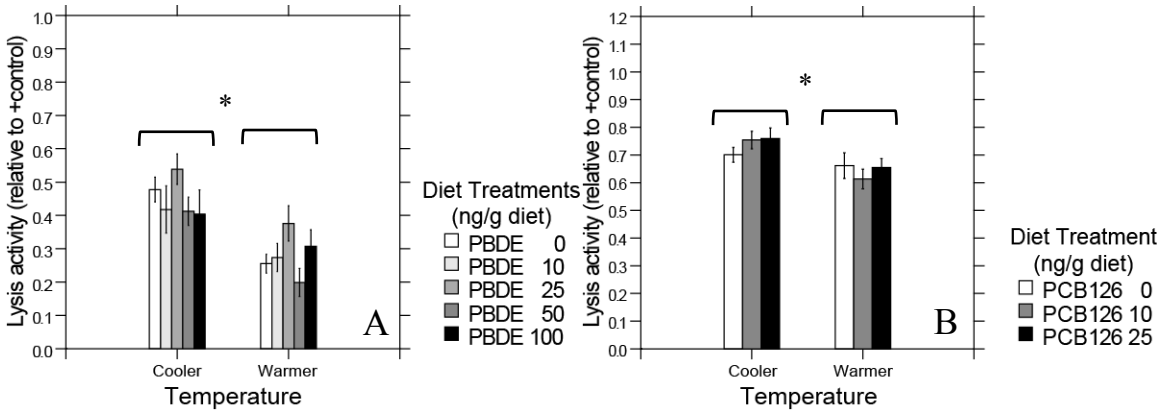

Figure 4: Lysis activity levels (percent of foreign erythrocytes lysed, relative to control). **A**: For PBDE exposure experiment, animals reared at 27°C showed lower lysis activity than animals reared at 18°C ($F = 30.8$, p-value < 0.001). There was a significant dietary effect: animals exposed to 25ng/g PBDE showed higher lysis activity than other dose levels, however it did not differ from control animals. This pattern held true at both temperatures, and there was no significant temperature and dietary treatment interaction. In **B**: For the PCB exposure experiment, animals reared at 27°C showed lower lysis activity than animals reared at 23°C ($F = 14.5$, p-value < 0.001). There was no significant dietary effect nor significant temperature and dietary treatment interaction. In both panels, the asterisks indicate there is significant difference between the bracketed groups.

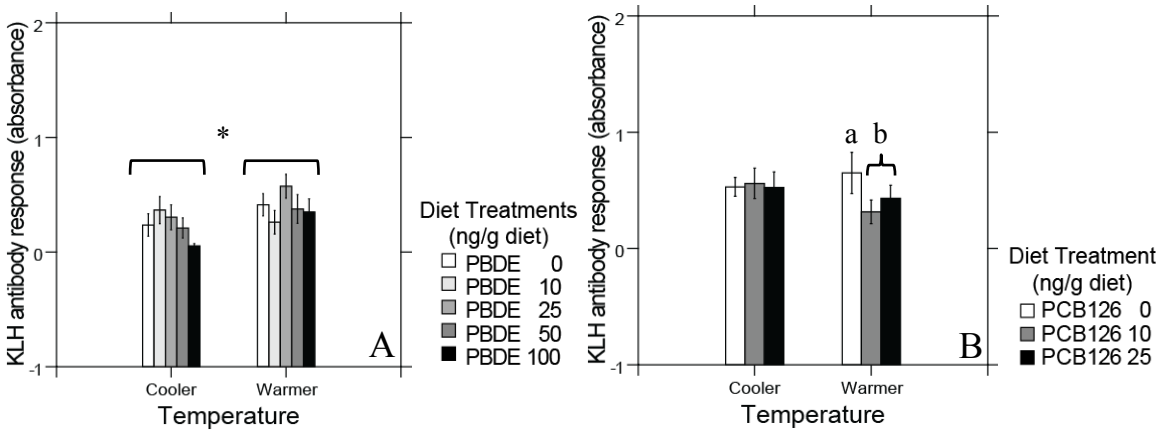

Figure 5: KLH-specific antibody levels after challenge with KLH injections 10 weeks and 14 weeks post metamorphosis.

A: For PBDE exposure experiment, animals reared at 27°C showed higher levels of KLH-specific antibody than animals reared at 18°C ($F = 4.63$, p-value < 0.05). There was no significant toxicant effect nor significant temperature and toxicant treatment interaction. The asterisks indicate there is significant difference between the bracketed groups.

B: For the PCB exposure experiment, there was no significant general temperature or toxicant effect on KLH-specific antibody levels. However, there was a significant temperature and toxicant treatment interaction (($F = 30.8$, p-value < 0.05). For animals reared at the warmer temperature, KLH-specific antibody levels were significantly lower in exposed tadpoles than control tadpoles ($F = 6.76$, p-value <0.05; signified by letters a *vs.* b), whereas there was no depression effect observed in animals reared at the cooler temperature.

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CHAPTER IV:

WARMER TEMPERATURE INTERACTS WITH POLYBROMINATED DIPHENYL ETHERS (PBDES) TO ALTER HORMONE PROFILES DURING METAMORPHOSIS IN *LITHOBATES PIPIENS*

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ABSTRACT

Amphibian populations have been declining, and climate change and exposure to environmental contaminants are thought to be involved. Higher water temperature accelerates larval development; however, its combined effects with contaminants and their influence on hormones during metamorphosis are poorly understood. The authors investigated changes in whole-body triiodothyronine (T3) and corticosterone concentrations in developing leopard frogs reared at 23 and 28°C on diets with 0, 6, and 37 ng/g of a technical mixture of polybrominated diphenyl ethers (PBDE; DE-71) from 10 d to 44 d (premetamorphosis to late climax; Gosner Stages 28 to 46). Unlike controls, PBDE-exposed tadpoles (6 ng/g) reared at 23°C failed to show any increase in T3 concentrations throughout metamorphosis, and exposed tadpoles reared at 28°C showed a lower peak at climax compared to controls. Corticosterone levels progressively increased throughout metamorphosis, but the levels were higher in PBDE-exposed tadpoles compared to controls at both temperatures. At the warmer temperature, corticosterone increase occurred earlier (at early climax) in controls and exposed tadpoles compared to tadpoles reared at the cooler temperature (late climax), coinciding with the faster development observed at 28°C. Tadpoles reared at 28°C were longer and developed faster than tadpoles reared at 23°C. At both temperatures, PBDE exposure decreased T3 and increased corticosterone concentrations, which can potentially impair developing tadpoles.

INTRODUCTION

Polybrominated diphenyl ethers (PBDEs) have been used extensively as flame-retardants in plastics, electronics, and textiles and are among the chemical pollutants reported in the Great Lakes food webs (Stapleton & Baker, 2003). Because they are not chemically fixed to the products on which they are applied, PBDEs can leach out of treated products into the environment. They are persistent and bioaccumulative, due to their lipophilic characteristic, and can biomagnify through the food chain(Fisk, Cymbalisty, Tomy, & Muir, 1998). Being endocrine disrupting chemicals, their presence in the environment may interfere with nontarget animals' hormone biosynthesis, metabolism, or mode of action and are associated with developmental and reproductive alterations in several wild amphibians(Bevan, Porter, Prasad, Howard, & Henderson, 2003; T. B. Hayes, 1998; Levy, Lutz, Kruger, & Kloas, 2004).

Metamorphosis is a key hormonally regulated process in amphibians' development and is mostly driven by thyroid hormones(Brown & Cai, 2007). Increasing thyroid hormone levels induce the primary morphological and physiological changes during this period(Fort et al., 2011). Corticosterone is also a key hormone in metamorphosis (T. Hayes & Wu, 1995; M. Wright et al., 1994), antagonizing the changes induced by the thyroid during early stages, but later synergizing with the rising level of thyroid hormones to accelerate metamorphosis in leopard frogs(M. Wright et al., 1994). In bullfrogs (*Rana catesbeiana*), the adrenal corticoids inhibited limb growth and epidermal cell proliferation during premetamorphosis and premetamorphosis (M. L. Wright, Proctor, & Alves, 1999). The depressive effect of corticoids during spontaneous metamorphosis was related at least partly to thyroid inhibition because hydrocortisone significantly reduced follicle cell height, lumen diameter, and cell proliferation in the thyroid. During induced metamorphosis in the same species, corticosterone antagonized the effect of low T4 concentrations on the limb. On the other hand, this adrenal steroid synergized with higher T4 concentrations, near climax levels, to promote limb growth and development(M. Wright et al., 1994).

Multiple studies have shown that exposure to PBDEs disturbed thyroid hormone homeostasis (Cheek, Kow, Chen, & McLachlan, 1999; Meerts et al., 2000). Tetrabromobisphenol A, also a brominated flame retardant, decreased T3 levels during metamorphic climax in wrinkled frog (*Rana rugosa*) tadpoles, suggesting an antithyroidal effect for these chemicals (Kitamura et al., 2005). Another congener, 2,2′, 3,3′, 4,4′, 5,5′, 6-nona brominated diphenyl ether (BDE206) antagonized thyroid hormone-regulated tail regression in *Xenopus laevis* (Schriks, Zvinavashe, Furlow, & Murk, 2006). The effects of PBDEs on corticosterone levels are less studied, although it is reasonable to postulate that stress-induced increases in corticosterone levels caused by chemical exposure could alter corticosterone-mediated events important for metamorphosis.

Endocrine alterations due to brominated compound exposure are consistent with previous studies from our laboratory, showing that northern leopard frog (*Lithobates pipiens*) tadpoles exposed via their diet to environmentally relevant concentrations of PBDEs, throughout larval development, showed diminished growth and delayed metamorphosis(Cary Coyle & Karasov, 2010), altered gonadal development(Van Schmidt, Cary, Ortiz-Santaliestra, & Karasov, 2012), and altered immune function(Cary, Ortiz-Santaliestra, & Karasov, 2014). Developmental impairments such as inhibited tail resorption and delayed metamorphosis were also observed in *Xenopus laevis* exposed to the commercial PBDE mixture DE-71(Balch, Velez-Espino, Sweet, Alaee, & Metcalfe, 2006).

Other environmental factors also influence the physiological responses of amphibians undergoing metamorphosis. Increased water temperature, for example, can increase thyroid hormone secretion in captive amphibians(M. L. Wright et al., 1999), which accelerates the growth (i.e., rate of body size increase) and development (i.e., rate of progression through developmental stages) of tadpoles in warmer conditions(Bellakhal, Neveu, Fartouna-Bellakhal, Missaoui, & Aleya, 2014).

Both chemical contamination and climate change are contributing to the worldwide decline in anuran amphibian populations(Collins & Storfer, 2003; Hooper et al., 2013). Yet, to our knowledge, no in vivo studies have investigated the combined effects of these factors together on spontaneous metamorphosis in any frog species. The aim of the present study was to investigate the effects of environmentally relevant concentrations of PBDEs, at normal (23 °C) and elevated (28 °C) water temperature, on hormone concentrations, growth (total length) and development (time to metamorphic climax) of *Lithobates pipiens* tadpoles throughout metamorphosis. We predicted that 1) increased temperature would increase T3 and corticosterone levels and accelerate growth and development during metamorphosis; 2) PBDE exposure would decrease T3 levels and reduce the rate of development; and 3) interactions would occur between temperature and PBDE that would alter hormone levels and tadpoles' growth and development.

MATERIALS AND METHODS

Animals and husbandry

We chose northern leopard frogs because they are native to North America and abundant in the Great Lakes ecosystem, although their populations may be impacted by climate change and pollutants in this region. Two newly fertilized egg clutches (same day) of *Lithobates pipiens* embryos were purchased from Nasco. Embryos from both clutches were immediately transferred into containers with laboratory-prepared water (36 embryos/container) and distributed in 2 different animal rooms set up for water temperatures at 23 ± 1 °C or 28 ± 1 °C. Laboratory water was prepared using an inline series of 1 μm sediment and carbon filters followed by ultraviolet sterilization. Water was changed every other day and any nonviable embryos were removed to minimize bacterial growth. After 6 d, the tadpoles became free-swimmers and reached Gosner Stage (GS) 25 (Gosner, 1960), and the exposure begun. All experimental procedures were approved by the University of Wisconsin's Institutional Animal Care and Use Committee.

Conditions of exposure

The major PBDE congeners in technical pentabromodiphenyl ether mixture (Great Lakes DE-71; Wellington Laboratories) are (percentage by weight, according to the manufacturer): BDE-47 (32.4%); BDE-99 (43.9%); BDE-100 (8.9%); BDE-153 (3.8%); and BDE-154 (3.3%). This mixture has been used extensively in the past decades and relevant concentrations have been found in Great Lakes biota samples, varying from 0.29 ng/g to 3.93 ng/g in shrimp to up to 86 ng/g to 156 ng/g in lake trout 1. Doses used in the present study were chosen based on these concentrations. Nominal dietary concentrations of DE-71 (0 ng, 6 ng, and 37 ng DE-71/g diet wet weight) were incorporated into the food, prepared according to Coyle and Karasov (Cary Coyle & Karasov, 2010) and Gleason et al. (Gleason, Yahn, & Karasov, 2016). 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; Harlan Teklad, catalog 2030). The mixture was left on a tray inside a chemical fume hood overnight to allow for acetone and toluene evaporation. The spiked dried rabbit chow was then mixed with agar (20 g/L), gelatin (14 g/L), and distilled water. The mixture was brought to boil for 1 min and then cooled to room temperature. The nutritional composition of the diet is shown in Gleason et al. The spiked diet was kept refrigerated at –4 °C or stored at –20 °C for future use.

Tadpoles (GS 25; $n = 450$) were randomly distributed into a closed system consisting of glass aquaria filled with 12 L of laboratory water and provided with air stones. In each animal room, dietary treatment groups (target concentrations: 0 ng, 6 ng, and 37 ng PBDE/g diet wet weight) were each replicated in 3 different aquaria ($n = 25$ tadpoles/tank). Light cycles were set at 14:10-h light:dark and water temperatures were set at 23 ± 1 °C in the cooler room and at 28 ± 1 °C in the warmer room. The temperature choice was based on previous data from our laboratory on typical water temperatures for frog husbandry ranging from 22 °C to 24 °C and on the expectation that climate may warm as much as 4 °C by the turn of the century in Green Bay(Veloz et al., 2012). Relative humidity was kept > 30%, with static renewal of the

aquaria water (> 80% water change) every other day, to provide satisfactory water quality, monitored weekly. The water was mixed to yield hardness of 150 mg/L to 400 mg/L as CaCO3, with routine water quality measurements throughout the experiment being: chlorine: nondetect; pH = 7.9 ± 0.2; nitrite = 0.1 ± 0.07 mg/L; total NH3 = 0.03 ± 0.02 mg/L; dissolved oxygen > 6.0 mg/L. Food was introduced to the tadpoles as soon as they were transferred to the aquaria (free-swimming stage), approximately 6 d posthatch. Tadpoles were fed ad libitum once a day (food mass was 5–10% of summed tadpole mass per day), and left over food from the previous feeding was syphoned every day prior to feeding. On exposure day 40, 5 to 6 tadpoles were removed from the different treatment tanks in each room for PBDE residue analysis. Tadpoles that reached GS 42 were removed from tanks and placed in individual 500 mL polypropylene containers (Thermo Fisher Scientific) with water until completion of metamorphosis (GS 46) at their respective treatment temperature (23 °C or 28 °C). Mortality rates were <1% in both rooms.

Sample collections and endpoints

The time schedule for each endpoint is shown in Table 1. The stages of development, according to Gosner (Gosner, 1960), were recorded twice a week in all tanks during the experimental period. To determine hormone concentrations, stage-matched tadpoles were collected on the same day, following the observation of at least 8 individuals sharing the same developmental stage. Specific developmental stages were chosen for presenting distinctive features that would enable the unambiguous staging and therefore minimize the error during sample collections: GS 28 (middle of the hind limb bud growth), GS 31 (foot paddle), GS 37 (all toes separated), GS 41 (forelimbs visible; vent tube gone), GS 42 (forelimbs emergence), and GS 46 (tail resorption and metamorphosis complete).

For each hormone sample collection, we measured body mass (g) and total length (cm) of each tadpole immediately after euthanasia with 2% tricaine methanesulphonate solution at pH 7. Whole

tadpoles were then snap frozen individually in cryo-vials using liquid nitrogen and stored at –80 °C until hormonal analysis.

Total length was considered as the length from the tip of the snout to the posterior end of the tail, measured when tadpoles were photographed and had their measurements taken. The same tadpoles had their developmental stages determined visually according to Gosner. The tadpoles were then placed back into their respective tanks. To avoid discrepancies, the same analyst performed growth and development endpoints throughout the experimental period. We decided not to use anesthesia when taking pictures or visually determining the stage to avoid chemical interference with their natural metamorphosis, and we minimized animal handling to avoid stress to the animals. Time to reach metamorphic climax (GS 42: forelimb emergence) was recorded for each tadpole.

Chemical analysis

We performed limited chemical analyses to confirm exposure differences, and several earlier studies demonstrated that PBDE kinetics are first order and rapid enough that tissue concentrations in tadpoles exposed for 34 d (the day of exposure we chose to collect the samples) correspond closely to those in the diets ((Cary Coyle & Karasov, 2010; Cary et al., 2014); W. Karasov, unpublished data). Diet and tissue samples were sent to ALS Environmental, ALS Group USA to measure PBDE concentrations. Chemical analysis (K1413603) was performed according to the laboratory's National Environmental Laboratory Accreditation Program-approved quality assurance program. Minimum detection level was 0.6 ng g−1 wet food or wet tadpole. For food samples, measured once on a random day from the stock recipients, values of total PBDEs (sum of all 6 congeners, ΣPBDEs) were close to the target values: measured 36.8 ng g−1 wet food versus target of 36, measured 6.13 versus target of 6, and nondetectable measures for the controls (n = single pools in each case; Table 2). Corresponding body burdens in pooled tadpoles exposed for 34 d at 23 °C were: 35.7 ng g−1 wet mass, 5.6 ng g−1 wet mass, and nondetectable (controls). For

tadpoles exposed for 34 d at 28 °C, the values obtained were: 75 ng g−1 wet mass, 5.1 ng g−1 wet mass, and nondetectable (n = 3–4 tadpoles; ≈7 g), from multiple tanks, in each pool). Lacking replicates, we cannot say whether the value of 75 ng g−1 wet mass is higher than the value in tadpoles raised at the lower temperature (35.7 ng g−1 wet mass) eating the same food. However, the pair of values (average 55.4 ng g−1 wet mass) confirms a higher planned exposure of tadpoles eating the higher PBDE food compared to those eating the lower PBDE food (tadpoles averaged 5.4 ng g−1 wet mass). Values for the 6 individual congeners are provided in Table 2.

T3 and corticosterone extraction

Triiodothyronine and corticosterone were extracted using Hersikorn and Smits methods(Hersikorn & Smits, 2011), which were adapted from methods developed by Brasfield et al. (Brasfield et al., 2004). Briefly, each tadpole was weighed and minced in an equal amount of homogenization buffer consisting of 1 mM 6-propyl-2-thiouracil in methanol, previously prepared and stored at –20 °C. A second volume of homogenization buffer was added, and the samples were homogenized with a tissue tearor (4 bursts of 15 s each) and vortexed for 1 min. Samples were then centrifuged at 2900 rpm at 4 °C for 10 min and the resulting supernatant was transferred to a glass tube. All tissue manipulations were performed over ice. The procedure was then repeated with the remaining pellet, and the collected supernatants were combined. For each tadpole supernatant, the total volume was quantified and divided into as many 150 µL aliquots as possible, and stored at –80 °C until required.

Enzyme immunoassay for T3 and corticosterone

Triiodothyronine concentrations were measured with enzyme immunoassay (EIA) kits for T3 (BioQuant – BQ104T, detection limit: 0.2 ng/mL) determinations in human serum/plasma. Corticosterone concentrations were measured with an EIA kit developed for measuring corticosterone in tissue culture, plasma, serum and fecal samples and has been validated with multiple animal species (Arbor Assays–

K014-H, detection limit: 18.6 pg/mL). Enzyme immunoassays are being used successfully for whole-body thyroid hormones and corticosterone determinations in aquatic animals (Croteau et al., 2009; Yu et al., 2010). All samples and standard curves were run in duplicate, and any samples with a coefficient of variation above 20% (4.4% of T3 samples and 3.8% of corticosterone) and any standard curves with less than 95% of confidence interval were re-analyzed, as specified in Hersikorn and Smits. Serial dilutions of extracts were used to validate the analysis.

Statistical analysis

Mean growth (total length), development (GS and time to metamorphic climax), and hormone concentrations from tadpoles reared at different temperatures, under different PBDE exposures, at different developmental stages, and all possible combinations were compared using analysis of variance followed by posthoc comparisons by the Tukey's test. Because no hormonal differences were detected between the 2 samples collected during premetamorphosis (GS 28 and GS 31) these samples were pooled for statistical analysis. Likewise, no hormonal differences were detected in samples collected during prometamorphosis (GS 37 and GS 41); therefore, they were also pooled for statistical analyses. This resulted in 4 distinct time points defined by the 4 developmental stages (premetamorphosis and prometamorphosis and early- and late metamorphic climax). Gosner stage data were log10 transformed prior to the analysis to become normally distributed. A value of $p < 0.05$ was considered as statistically significant. All values were expressed as the mean ± standard error of the mean. Statistical analyses were conducted using Statistica 12 (StatSoft).

RESULTS

T3 concentrations

Whole-body T3 concentrations were assessed in tadpoles reared at 23 °C (Figure 1A) and 28 °C (Figure 1B) during 4 metamorphic stages: premetamorphosis (GS 28 and GS 31), prometamorphosis (GS 37 and GS 41), early metamorphic climax (GS 42), and late metamorphic climax (GS 46). Of the 3 factors tested (metamorphosis stage, temperature, and PBDE treatment) metamorphic stage and temperature showed overall significant differences in T3 levels. Also, there were some interactions with PBDE treatment that were also significant (Supplemental Data, Table S1). Whole-body T3 levels in control tadpoles rose to a peak at early climax and declined at late climax, which is expected during amphibian metamorphosis (Brown & Cai, 2007). The peak was significantly higher, relative to the value premetamorphosis, at 28 °C than at 23 °C . This pattern was muted in tadpoles fed diets with PBDEs. In those, the peak at early climax was lower and not significantly higher than values at premetamorphosis in at least 1 of the exposed groups at both 23 °C (PBDE6) and 28 °C (PBDE37).

Corticosterone concentrations

Whole-body corticosterone concentrations were assessed in tadpoles reared at 23 °C (Figure 2A) and 28 °C (Figure 2B) during the same metamorphic stages. All the factors tested (temperature, metamorphosis stage, and PBDE treatment) showed significant differences for corticosterone levels (Supplemental Data, Table S1). Corticosterone levels peaked earlier at 28 °C (peak at early climax) than at 23 °C (peak at late climax). Corticosterone levels were significantly higher in PBDE-exposed animals than in controls.

Growth and development

Total length and Gosner Stage were obtained after 7 d, 14 d, and 21 d of exposure in tadpoles reared

at 23 °C (Figure 3A) and at 28 °C (Figure 3B). In control animals, both parameters showed significant increases as days post-hatch increased, reflecting tadpoles' natural growth and development. Both parameters showed significantly higher values in tadpoles reared at the warmer temperature (Supplemental Data, Table S2). Growth and development were not significantly affected by PBDE exposure in tadpoles assessed up to 28 d at both temperatures.

Time to metamorphic climax was decreased in tadpoles reared at the warmer temperature, because they needed fewer days to reach GS 42 (forelimb emergence; Figure 4) compared to those reared at the cooler temperature. Polybrominated diphenyl ethers-induced differences in time to metamorphic climax were not observed during this preclimax period.

DISCUSSION

Hormonal profile: T3

It is generally known that the progression of anuran metamorphosis from tadpole to frog is under complex hormone control, and that the drastic internal and external transformations that take place during different stages are mostly driven by the rise in thyroid hormones (Fort et al., 2011). However, many other endocrine secretions synergize with or antagonize the action of thyroid hormone, including glucocorticoids (M. Wright et al., 1994).

Our results showed increasing T3 concentrations as tadpoles progressed from premetamorphosis to metamorphic climax under both temperatures in nonexposed tadpoles. These findings corroborate the previously described T3 peak at early metamorphic climax described for most amphibians (Brown & Cai, 2007; Denver, 1993). In general, premetamorphosis is an early larval development period that includes hind limb bud development and usually takes place under low thyroid hormone levels. During prometamorphosis, differentiation of digits and a rapid growth of the limbs occur, and rising levels of

thyroid hormone are observed. The initial values obtained for *L. pipiens* were higher than those obtained for control tadpoles at GS 29 and 34 reported by Croteau et al. (Croteau et al., 2009), but our values at the different stages were similar to those reported by Krain and Denver (Krain & Denver, 2004) for whole-body T3 concentrations in *Xenopus laevis* throughout metamorphosis.

Although control tadpoles reared at both temperatures showed increasing levels of T3 from premetamorphosis to early climax, the warmer rearing temperature resulted in an increased T3 elevation (187%) compared to increases observed at the cooler temperature (62%). The warmer temperature also caused an earlier increase in corticosterone levels. The association of faster development and higher T3 and corticosterone at warmer temperatures is consistent with the idea that accelerations in metamorphosis might be driven by higher T3 and corticosterone concentrations. These findings agree with the pattern of faster development and precocious elevation of whole-body thyroid hormones and corticosterone levels in response to drying conditions and warmer temperatures (R. J. Denver, 1998; Walsh, Downie, & Monaghan, 2008), indicating that the neuroendocrine stress axis may play a central role in developmental plasticity (Boorse & Denver, 2003). When exposed to higher temperatures, a higher thyroid response to ovine TSH was found in *Rana catesbeiana* (M. L. Wright et al., 1999). The present study's findings are consistent with this experiment, as we showed increased thyroid hormone and corticosterone concentrations in tadpoles that underwent metamorphosis acceleration due to the 5 °C higher water temperature. Considering that the expectation is that climate may warm as much as 4 °C by the turn of the century in Green Bay(Veloz et al., 2012), the additional exposure to endocrine disruptors at critical life stages may impact the adaptive capacity of amphibians to changing environmental conditions (Hooper et al., 2013).

Exposure to DE-71 had a suppressive effect on the T3 profile during metamorphosis. We found that exposed tadpoles showed lower T3 levels at both temperatures. In fact, tadpoles from 3 of 4 DE-71

exposed groups (6 ng/g and 37 ng/g at both temperatures) were found to have significantly lower T3 levels compared to unexposed controls. Several other studies have shown reduced thyroid function following BDEs exposure in rats, as reviewed in Legler (Legler & Brouwer, 2003). This observed impairment of thyroid hormone homeostasis may involve thyroid gland function and regulation, thyroid hormone metabolism, and/or thyroid hormone transport mechanisms (Brouwer et al., 1998). Some recent findings show inhibition of deiodinase enzymes (which are responsible for activating and inactivating thyroid hormones in peripheral tissues) in PBDE-exposed fish (Noyes, Hinton, & Stapleton, 2011) and antagonism to thyroid hormones in mammals (Kitamura et al., 2005), although the same authors showed PBDEs' agonistic effects earlier, also in mammals (Kitamura, Jinno, Ohta, Kuroki, & Fujimoto, 2002). In amphibians, PBDE formulations antagonized T3-induced tail resorption in *Xenopus laevis* tadpoles in isolated tissues both in vitro (Schriks et al., 2006) and in vivo (Balch et al., 2006). These studies illustrate the complexity of thyroid signaling and the several potential alterations to this process. Recent studies indicate that although thyroid hormone receptors and cofactors are the same in both mammals and amphibians, the sensitivity to thyroid hormone and its receptors may be different in amphibians (Brown & Cai, 2007). Furthermore, other hormones, such as corticoids, prolactin and growth hormone, can play important roles controlling growth and development during frog metamorphosis (M. Wright et al., 1994).

Hormonal profile: Corticosterone

Glucocorticoids play critical roles throughout vertebrate life history (Kulkarni & Buchholz, 2012). Developmental roles include maturation of various organs and influences on the organism physiology, metabolism, immune function, neural function, behavior, and reproduction (Wada, 2008). Corticosterone is the main product secreted by amphibian interrenal glands, and because both thyroid hormone and corticosterone are developmental hormones acting together on some of the same organs, some interaction is expected (Kulkarni & Buchholz, 2012). In amphibian metamorphosis, this interaction seems to be

crucial yet complex: in the absence of thyroid hormones during premetamorphosis, corticosterone inhibits development, but in the presence of thyroid hormone during late prometamorphosis and metamorphic climax, corticosterone synergizes with thyroid hormone to accelerate development (Kulkarni & Buchholz, 2012). The levels of both hormones in most amphibians studied are lower at early larval stages; therefore, it has been suggested that corticosterone levels are not critical until they are high enough to potentiate thyroid hormone action (M. Wright et al., 1994). Because thyroid hormone alone seems insufficient to complete metamorphosis, corticosterone concentrations are expected to increase at the end of the process (M. Wright et al., 1994).

Our results showed an increase in corticosterone concentrations during metamorphosis in control animals reared at both temperatures, although differences in corticosterone profile were observed depending on the rearing temperature. At the cooler temperature, we found a 60% increase in corticosterone concentrations at late climax (GS 46), whereas at the warmer temperature the hormone levels increased and reached significantly higher levels earlier, at early climax (GS 42). These differences in corticosterone concentrations in *L. pipiens* suggest that higher temperatures affect corticosterone hormone profiles during metamorphosis. Considering its potential to synergize with high thyroid hormone levels, higher corticosterone earlier in metamorphic climax might explain the acceleration of metamorphosis under the warmer temperature. This acceleration is demonstrated in the present study by the shorter amount of time tadpoles took reach GS 42 when reared at 28 °C.

Overall, corticosterone concentrations were higher in the PBDE-exposed tadpoles compared to controls at both temperatures. This suggests that even low, environmentally relevant concentrations of PBDEs might affect corticosterone levels during metamorphosis, especially at warmer temperatures, where the increase in corticosterone in exposed tadpoles also occurred earlier. Increased corticosterone concentrations in exposed tadpoles reared at both temperatures also might explain why we did not find

delayed metamorphosis following PBDE exposure. Corticosteroids synergized with thyroid hormone during prometamorphosis in *Rana pipiens* and prolactin also augmented the action of T4 to bring about rapid hindlimb growth (M. Wright et al., 1994). Elevated corticosterone levels observed in the present study might be compensating for the observed lower T3 levels that might otherwise delay development. Elevated corticosterone concentrations were reported following a subchronic DE-71 exposure in mouse plasma, although the authors used much higher concentrations (up to 1000 mg/kg) (Kulkarni & Buchholz, 2012). Corticosterone is known to produce physiological changes to enhance survival in the face of adverse environmental conditions. Chronically high corticosterone concentrations, however, can have negative effects on an animal's health. Another possible explanation for the lack of significant delay in metamorphosis in exposed tadpoles with low whole-body T3 concentrations would be a mimic effect of DE-71, because brominated flame retardants are structurally similar to thyroid hormones (Veldhoen, Boggs, Walzak, & Helbing, 2006). At low concentrations (100 nM), tetrabromobisphenol A exposure in pacific tree frog tadpoles significantly increased brain thyroid receptor TRα transcription in the presence of T3 (Veldhoen et al., 2006). Other molecular studies are needed to better understand how DE-71 affects receptors and enzymes transcriptions at low, environmental concentrations in *L. pipiens*.

Growth, development, and timing of metamorphosis

Tadpoles reared at 28 °C were bigger and more developed when compared to tadpoles reared at 23 °C, and also needed a shorter development time to reach metamorphic climax. When exposed to PBDE, tadpoles did not show significant changes on developmental parameters at either temperature. Previous studies from our laboratory reported decreased growth and delayed time to metamorphosis for *L. pipiens* tadpoles reared at 23 °C and exposed to 7 ng/g to 277 ng/g of DE-71 (Cary Coyle & Karasov, 2010). These effects were observed during early prometamorphosis, which might suggest that those effects are hormone-independent, because thyroid hormone levels are commonly low during these initial stages.

Exposure to much higher PBDE concentrations caused significant inhibition of tail resorption and delayed metamorphosis in *Xenopus laevis* (Balch et al., 2006), and slowed development in *Xenopus tropicalis* (Carlsson, Kulkarni, Larsson, & Norrgren, 2007).

Given the present study's results, we conclude that both PBDE exposure and temperature affected thyroid hormone and/or corticosterone profiles during metamorphosis in *L. pipiens*, although temperature resulted in larger alterations of growth and development in tadpoles during metamorphosis. Temperature was shown to be an important factor affecting tadpoles' hormonal profiles, because we observed an association between the observed acceleration in metamorphosis and higher T3 and earlier corticosterone elevation at the warmer temperature. In addition, interactions between PBDE exposure and temperature seem apparent, because thyroid hormone profile during metamorphosis was affected in a distinctive way, depending on the temperature at which tadpoles were raised. Although we did not find any overall significant alterations in growth and development in PBDE-exposed tadpoles, the hormonal changes found in the present study support earlier findings of delayed metamorphosis in PBDE-exposed tadpoles reared at 23 °C (Cary Coyle & Karasov, 2010; Cary et al., 2014). Further investigations are necessary to address the implications of these changes for the species' reproduction and survival considering a continuous contaminant exposure under a warming climate scenario.

TABLES AND FIGURES

Table 1. Sampling schedule for the different endpoints

Endpoints	Samplings of groups of treated animals (0 ng/g, 6 ng/g, and 37 ng/g reared at 23 °C and 28 °C)			
T3 and corticosterone concentrations[1]	Premetamorphosis (GS 28 and GS 31)	Prometamorphosis (GS 37 and GS 41)	Early Climax (GS 42)	Late Climax (GS 46)
TL[2] and GS[3]	7 DE	14 DE	21 DE	
Chemical analysis[4]	34 DE			

Notes:

[1]Triiodothyronine (T3) and corticosterone concentrations ($n = 8–16$ individuals per group).

[2]Total length (TL; $n = 12$ individuals per group).

[3] Gosner Stage (GS; $n = 12$ individuals per group).

[4]Chemical analysis (polybrominated diphenyl ethers concentrations assessment; $n = 3–4$ tadpoles per group).

DE = Days of exposure.

Table 2. Values of individual polybrominated diphenyl ether (PBDE) congeners from DE-71 obtained in the diet and in tissues from tadpoles reared at 23 °C or 28 °C exposed to 0 ng/g (control), 6 ng/g, or 37 ng/g DE-71 diet wet weight[5,6]

	Diet			**Tadpoles at 23 °C**			**Tadpoles at 28 °C**		
	CTL	**6**	**37**	**CTL**	**6**	**37**	**CTL**	**6**	**37**
PBDE47	ND	1.4	12.0	ND	ND	ND	ND	ND	24.0
PBDE85	ND	ND	1.7	ND	ND	1.4	ND	ND	1.8
PBDE99	ND	3.8	16.0	ND	4.5	27.0	ND	5.1	36.0
PBDE100	ND	0.9	3.6	ND	1.1	5.6	ND	ND	8.0
PBDE153	ND	ND	2.1	ND	ND	ND	ND	ND	3.0
PBDE154	ND	ND	1.4	ND	ND	1.7	ND	ND	2.2
ΣPBDE	ND	6.1	36.8	ND	5.6	35.7	ND	5.1	75

Notes:

[5]*n* = 1 pool of 4 to 5 animals for each sample, randomly taken from multiple tanks.

[6]The following congers were measured and showed a nondetectable level throughout all treatment groups: PBDE17, PBDE28, PBDE66, PBDE138, PBDE183. Numerical values in the table have units of ng g−1 wet mass diet or tadpole.

CTL = control; ND = nondetectable.

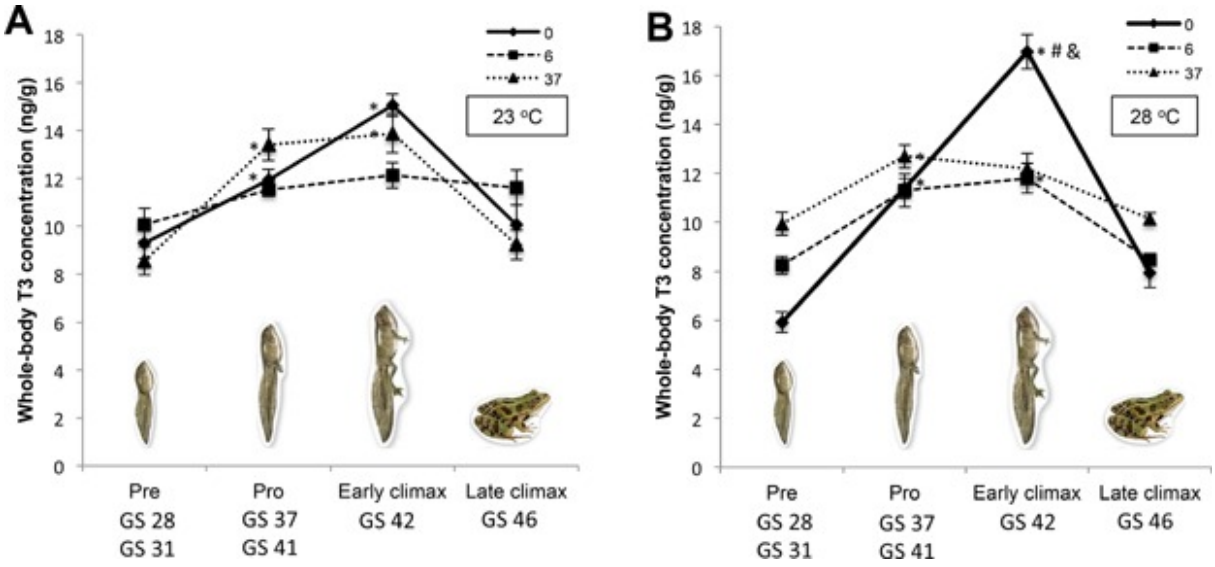

Figure 1. Whole-body triiodothyronine (T3) levels (ng g−1) in tadpoles reared at 23 °C (**A**) and 28 °C (**B**) chronically exposed to 0, 6, and 37 ng/g of dietary polybrominated diphenyl ethers (PBDE; DE-71). Stage-matched tadpoles were sampled during premetamorphosis (Gosner stage [GS] 28 and GS 31; Pre); prometamorphosis (GS 37 and 41; Pro); early metamorphic climax (GS42; Early climax); and late metamorphic climax (GS 46; Late climax). (n = 8–16 per group). *Significantly different from premetamorphosis tadpoles in the same treatment; #Significantly different versus early climax control tadpoles reared at 23 °C; &Significantly different versus early climax exposed tadpoles reared at 28 °C.

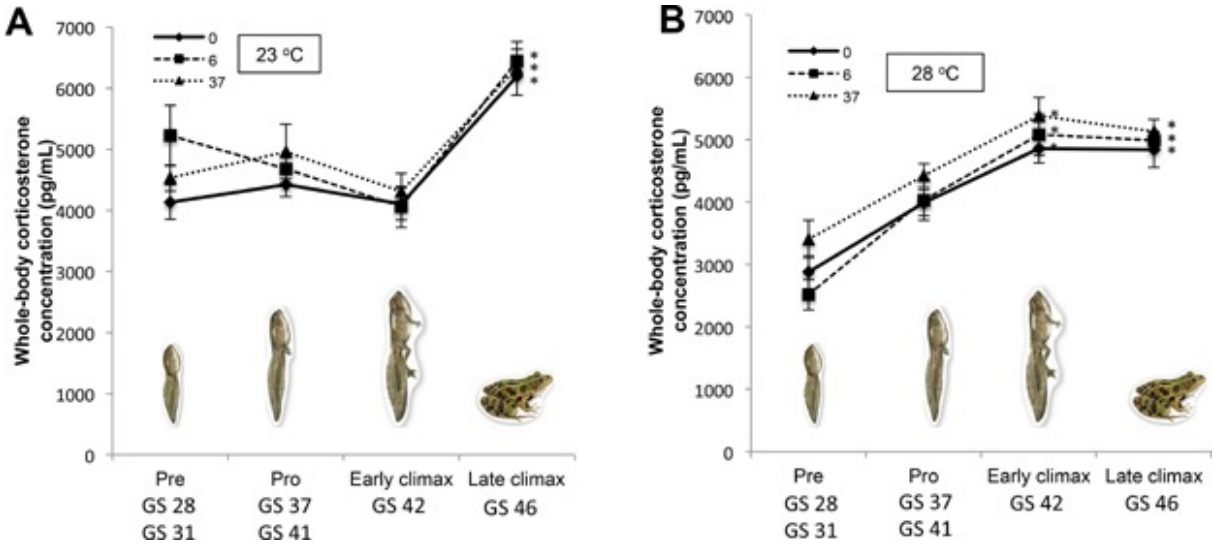

Figure 2. Whole-body corticosterone levels (pg/mL) in tadpoles reared at 23 °C (**A**) and 28 °C (**B**) chronically exposed to 0 ng, 6, and 37 ng/g of dietary polybrominated diphenyl ethers (PBDE; DE-71). Stage-matched tadpoles were sampled during premetamorphosis (Gosner stage [GS] 28 and GS 31; Pre); prometamorphosis (GS 37 and 41; Pro); early metamorphic climax (GS42; Early climax); and late metamorphic climax (GS 46; Late climax). (n = 8–16 per group). *Significantly different from premetamorphosis tadpoles in the same treatment.

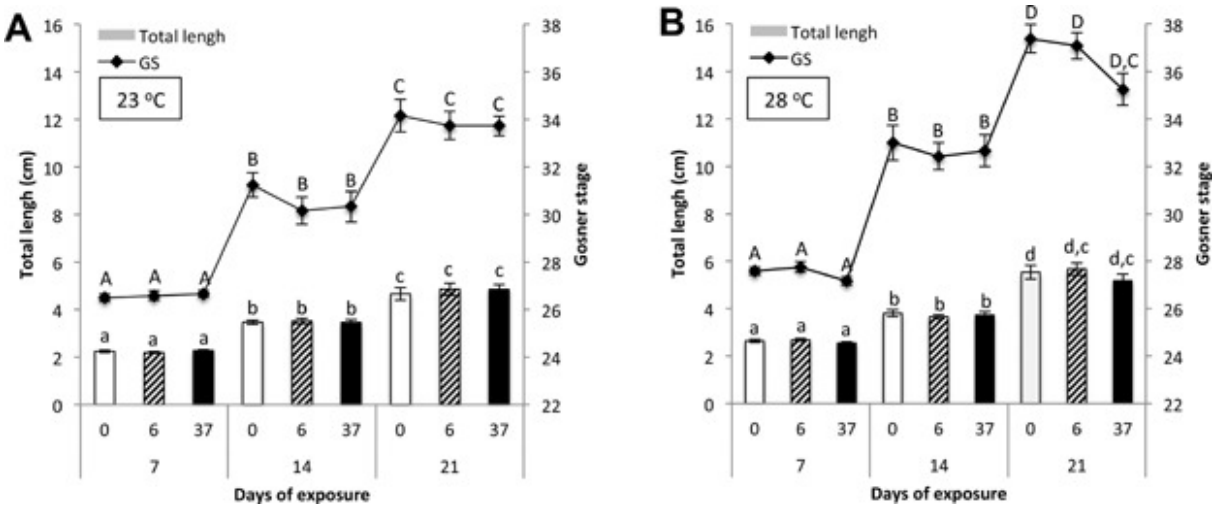

Figure 3. Growth (Total length [cm]; bars) and development (Gosner stage [GS]; lines) of tadpoles reared at 23 °C (**A**) and 28 °C (**B**) after 7, 14, and 21 days of exposure under 0, 6, and 37 ng/g of dietary polybrominated diphenyl ethers (PBDE; DE-71) exposure (n = 12 per group). Different letters represent significant differences.

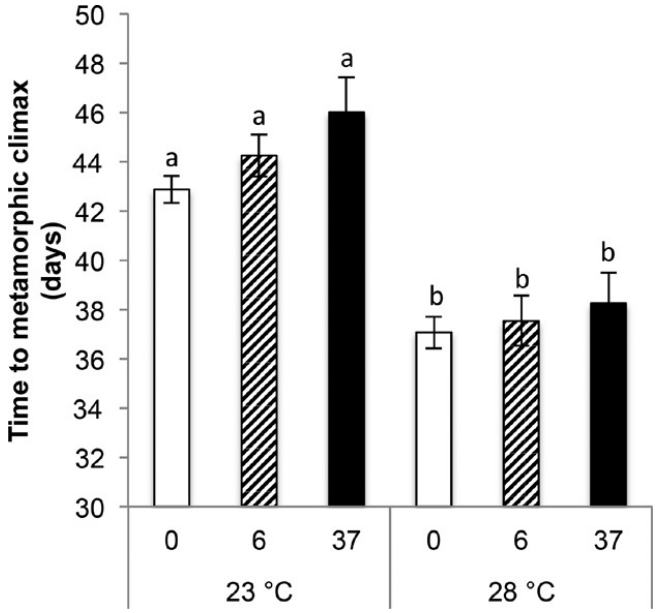

Figure 4. Time to metamorphic climax (Gosner stage [GS] 42; days of exposure) in tadpoles reared at 23 °C and 28 °C for each treatment under 0 ng g−1, 6 ng g−1, and 37 ng g−1 of dietary polybrominated diphenyl ethers (PBDE; DE-71) exposure (n = 8–15 per group). Different letters represent significant differences.

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