Title: Interactions between a Small Chronic Increase in Diel Water Temperature and Exposure to a Common Environmental Contaminant on Development of Arizona Tiger Salamander Larvae

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Interaction between Temperature Increase and Perchlorate on Development

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ABSTRACT

Global climate change leading to increased temperatures may affect shifts in physiological processes especially in ectothermic organisms. Temperature-dependent shifts in developmental rate in particular, may lead to life-long changes in adult morphology and physiology. Combined with anthropogenic changes in the chemical environment, changes in developmental outcomes may affect adult functionality. The purpose of this study is to determine 1) if small increases in diel water temperature affect the development of Arizona tiger salamander (*Ambystoma tigrinum nebulosum*) larvae, and 2) if this change interacts with exposure to the common environmental thyroid disrupting compound, perchlorate. Larvae between Watson and Russell developmental stages 8-13 were exposed to ammonium perchlorate (AP) at doses of 0, 20 or 200 ppb and then raised at either ambient or a 0.9°C elevated above ambient temperature for 81 days in outdoor enclosures. During the first 5 treatment weeks, AP treatment induced slower development and smaller snout-vent length (SVL) of exposed larvae, but only in the elevated temperature group. During the later stages of development, the small increase in temperature, regardless of AP treatment, tended to decrease the time to metamorphosis and resulted in a significantly smaller body mass and worse body condition. Our results suggest that even small diel water temperature increases can affect the developmental process of salamanders and this shift in the water temperature may interact with a common environmental contaminant.

Key words: climate change, endocrine disrupting compound, ammonium perchlorate, metamorphosis, *Ambystoma tigrinum nebulosum*

1. Introduction

Global warming can affect multiple levels of amphibian life history including growth, reproduction, and mortality, in addition to larger-scale population and community fluctuations (Lee et al., 2011; McCain and Colwell, 2011; Blaustein et al., 2012; Caruso et al., 2014). The effect of global warming may be of particular importance during early development and/or during transitions between habitats, when animals are vulnerable and often living at the limit of homeostatic or physiological tolerance ranges (Duarte et al., 2012; Arrighi et al., 2013). Global warming may also interact with endocrine disrupting compounds and other contaminants to affect the impacts of exposure either directly or through changes in toxicokinetics (Honkanen and Kukkonen, 2006; Schiedek et al., 2007; Noyes et al., 2009; Kim et al., 2010; Hooper et al., 2013). Recent studies investigating these interactions have shown both negative and positive impacts of exposure to temperature fluctuations together with environmental exposure to micropollutants (Morgan et al., 2001; Rohr et al., 2011).

Further, understanding the potential impacts of global warming as a mechanism contributing to declining amphibian populations is critical to the conservation of this threatened clade (Blaustein et al., 2011; Hof et al., 2011; Li et al., 2012). Improving our knowledge regarding the interaction between the human-induced changing chemical environment and the stressors involved in climate change (Hof et al., 2011; Relyea and Hoverman, 2006; Rohr et al., 2008) will help to identify regions where amphibians may be under particularly high threats from environmental change. Results from recent studies demonstrate that shifts in temperature alter amphibian responses to toxic chemicals (Rohr et al., 2011; Broomhall, 2002), but the underlying mechanisms driving interactions between global warming and exposure to common contaminants is not well understood.

Studies simulating environmental conditions and evaluating the impacts of global warming are important to predict global warming effects in the field. For example, diel temperature fluctuations may more closely model changes in global warming patterns than those studies utilizing constant temperature settings (Arrighi et al., 2013; Kaplan and Phillips, 2006; Paaijmans et al., 2010; Niehaus et al., 2012). Furthermore, small temperature increases, as opposed to larger ones, result in more realistic models that represent the immediate outcomes occurring under current natural conditions. The Intergovernmental Panel on Climate Change (IPCC) and the National Climatic Data Center (NCDC) have reported that over the last century, temperatures have increased globally by slightly less than 1 °C (IPCC, 2007; NCDC, 2013). However, studies incorporating conditions where only small changes in temperature are evaluated are still rare. Evaluation of a small, but consistent change in temperature will provide insight into the more immediate outcomes of global warming on ectothermic animal development. Furthermore, testing interactive effects between small increases in temperature and exposure to sublethal doses of common contaminants will provide insight into the effects of multiple environmental shifts representing potential stressors to populations.

Ammonium perchlorate (AP) is a thyroid hormone disruptor that interferes with normal iodine uptake by the thyroid gland (Fisher et al., 2012; Leung et al., 2014). It is widely used as an oxidizer in solid rocket fuels and is detected at levels up to 820 µg/L in rivers, lakes, and municipal water supplies (Brandhuber et al., 2009). A wide range of studies using low doses of AP have demonstrated its adverse effects on growth, gonadal and sexual development in fish and birds, as well as metamorphosis in frogs (Mukhi et al., 2007; Patiño et al., 2003; Bernhardt et al., 2006; Furin et al., 2015a, 2015b; Gardell et al., 2015; Petersen et al., 2015; Anne McNabb et al., 2004; Goleman et al., 2002; Ortiz-Santaliestra and Sparling, 2007). Additionally, AP has been demonstrated to affect gene expression involved in thyroid hormone biosynthesis in rats (Wu et al., 2012).

Early development and metamorphosis in most amphibians are critical processes in their life history (Dodd and Dodd, 1976). It is well known that they are dependent on various environmental factors, such as temperature and food availability, and that thyroid hormones are a major hormone involved in these processes (Smith-Gill and Berven, 1979; Larras-Regard et al., 1981; Tata, 2006). Considering the disruptive effects of AP on the thyroid hormone system and reports about increased temperature following recent global warming, studies deciphering potential impacts of sublethal doses of ammonium perchlorate in combination with increased temperature treatments on development and metamorphosis of urodele amphibians are necessary.

In this study, we used the Arizona tiger salamander (*Ambystoma tigrinum nebulosum*) as a model to investigate the developmental effects of a realistic early global warming upward shift in diel water temperature. We also determined the potential interactive action of temperature with exposure to sublethal doses of the common contaminant, ammonium perchlorate, on the development of Arizona tiger salamander larvae in outdoor enclosures.

2. Materials and methods

2.1. Animal collection and maintenance

For this study, using a dip net and a seine, a total of 180 Arizona tiger salamander (*Ambystoma tigrinum nebulosum*) larvae were collected from three different ponds (100 individuals from Snowbowl Tank, 50 from Beech Tank and 30 from Stagecoach Tank, Coconino Co., AZ) on June 2, 2012. The larvae were then transported to NAU Department of Biological Sciences and housed in 38 L aquaria (48 x

27 x 30 cm) containing 7 L of dechlorinated tap-water (Microbe-lift dechlorinator, Ponds Inc., Elgin, IL, USA) and 7 L of water collected from the above sites. Each aquarium, housed in an outdoor courtyard and exposed to local temperature and daylight patterns, contained fewer than 20 larvae.

A day after collection the baseline developmental stage and snout to vent length (SVL) were determined for each larva. All larvae greater than 30 mm SVL or that exhibited a cannibalistic morphology (Hoffman and Pfennig, 1999) were removed from the study. For the developmental staging, we used the stage definitions for the long-toed salamander, *A. macrodactylum krausei* (Watson and Russell, 2000) which defined stages 8 to 12 based on the hind limb formation, 12 to 15 based on the number of hind limb digits, 16 to 18 based on the relative length of hind limb digits and 19 to 22 based on metamorphosing process, mainly gill reduction. To further improve accuracy of the developmental staging in this study, we added 3 additional stages between stages 16 and 19 as follows: Stage 16.5: the length of the 2nd digit on hind foot is longer than the 4th digit, but shorter than 3rd digit; Stage 17.5: the length of the 4th digit on hind foot is longer than the 2nd digit, but shorter than the 3rd digit; Stage 18.5: the length of 4th digit of hind foot is longer than 3rd digit. Last, when the left and right hind feet were in different stages, we assigned the earlier developmental stage. To measure SVL, each larva was photographed (WB150F, Samsung, Seoul, Korea) in a transparent acrylic box (8.5 x 2 x 4 cm), partially filled with water and containing a ruler at the base and posterior wall of the box. The SVL was calculated from each photo using image J (1.45s, National Institutes of Health, USA).

Larvae were distributed evenly based on developmental stage (stages 8-13), into a total of 18 38 L aquaria. This distribution yielded 8 – 9 larvae per aquarium (mean $\pm$ SE = stage 12.7 ± 1.1 , $n = 18$). Each aquarium was filled with 14 L of dechlorinated tap water and equipped with a charcoal water filter which cycled for one hour (340 gallon/hour), four times daily. At the pH of the treatment water, perchlorate would only minimally be adsorbed by the filter (Fang et al., 2014), and concentrations were reestablished with each water change. All aquaria were placed on a four-shelve cage rack (180 x 40 cm shelves, 160 cm

high; 6 aquaria/ shelf) in a shady courtyard encompassed by four story walls. The top shelf was covered by a wire mesh cover to prevent leaf fall. During rainstorms, the top of the wire mesh of each aquarium was covered with a piece of acrylic which was removed when the rain ceased.

This study was conducted under the approval of Northern Arizona University IACUC protocol # 11-006.

2.2. Water temperature manipulations and ammonium perchlorate treatments

Fig. 1

In order to determine whether there was an interaction between temperature and thyroid disruption, we treated animals to two different temperature regimes in combination with ammonium perchlorate exposure. The temperature treatments were 1) ambient water temperature at the courtyard site as a control (ambient temperature group) and increased water temperature treatment (increased temperature group) achieved by placing water aquarium heaters (25W, Hydor THEO submersible aquarium heater, Hydor, Sacramento, CA, USA) directly into the aquaria. We turned the heaters on twice a day for 2 hrs, starting 2 hrs before the lowest and highest temperature time points based on the daily temperature data of the last 7 days from a local weather center (<http://weather.gladstonefamily.net/>). This heating method increased both minimum and maximum temperatures in the increased temperature treatment aquaria while keeping daily fluctuation patterns resembling those of the ambient temperature treatment groups (Fig. 1A, B). For each nominal concentration of ammonium perchlorate (AP; Sigma-Aldrich, USA), a stock dilution was made in 6 ml tap water and delivered to each aquarium in 500 µl dechlorinated tap water for a final dilution of 0, 20 ppb and 200 ppb (AP group). These concentrations were based on previous studies which demonstrated effects on thyroid hormone-dependent metamorphic endpoints in *Xenopus laevis* tadpoles exposed to between 20 ppb and 14,140 ppb AP (Goleman et al., 2002; Brausch et

al., 2010). The concentrations chosen are within the higher ranges reported from surface and groundwater sites providing drinking water resources in the USA (reviewed in Brandhuber et al., 2009). A new stock was made weekly and kept at 4 °C before delivery at the targeted concentrations into the aquaria.

Perchlorate concentrations remain stable for more than 90 days in water samples (Stetson et al., 2006).

Each of the six treatment groups had three replicate aquaria for a total of 18 aquaria. We randomly assigned a treatment type to each aquarium by tossing a die. Treatments began on June 4, 2012 and ended on August 23, 2012 (81 days total). The water temperature of one replicate of each treatment group ($n = 6$) was recorded every 30 minutes using a HOBO pendant data logger (UA-001-08, Onset, Bourne, MA, USA) from June 4 to July 30, 2012. Dissolved oxygen (DO) and pH levels in each aquarium were monitored weekly between June 8, 2102 to August 14, 2012 using field DO (DO 110 series, Oakton Instruments, Vernon Hills, IL, USA) and pH (pH 110 series, Oakton Instruments, Vernon Hills, IL, USA) meters. We changed the half of the water (7 L) once a week for weeks 0-3 and 9-12 and twice a week during weeks 4-8 because the animals were larger and none had been removed from the tanks at that time as a result of metamorphosis. All aquaria were re-dosed with the appropriate amount of AP to maintain the originally assigned nominal concentration after each water change. The larvae were fed once a day with a 50 ml mixture of brine shrimp (Brine Shrimp Direct, Ogden, Utah, USA) and frozen blood worms (Doctors Foster and Smith, Rhinelander, WI, USA). Uneaten food from the previous day was siphoned from each aquarium prior to the next feeding.

If cannibalism occurred more than once in any aquarium, the cannibalistic individual was isolated from the remaining larvae by placing the animal in a small acrylic container (23 x 15 x 15 cm) within the main aquarium until metamorphosis. This measure was necessary for three of the aquaria, each containing only one cannibalistic individual (0 ppb AP/ambient temperature, 0 ppb AP/increased temperature and 200 ppb AP/increased temperature groups).

2.3. Measurement of larval developmental stage and growth

Once a week, we staged all larvae, measured their SVL as described above, and then returned the animals to their aquaria. When they reached developmental stage where the gills had lost most of their filaments (developmental stage 19), larvae were removed from the aquaria to prevent death from drowning. At this stage, the larvae were considered to have undergone metamorphosis. Total body mass was measured to the nearest 0.1 g using a digital balance (Ohaus Corporation, Florham Park, NJ, USA). SVL was measured as described above.

2.4. Data handling and statistical analysis

For water temperature analyses, we calculated the daily mean, minimum and maximum water temperatures for all treatments. Data for temperature, pH and DO are reported for aquaria during the period between the start of the study and July 30; thereafter, enough animals had undergone metamorphosis that there were several empty aquaria. Also, because some larvae started metamorphosis at or just after week 5 of treatment, we used the weekly developmental stages and SVL measurements collected during treatment weeks 0 – 5 (June 4 – July 9) to assess larval growth. We calculated weekly mean values of developmental stage and SVL for each replicate aquarium because we did not discriminate individuals during measuring weekly developmental stages and SVL over different treatment weeks. To analyze the weekly developmental data, we used the numeric developmental stage as a value. Since all larvae did not complete metamorphosis by the final collection, we determined the number of exposure days it took for 10, 30, 50 and 70% of the individuals in each treatment group to undergo metamorphosis. We did not include 90% metamorphosis dates in the analysis because the metamorphosis

rate in two of the aquaria was 79% at time of collection. For the data of body mass, SVL, and condition factor (body mass/ SVL*100) at metamorphosis, we could get individual data so that we did not produce mean values for each replicate aquarium. Since a previous study (Álvarez and Nicieza, 2002b) showed that the body mass at metamorphosis varied in different phases of metamorphosis under conditions of elevated temperatures, we allocated metamorphosed individuals in each replicate aquarium into 0 - 25%, 26 - 50%, 51 - 75%, and 76 - 100% group for comparison based on the completed order of metamorphosis. This binning was used to detect possible treatment effects on the body mass and SVL at a specific phase of metamorphosis.

We used a Fisher's exact test or Chi-square test to determine if there were significant differences in a) the number of larvae which were predated by other larvae or b) the number of un-metamorphosed larvae between temperature groups or among AP groups, respectively. The differences between mean, minimum and maximum temperature between ambient and increased temperature group were analyzed using an independent t-test because the data were normal (Shapiro-Wilk normality test, $p > 0.05$). Replicate aquaria means across time for each treatment were used in a two-way repeated analysis of variance (ANOVA) using a general linear model (GLM) followed by a Tukey's pairwise comparison for the analysis of pH, DO, weekly developmental stage, SVL and time to metamorphosis. When an interaction between temperature and AP treatments was significant, we repeated the same procedure separately to know the effects of AP treatment within each ambient and increased temperature group, followed by a Tukey's pairwise comparison, and to know the effects of temperature treatment within each control and 20 ppb and 200 ppb AP group. For the analyses of the body mass, SVL, and body condition of larvae at metamorphosis, we applied a three-way analysis (ANOVA) of variance using a GLM model which included the metamorphosis phase as an independent variable. When an interaction between temperature and AP treatments and the metamorphosis phase was significant, we separately analyzed temperature and AP effects on body mass, SVL, and body condition within each metamorphosis phase

using either Kruskal-Wallis for AP effects or independent t-test for temperature effects. All statistical analyses were done in SPSSPC (v. 20.0).

3. Results

3.1. Water temperature

During a 57 day period (June 4 – July 30, 2012), the water temperature differences between the two temperature groups were significantly different (mean daily difference was $0.9\text{ }^{\circ}\text{C}$: mean $\pm$ SD = $18.37 \pm 0.31\text{ }^{\circ}\text{C}$ for ambient; $19.27 \pm 0.36\text{ }^{\circ}\text{C}$ for the increased temperature treatment; $t = 3.31$, $df = 4$, $p = 0.030$; mean daily difference in minimum water temperatures was $1.0\text{ }^{\circ}\text{C}$: $15.33 \pm 0.30\text{ }^{\circ}\text{C}$ for ambient; $16.33 \pm 0.32\text{ }^{\circ}\text{C}$ for the increased temperature treatment; $t = 3.90$, $df = 4$, $p = 0.018$; the mean daily difference in maximum water temperatures , was $1.5\text{ }^{\circ}\text{C}$: $21.08 \pm 0.33\text{ }^{\circ}\text{C}$: $22.60 \pm 0.41\text{ }^{\circ}\text{C}$, $t = 5.04$, $df = 4$, $p = 0.007$) (Fig. 1). During temperature and AP treatments, the average weekly pH ranged between 6.33 and 7.68, and the average DO ranged from 2.64 and 5.86 (mg/L). For the 57 day period, , neither pH nor DO varied significantly between two temperature groups (pH, $p = 0.536$; DO, $p = 0.289$) or the AP groups (pH, $p = 0.789$; DO, $p = 0.554$). After day 57, some tanks had all of the larvae complete metamorphosis. The temperature regime was maintained for a total of 81 days, and again, there were no significant differences in among treatment groups in pH or DO.

3.2. Survivorship

Among 155 larvae, 11 died due to predation by other larvae and one larva died as a result of a handling error (7.7% mortality). There were no differences in the amount of cannibalism between the two temperature groups or between different AP groups ($p = 0.75$, $p = 0.84$, respectively).

3.3. Early development and growth

Fig. 2

During the early prometamorphic developmental period AP treatment, but not temperature, affected the timing of development. Specifically, there was a significant interaction effect between the AP and temperature treatment ($F_{2,12} = 4.33$, $p = 0.039$; Fig. 2A, B). Further evaluation revealed that AP treatment significantly increased developmental time in the increased temperature group ($F_{2,6} = 7.30$, $p = 0.025$), but not in the ambient temperature group ($F_{2,6} = 0.62$, $p = 0.572$). In the increased temperature group, compared to controls, the developmental stage in the 20 ppb AP treatment group trended towards being lower ($p = 0.058$) and the 200 ppb AP treatment was at a lower development stage ($P = 0.009$). There was no significant difference between the two AP groups ($p = 0.198$). In a separate analysis of the effects on development stage by different temperature treatment within each AP treatment, no significant effects were found (control: $p = 0.102$; 20 ppb: $p = 0.568$; and 200 ppb: $p = 0.140$).

Also during this time period, AP, but again, not temperature affected growth. Specifically, for SVL the interaction between temperature and AP treatments was significant ($F_{2,12} = 4.10$, $p = 0.044$; Fig. 2C, D) with AP having effects at again in the increased temperature treatment ($F_{2,6} = 9.43$, $p = 0.014$), but not in the ambient temperature group ($F_{2,6} = 0.57$, $p = 0.593$). In the increased temperature group, compared to the 0 AP controls, the SVLs of the 20 and 200 ppb AP groups were smaller ($p = 0.008$, $p = 0.012$,

respectively). There was no difference between two AP groups ($p = 0.917$). In a separate analysis of the effects of different temperature treatments within each AP group, no differences were found (controls: $p = 0.139$; 20 ppb: $p = 0.206$; or 200 ppb: $p = 0.248$).

3.4. Late development, growth, and metamorphosis

Fig. 3

Larvae began metamorphosing on treatment day 36, and by treatment day 81 when we ended the study, only seven larvae had not completed metamorphosis. The number of remaining un-metamorphosed larvae between the two temperature groups and among AP groups was not significant ($p_{\text{temp}} = 0.72$, $p_{\text{AP}} = 0.14$).

The time until animals reached metamorphosis tended to be affected by temperature, but not by AP treatment. The time it took to reach 10, 30, 50 and 70% of individuals reaching metamorphosis tended to be shorter in the increased temperature group than in ambient temperature group ($F_{1,12} = 4.32$, $p = 0.060$; Figure 3A,B), but it was not different among AP groups ($F_{2,12} = 1.78$, $p = 0.21$). There was no significant interaction between temperature and AP treatments ($F_{2,12} = 2.27$, $p = 0.146$).

Fig. 4

Both the AP and temperature treatment did not affect the body mass ($p_{\text{AP}} = 0.63$, $p_{\text{temp}} = 0.14$), SVL ($p_{\text{AP}} = 0.63$, $p_{\text{temp}} = 0.30$), and body condition ($p_{\text{AP}} = 0.72$, $p_{\text{temp}} = 0.14$) of metamorphosed individuals, but those measurements, not surprisingly, differed significantly as the animals progressed through metamorphosis ($p < 0.01$ for all cases). There was a trend in the interaction effect between the metamorphosis phase and temperature treatment on body mass ($F_{3,134} = 2.41$, $p = 0.071$), and there was a significant effect between the two variables on condition factor ($F_{3,134} = 2.78$, $p = 0.045$), and between the metamorphosis phase and AP treatment on SVL ($F_{6,134} = 2.71$, $p = 0.017$). Further evaluation revealed that increased temperature treatment significantly decreased body mass ($t = 2.19$, $df = 32$, $p = 0.036$; Fig. 4A) and condition factor ($t = 2.47$, $df = 32$, $p = 0.019$; Fig. 4B) during the 51-75% metamorphosis phase,

but not during other phases ($p > 0.05$ for all cases). In a separate analysis of the AP treatment effects on body mass, SVL, and body condition, no differences were found at any of the metamorphosis phases ($p > 0.05$ for all cases; Figure 4C,D).

4. Discussion

The recognition that temperature has effects on physiological function and developmental rate is not new and has been studied since the 1800's (Moore, 1939). However, the finding that the rate of development shifts during different developmental stages (Rombough, 2003), suggests factors that interact with temperature-dependent developmental rate, such as those that affect endocrine function, also play an important developmental role. This study, conducted in outdoor enclosures, examines the combined effects of small diel temperature increases and exposure to sublethal doses of ammonium perchlorate on Arizona tiger salamander development. In this study, a small increase in temperature affected early developmental and growth, but only when the animals were also exposed to the thyroid hormone synthesis inhibitor, ammonium perchlorate (Goleman et al., 2002; Fisher et al., 2012). Increased temperature alone also affected longer term development and growth. These results suggest that even small shifts in climate may affect the developmental process of salamanders under natural conditions both alone and combined with exposure to environmental pollutants, and each has effects at different stages of development.

The small changes in water temperature applied in this study could reasonably represent recent temperature shifts associated with climate change. In our study, we increased the daily mean water temperature on average 0.9°C , which is similar to the 0.74°C increase in temperature during last century reported by the IPCC (IPCC, 2007) and the 0.72°C increase per century in the USA reported by the

NCDC (NCDC, 2013). Furthermore, not only are mean daily temperatures shifting with climate change, but also the daily fluctuation in temperature patterns are changing. The temperature fluctuation pattern used in this study is similar to diel temperature fluctuations seen in Europe since 1990 (Wild et al., 2007; Makowski et al., 2008). These reports suggest that animals in the field have already faced shifts in temperature similar to the changes implemented in this study.

Several studies have shown that different diel temperature fluctuation ranges differentially affect physiological performance, thermal physiology, developmental rate and morphology of amphibians (Měráková and Gvoždík, 2009; Arrighi et al., 2013; Niehaus et al., 2012). For example, development rate increases with increased static temperature in *Bombina orientalis* (Arrighi et al., 2013). Similar results are found in striped marsh frogs (*Limnodynastes peronii*) (Niehaus et al., 2012). Furthermore, in both studies, the effect of temperature is moderated, though not eliminated, if the animals are raised in a fluctuating diel temperature environment. Shifts in temperatures and diel temperature fluctuation patterns not only affect developmental speed, but also physiological performance as is found in the newt *Triturus alpestris* (Měráková and Gvoždík, 2009). These studies demonstrate that developmental timing in these ectothermic vertebrates can be affected not just by the average daily temperature, but also by the variation in diel temperatures. The results make predicting developmental impacts from global climate change a challenge.

Temperature impacts development and the timing of metamorphosis through affecting the hypothalamic-pituitary-thyroid axis. High temperature exposure (5 – 16 °C high) induce both higher activity of thyroid glands in *Hynobius retardatus* (Moriya, 1983) and earlier metamorphosis in several anuran species (Smith-Gill and Berven, 1979; Álvarez and Nicieza, 2002b) and in *A. tigrinum* (Bizer, 1978). Although direct effects of small temperature changes on thyroid hormone physiology have not been studied, exposure to very low temperatures reduces transport of intracellular thyroxine into cells (Griswold et al., 1972), and affects binding of thyroxine to its plasma binding proteins (Murata and

Yamauchi, 2005). Furthermore, exposure to low temperatures down-regulates deiodinase II gene, an enzyme important to thyroid hormone production (Mochizuki et al., 2012). Also, in a turtle, *Pseudemys scripta*, exposure to high temperatures increases T4 production from thyroid glands *in vivo* (Licht et al., 1989). Therefore, the limited temperature increase applied in this study might shift developmental rate for metamorphosis by moderately changing production of thyroid hormone and thyroid hormone uptake of various tissues.

Interacting with the complex nature of physiological and developmental response to climate change is the rapidly changing chemical environment that many organisms face. Perchlorate is a common environmental pollutant (Smith et al., 2001; Brandhuber et al., 2009), and affects amphibian development through inhibition of thyroid hormone production (Goleman et al., 2002; Tietge et al., 2010). This inhibition can lead to developmental delays in many thyroid hormone-dependent tissues ultimately causing a prolonged period in the larval stage (Opitz et al., 2009). Even environmentally relevant 59 ppb perchlorate exposure in *Xenopus laevis* inhibits thyroid-hormone dependent aspects of metamorphosis such as T4 production and affects thyroid gland morphology (Goleman et al., 2002). These results suggest that perchlorate in the environment may interfere with development of amphibians in natural settings and can function as a model thyroid targeting pollutant for evaluating the interactions between thyroid disrupting pollutants and climate change. We find that environmentally relevant AP exposure affects early stage development in *A. tigrinum*, but only when also animals are exposed to the higher average temperature regime. Alternatively, temperature, but not AP affects the longer term developmental outcomes, suggesting that at these concentrations of AP, the animals may be able to compensate and increase their own thyroid hormone synthesis and/or secretory capacity to meet the challenges of AP exposure. Because much higher concentrations of thyroid hormone are produced and utilized during metamorphosis than during early developmental periods in *A. tigrinum* (Larras-Regard et al., 1981), decreased amounts of thyroid hormones caused by the low dose AP treatment at this stage may

not have been sufficient to induce lasting significant AP treatment effects on the later stages of development.

One other study has investigated the interaction between perchlorate and temperature on thyroid-dependent endpoints (Lee et al., 2010). In this study, adult fish (*Oryzias latipes*) exposed to ppm perchlorate exhibit shifts in expression of thyroid hormone receptors and deiodinase 2 as well as decreased levels of T4, but not T3. The effects on the thyroid hormone signaling pathways were greater when animals were exposed at higher temperatures rather than lower temperatures. This short term adult exposure to high concentrations of perchlorate led to a decrease in fertility, especially at higher temperatures. Our finding of a development effect resulting from the interaction between mild temperature elevations and thyroid disruption by a common environmental contaminant suggests that we may not be able to predict the overall physiological outcomes of either of these changing environmental factors by investigating them individually.

Only a few reports have evaluated the interactions between temperature shifts and chemical exposure, and their findings reveal that a wide range of physiological parameters are effected. For example, the estrogenic gene expression response of zebrafish following exposure to ethynyl estradiol and/or nonylphenol is exaggerated at elevated temperatures (Jin et al., 2010). Trout exposed to ammonia or acid pollution show shifts in growth and appetite when also exposed to small changes in temperature (Morgan et al., 2001). *Daphnia magna* demonstrate increased overtly toxic responses at higher temperatures compared to lower when also exposed to several common pharmaceuticals commonly found in the environment (Kim et al., 2010).

Other aspects of environmental quality may also interact with contaminant exposure. For example, 60 ppb perchlorate exposure disrupts *Xenopus laevis* tadpole development, but only when the animals are exposed in FETAX, a standard laboratory media (Brausch et al., 2010). When animals are exposed in natural surface water, they showed no effect from AP exposure. Similar results are found when the

rapidly developing species, *Spea multiplicata*, is exposed to 60 ppb perchlorate in surface waters suggesting that either something in the water resource interferes with the animals' response to perchlorate or something in this particular water resource affects perchlorate's bioavailability (Brausch et al., 2010). Again, these data suggest that a strong systems level approach will be needed to begin to ascertain how anthropogenically induced environmental change will impact wildlife health.

Changes in metamorphosis timing could affect a metamorphic size in these salamanders. Our results demonstrate that the body mass and body condition during the later phase of metamorphosis were significantly smaller in those individuals within the increased temperature group than in those individuals within the ambient temperature group. Higher temperature exposures resulting in early metamorphosis at a smaller body size has been previously reported in an amphibian species (Álvarez and Nicieza, 2002b). In particular, exposure to a 5 or 10 °C temperature increase over ambient during development induces an increase in body mass of developing individuals (*Discoglossus galganoi*) during the pro phase of metamorphosis, but it decreases the body mass as the animals progress through metamorphosis. One possibility is that the elevated temperature, shifts energetic resources towards increasing the differentiation rate much more than the growth rate and results in an overall smaller body size at metamorphosis. Our current result is consistent with this possibility since we find that during the later phase of metamorphosis, smaller and lower body condition individuals were produced in animals exposed to the higher temperature regime. In amphibians small body size or low energy reserves at metamorphosis has been demonstrated to decrease physical performance, survival or fecundity (Smith, 1987; Semlitsch et al., 1988; Álvarez and Nicieza, 2002a; Ficetola and De Bernardi, 2006), suggesting that the effects of increased water temperature on metamorphic timing could be costly at later life history stages. Even if the size difference recovers during a terrestrial life stage as seen in American toads, exposed to carbaryl, a common pesticide (Distel and Boone, 2009), the compensatory processes should be associated with other various costs such as a delay in the timing of first reproduction (reviewed in Ficetola and De Bernardi, 2006).

Overall, our results on developmental outcomes following the interaction between exposure to shifts in temperature and exposure to a thyroid hormone-related pollutant have two important implications: 1) small increases in water temperature may shorten the developmental period, and 2) interactive effects between increased water temperature and endocrine disrupting compound exposure could be dependent on the levels of a specific contaminant at the time of exposure. How these changes in development lead to functional shifts throughout an organism's life will be difficult to predict without longer term data sets investigating the fitness outcomes resulting following these interacting exposure studies.

5. Conclusions

In our study, we applied small temperature increase, less than 1 °C, comparable to actual temperature increases reported in the current environment as a result of global warming. We also included diel fluctuations in temperature by housing the tiger salamander larvae in outdoor enclosures. These measures will aid in approximating how recent climate temperature changes can affect individual developmental outcomes within natural populations. Overall our results revealed two important points. First, even small increase in water temperature can intensify disruptive effects of a common thyroid hormone disruptor (AP) on early larval growth and affect metamorphic endpoints such as time to metamorphosis and body mass and SVL at metamorphosis in Arizona tiger salamander larvae. Second, interactive effects between water temperature and exposure to a sublethal dose of a contaminant can vary due to developmentally and possibly, seasonally fluctuating hormone levels. Further studies investigating overall population impacts by such small developmental disruptions and testing potential shifting in dose-response curves of various endocrine disrupting chemicals following small temperature manipulations could further increase our

understanding how global warming can actually affect ectothermic vertebrates in their natural environments.

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Figure legends

Figure 1. Daily temperature change patterns in representative aquaria. **A)** A one-day (July 9 – 10) temperature change pattern extracted from an aquarium within the 20 ppb AP/ ambient temperature group (solid line) and one aquarium within the 0 ppb control/ increased temperature group (dotted line). The arrows indicate the time (Mountain Standard Time) at which aquarium heaters turned on. **B)** Averaged high, low and mean (thick black or gray lines) daily temperature from June 4 to July 30 in one aquarium from the 20 ppb AP /ambient temperature group and one from the 20 ppb AP/increased temperature group. The arrow indicates the date when individuals began metamorphosis.

Figure 2. Weekly developmental stage and snout-vent length (SVL) shifts during early development. In the increased temperature groups, the weekly developmental stage and snout-vent length (SVL) within the 20 and 200 ppb AP groups were smaller than those of the control group ($p_{0,20 \text{ ppb}} = 0.058$, $p_{0,200 \text{ ppb}} = 0.009$ for the developmental stage, **B**; $p_{0,20 \text{ ppb}} = 0.008$; $p_{0,200 \text{ ppb}} = 0.012$ for SVL, **D**), but these measurements were not different in ambient temperature groups ($p_s > 0.05$, **A**, **C**). Data are presented as the mean $\pm$ SE.

Figure 3. Effects of temperature and AP treatment on the percentage (%) of individuals metamorphosed. The time needed to reach metamorphosis (10, 30, 50 and 70 %) tended to be shorter in the increased temperature group (**B**) than in ambient temperature group (**A**; $p = 0.060$) although effects on the time to reach metamorphosis by AP treatments were not significant ($p = 0.146$). Data are presented as the mean $\pm$ SE.

Figure 4. The effect of temperature (A and B) and AP (C and D) treatment on body mass and condition factor as individual progress through development. The body mass and body condition of individuals during the 51–75% phase of metamorphosis in the increased temperature group was smaller than the body mass and body condition of individuals in the ambient temperature group ($p = 0.036$; $p = 0.019$, respectively). There was no significant effect on body mass or body condition by AP treatment during the any phases of metamorphosis ($p_s > 0.05$). Data are presented as the mean $\pm$ SE.

Figure 1

Figure 2

Figure 3

Figure 4