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# Specific time of exposure during tadpole development influences biological effects of the insecticide carbaryl in green frogs (*Lithobates clamitans*)

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ABSTRACT

The orchestration of anuran metamorphosis is initiated and integrated by thyroid hormones, which change dynamically during larval development and which may represent a target of disruption by environmental contaminants. Studies have found that some anurans experience increased rates of development when exposed to the insecticide carbaryl later in larval development, suggesting that this insecticide could affect thyroid hormone-associated biological pathways. However, the time in development when tadpoles are sensitive to insecticide exposure has not been clearly defined nor has the mechanism been tested. In two separate studies, we exposed recently hatched green frog (*Lithobates clamitans*) tadpoles to a single, three day carbaryl exposure in the laboratory at either 2, 4, 8, or 16 weeks post-hatching. We examined the impact of carbaryl exposure on mRNA abundance patterns in the brains of frogs following metamorphosis months after a single three day exposure (experiment 1) and in tadpole tails three days after exposure (experiment 2) using cDNA microarrays and quantitative real time polymerase chain reaction (QPCR) analyses. For tadpoles reared through metamorphosis, we measured tadpole growth and development, as well as time to, mass at, and survival to metamorphosis. Although carbaryl did not significantly impact tadpole development, metamorphosis, or survival, clear exposure-related alterations in both tail and brain transcript levels were evident when tadpoles were exposed to carbaryl, particularly in tadpoles exposed at weeks 8 and 16 post-hatching, indicating both short-term and long-term alterations in mRNA expression. These results indicate that carbaryl can have long-lasting effects on brain development when exposure occurs at sensitive developmental stages, which may have implications for animal fitness and function later in the life cycle.

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## 1. Introduction

Pesticides and pharmaceuticals have demonstrated potential to alter thyroid hormone (TH) action in cell culture models and whole organisms, including humans (Branchi et al., 2003; Brucker-Davis, 1998; Karmaus, 2001; Winneke et al., 2002; Zoeller, 2005, 2006). Exposure to these endocrine disruptors can alter production or breakdown of THs or modulate hormone function leading to aberrations in developmental processes or homeostasis (Capen, 1996; Crump et al., 2002; Helbing et al., 2006, 2007; Veldhoen and Helbing, 2005; Veldhoen et al., 2006a,b,c). Arguably, few TH-associated developmental events in nature are more dramatic than the metamorphosis of an aquatic tadpole into a frog (Shi, 2000). This complex biological process involves drastic changes in morphology, physiology, behavior, and habitat (Duellman and Trueb, 1994; Moran, 1994; Tata, 1996; Wilbur, 1980). Biochemical changes that accompany larval development may result in sensitivity to environmental contaminants during certain key periods of larval development, but not at others. Because appropriate TH function is integral to metamorphic transformation, larval anurans are excellent models for detecting environmental contaminants that disrupt normal TH-associated processes (Crump et al., 2002; Helbing et al., 2006; Howe et al., 2004; Veldhoen et al., 2006a,b).

A major component of anuran metamorphosis is the establishment of coordinated gene expression programs dependent on hormone action that establish tissue- and stage-specific transcriptomes and resultant proteomes required for developmental progression. This genomic function of TH is highly conserved across vertebrates and is mediated through thyroid hormone receptors (TRs) binding to promoter regions of TH-responsive gene and altering mRNA transcription activity (Buchholz et al., 2004; Harvey and Williams, 2002; Shi, 2000). The two TR isoforms (TRα and TRβ) are expressed across a diversity of anuran tissues (Hammond et al., in press; Hogan et al., 2007; Shi, 2000). TRα is produced constitutively throughout development, while TRβ displays marked induction during the early stages of tadpole metamorphosis (Denver, 1998; Shi, 2000). The normal expression levels and activity of TRs can be influenced by exposure to endocrine disrupting

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chemicals and examples of altered TH-associated mRNA abundance profiles have been described in several anurans (Crump et al., 2002; Helbing et al., 2006; Howe et al., 2004; Veldhoen et al., 2006a,b).

Pesticide-use is a widespread environmental issue that affects species in protected habitats and areas of human use because pesticides disperse from an application site through air and water to nearby and distant locations (Bidleman, 1999; LeNoir et al., 1999; Solomon et al., 1996; van Dijk and Guicherit, 1999; Vitousek et al., 1997). The biological effects and consequences of pesticide exposure are often poorly predicted or unanticipated, in part because of a limited understanding of mode of action, but also because differential effects may arise depending on the specific timing of exposure during development. For instance, female human fetuses proved to be particularly sensitive to exposure to diethylstilbestrol taken by their mothers resulting in increased risk to rare vaginal and cervical cancers decades later, even though mothers do not have greater risks to these cancers (Giusti et al., 1995). Recent studies have suggested that the effects of time of exposure may change during amphibian development, with some life stages more sensitive than others. For instance, Bridges (2000) found that insecticide exposure of eggs of southern leopard frogs (*Lithobates sphenocephalus*) did not have strong effects on survival or hatching, but impacted size at metamorphosis many months later, suggesting that there were long-term consequences to exposure of eggs. Studies with amphibians have also shown that pesticide exposure later in development increased mortality in some species (Boone and Semlitsch, 2001; Marian et al., 1983; Relyea, 2009), while leading to precocious metamorphosis in others (Boone et al., 2001; Boone and Bridges, 2003). For instance, Boone et al. (2001) and Boone and Bridges (2003) found that green frog (*L. clamitans*) tadpoles exposed to multiple doses of the insecticide carbaryl had a greater probability of reaching metamorphosis in a single season than individuals not exposed to the insecticide, but this study was not designed to distinguish between effects of multiple exposure versus effects of exposure later in development. They hypothesized that changes in the food web, which resulted in greater algal food resources, increased the likelihood of precocious metamorphosis (Boone et al., 2001, Boone and Bridges, 2003); however, it is possible that carbaryl exposure stimulated metamorphosis through changes in the thyroid hormone axis, because ability to respond to environmental cues increases with development (Denver, 1997).

Our objective was to investigate if green frog (*L. clamitans*) tadpoles were differentially impacted by transient exposure to the insecticide carbaryl based on when exposure occurred during development at approximately 2, 4, 8, or 16 weeks post hatching. In two separate studies, we examined the effects of time of exposure on mRNA abundance patterns in the brain of recently metamorphosed green frogs (experiment 1) and in the tadpole tail three days after exposure (experiment 2); additionally, we examined the effects of time of exposure on tadpole growth and metamorphosis (experiment 1) to link biochemical changes with traits correlated with fitness. We hypothesized that timing of carbaryl exposure during larval developmental would influence the likelihood of both short-term and legacy effects. We predicted that early exposure to carbaryl would have fewer effects on anurans than exposure later in development, because of a greater association of TH activity in later, post-embryonic development.

## 2. Materials and methods

### 2.1. Animal collection and chemicals

Four partial egg masses of green frogs (*L. clamitans*) were collected from Boesel Pond in the Miami University Natural Areas (Oxford, Butler Co., OH) on 25 June 2008 (experiment 1) and 16–19 June 2009 (experiment 2) for the "long-term" and "short-term" assay, respectively. Tadpoles were held in the laboratory at 20 °C on a 16:8 light:dark cycle. Tadpoles hatched within five days of collection and clutches within each experiment were mixed to homogenize genetic variation across treatments before commencing the experiments. A 10 g/L stock solution of carbaryl was prepared by dissolving 4.44 g Sevin (22.5% carbaryl; Garden Tech) in 100 mL distilled water. The stock solution was applied at $10^4$-fold dilution into the exposure beakers resulting in exposure to 1 mg/L carbaryl, which represents a realistic field concentration (Norris et al., 1983; Peterson et al., 1994). Carbaryl concentrations were confirmed twice at Mississippi State Chemical Laboratory (Mississippi State, MS) and indicated that concentrations expected to be 1 mg/L ranged between 0.78–0.88 mg/L at 1 h, 0.65–0.76 mg/L at 24 h, and 0.24–0.40 mg/L at 72 h.

### 2.2. Animal experimental treatments

In two separate experiments, we examined both the long-term consequences of a single, three day exposure to 1 mg/L carbaryl on metamorphosed frogs (experiment 1), as well as the short-term effects of the same exposure scenario on tadpoles (experiment 2). Initially, the short-term assay was planned to coincide with the long-term assay, but the 2 mm circular biopsy tissue samples we took were insufficient for analysis; therefore, a short-term assay was repeated the following year with whole individuals sacrificed at the end of exposure for subsequent analysis. The duration of the experimental period was from 8 July 2008 (experimental day 1) until 21 July 2009 (experimental day 337) for the long-term assay (experiment 1) and 7 July (experimental day 1) to 22 October 2009 (experimental day 108) for the short-term assay (experiment 2). One free-swimming Gosner stage 25 (Gosner, 1960) tadpole was added to each 1 L or 2 L glass beaker containing 1 L or 1.5 L of aged tap water (pH 8.3, dissolved oxygen 7.9 mg/L) in the long-term and short-term assay, respectively; in the long-term assay, tadpoles were moved to 2 L beakers containing 1.5 L aged tap water on experimental day 115 after the 16-week carbaryl exposure treatment was applied to maintain water quality and to provide extra room for growing tadpoles to swim. Water was changed in the beakers every two to three days throughout the experiment and tadpoles were fed ground Tetramin® Tropical Fish Flakes after each water change ad libitum.

*Experiment 1:* For the long-term assay, tadpoles were exposed to a single, three day exposure of 1 mg/L carbaryl at experimental week 2, 4, 8, or 16 (experimental days 14, 28, 56, or 112; tadpoles were approximately the same age post-hatching as those used in experiment 2). Each treatment was replicated 40 times and was randomly assigned to beaker. At experimental weeks 9 and 17, Gosner developmental stage and tadpole mass was determined for each tadpole. Tadpoles continued development with most individuals reaching Gosner stage 46 by 48 weeks. Beakers were checked daily for emergence of one or more front forelimbs (Gosner stage 42) at which time animals were removed and placed in plastic containers that were inclined at an angle to allow for escape from water. At tail resorption (Gosner stage 46), each metamorph was weighed, and time to metamorphosis and survival to metamorphosis were determined. All animals that reached Gosner stage 46 were placed in shoebox containers for 24 h with two wet paper towels and 20 crickets to determine feeding ability (results reported in Davis et al., 2011) and subsequently euthanized in 1% buffered tricaine methanesulfonate (Western Chemical, Ferndale, WA) to determine long-term impacts of carbaryl exposure on the brain transcriptome. Euthanized animals were immediately dissected and brain tissue was removed and placed in 1 mL of RNA*later* (Life Technologies Corp., Carlsbad, CA). Samples were stored for 24 h at 4 °C and subsequently transferred to −70 °C prior to isolation of total brain RNA.