Evaluating the Biological Effects and Safety of Exposure to Low, Putatively Safe Concentrations of the Common Pesticide Chlorpyrifos Using an Anuran Model

Sara McClelland

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LOW, PUTATIVELY SAFE CONCENTRATIONS OF THE COMMON PESTICIDE
CHLORPYRIFOS USING AN ANURAN MODEL

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the degree of Doctor of Philosophy

By
Sara Jeanine McClelland

August 2020
EVALUATING THE BIOLOGICAL EFFECTS AND SAFETY OF EXPOSURE TO LOW, PUTATIVELY SAFE CONCENTRATIONS OF THE COMMON PESTICIDE CHLORPYRIFOS USING AN ANURAN MODEL

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ABSTRACT

EVALUATING THE BIOLOGICAL EFFECTS AND SAFETY OF EXPOSURE TO LOW, PUTATIVELY SAFE CONCENTRATIONS OF THE COMMON PESTICIDE CHLORPYRIFOS USING AN ANURAN MODEL

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Sara Jeanine McClelland

August 2020

Dissertation supervised by Sarah K. Woodley

In the United States, over 1.1 billion pounds of pesticides are applied annually. Unfortunately, these pesticides often contaminate natural habitats and have effects on the health of humans and other species. Chlorpyrifos (CPF) is an organophosphorous insecticide that contaminates surface waters in the US. CPF, like other organophosphate pesticides, functions by irreversibly inhibiting acetylcholinesterase, the enzyme that degrades the neurotransmitter acetylcholine. There is also evidence that CPF affects neurodevelopmental processes below thresholds that affect AChE inhibition. However, few studies have assessed the impacts of CPF at such low doses. The goal of my dissertation was to test the effects of very low-dose CPF exposures on a variety of biological parameters including body morphology, hormone levels, and brain development in Northern Leopard Frogs (Lithobates pipiens), a common vertebrate
model. Understanding how low-dose CPF exposures impact animals is a point of concern because many pesticides, including CPF, are found in surface waters at sublethal concentrations currently considered safe by the EPA. Previous work showed that there were changes in brain shape in tadpoles exposed to low doses of CPF in artificial ponds (mesocosms), but it was unclear if changes were caused by direct exposure to CPF or by indirect effects of CPF mediated through community structure and food availability. To determine whether effects resulted from direct CPF exposure or from disruption of the food web due to a pesticide-induced decline in zooplankton, I examined the impacts of CPF on amphibian development in mesocosms with communities of either CPF-sensitive or CPF-resistant zooplankton. I found that CPF directly impacted brain shape. I then determined if responses to low doses of CPF were replicable in a controlled laboratory study. I tested responses to the lowest, most commonly encountered doses of CPF and found impacts on neurodevelopment, behavior, and neuroendocrine processes, demonstrating functional consequences of low-dose exposures in Northern Leopard Frogs. Both the neurodevelopmental and neurobehavioral effects of CPF occurred in a nonmonotonic dose response. This provided evidence that low doses of CPF impact animals in ways that are not always straightforward and easy to determine. One reason that we may see complex effects of CPF exposures is that CPF is likely impacting the body through numerous different mechanisms. One possibility is that CPF is activating the hypothalamus-pituitary-adrenal/interrenal axis (HPA/I) and the increasing concentrations of circulating corticosterone (CORT) are causing biological changes in animals. To determine the role CORT plays in low-dose CPF exposures, I exposed tadpoles to either a vehicle control, CPF, CORT, or CPF+MTP (metyrapone [MTP], a
CORT biosynthesis blocker). Results did not support the hypothesis that the effects of CPF were mediated through CORT. While CPF and CORT both impacted relative brain shape, they did so in different ways. Also, tadpoles exposed to CPF and CORT also had differing hormone profiles, pigmentation, and relative body shape. However, there was a trend for animals exposed to CPF+MTP to have reduced neurological effects, suggesting that MTP may have other impacts beyond inhibiting CORT synthesis. In addition, MTP could represent a potential means of mitigating the neurological effects of CPF. More research is needed to determine how the neurological impacts of CPF are modulated, as well as investigating whether MTP might ameliorate the effects of low dose CPF exposures. This research provides a better understanding of how low, ecologically relevant concentrations of CPF are impacting vertebrate development. This work also provides new insights for conservation and management strategies of animals living in habitats with organophosphate contamination.
ACKNOWLEDGEMENTS

"It’s been a long road, getting from there to here
It’s been a long time, but my time is finally near
And I will see my dream come alive at last"
- Star Trek: Enterprise, Theme Song
Written by Diane Warren

As the saying goes, "it takes a village." This has never applied more than to successfully completing a doctoral program and writing a dissertation, and I want to truly thank everyone who was a part of my village.

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Figure 4.7. Corticosterone Concentration. Tadpoles exposed to CORT had physiologically realistic increases in water-borne CORT concentrations. These concentrations were higher than all other treatments. Tadpoles exposed to CPF+MTP had slightly higher CORT concentrations than tadpoles exposed to CPF alone, however, neither were different than the controls. Mean +/- SEM is graphed. Points labeled with different letters are significantly different, p<0.05........................................................................................................141

Figure 4.8. Pigmentation. Tadpoles exposed to CORT had less skin pigmentation than other treatment groups. Mean +/- SEM is graphed. Points labeled with different letters are significantly different, p<0.05........................................................................................................142

Figure 4.9. Tadpoles that were exposed to CORT during development were found more often with their bodies held vertically in the water column than all other treatments. Mean +/- SEM is graphed. Points labeled with different letters are significantly different, p<0.05........................................................................................................143

Figure 4.10. Body Mass Adjusted Body Morphology (A) Principal Component (PC) 1. Tadpoles that were exposed to CORT during development had relatively longer bodies, relatively shorter, thinner, and wider tails, and thicker tail muscles. Tadpoles that were exposed to CPF+MTP during development had relatively longer bodies, relatively shorter, thinner, and wider tails, and thicker tail muscles than controls, but were not different than tadpoles exposed to CPF or MTP alone. (B) PC 2. There was no effect of treatment on tadpole body depths or width. Mean +/- SEM is graphed. Points labeled with different letters are significantly different, p<0.05........................................................................................................144

Figure 4.11. Brain Mass Adjusted Brain Morphology (A) Principal Component (PC) 1. Tadpoles that were exposed to CPF during development had relatively longer and wider telencephala, optic tecta, and diencephala, and wider medullas. (B) PC 2. Tadpoles that were exposed to CORT during development had relatively longer medullas and olfactory bulbs. +/- SEM is graphed. Points labeled with different letters are significantly different, p<0.05........................................................................................................145
LIST OF ABBREVIATIONS

AChE: Acetylcholine Esterase
ACTH: Adrenocorticotropin Hormone
ANOVA: Analysis of Variance
CF: Body Condition Factor
CORT: Corticosterone
CPF: Chlorpyrifos
CRF: Corticotropin Releasing Factor
EMM: Estimated Marginal Mean
HPA/I: Hypothalamus Pituitary Adrenal/Interrenal
HPT: Hypothalamus Pituitary Thyroid
IACUC: Institutional Animal Care and Use Committee
IQ: Intelligence Quotient
KMO: Kaiser Meyer Olkin Test
LOAEL: Lowest Observed Adverse Effect Levels
MANCOVA: Multiple Analysis of Covariance
MS-222: Tricaine Methanesulfonate
MTP: Metyrapone
NOAEL: No Observed Adverse Effect Levels
PC: Principle Component
PCA: Principle Component Analysis
USEPA: United States Environmental Protection Agency
Chapter 1

Introduction

Overview

Pesticides are used world-wide to kill or control pests, benefiting humans by increasing crop yields, decreasing the number of disease-vectors, and aiding in managing transportation throughways and business properties. In the United States, pesticide use is so prolific that over 1.1 billion pounds of pesticides are applied annually with an expenditure greater than $13.8 billion in 2012 (Atwood and Paisley-Jones 2017). Unfortunately, pesticides have also been shown to contaminate natural habitats affecting the health of humans and other species (Slotkin 2004; Rauh et al. 2006; Bernabò et al. 2011; Rauh et al. 2012; Stone et al. 2014; U.S.EPA 2015). Sources of pesticide pollution include atmospheric drift, direct application, run-off, leaching, erosion, upstream sources, and spills (Giesy et al. 1999). Further, periods of rainfall have been shown to increase the amount of pesticides that are washed from agricultural sources into natural habitats resulting in spikes in concentration and number of pesticides found in wetlands (Donald et al. 1999).

One of the most applied pesticides and the most applied insecticide is the organophosphate chlorpyrifos (Grube et al. 2011). Chlorpyrifos (CPF), like other organophosphate pesticides, functions by competitively binding and irreversibly inhibiting acetylcholinesterase, the enzyme that degrades the neurotransmitter acetylcholine. Interestingly, organophosphates are also naturally occurring in freshwater cyanobacterial algal blooms in North America (Fiore et al. 2020). Exposure to organophosphates results in a buildup of acetylcholine resulting in continued stimulation
of the nervous system (Slotkin 2004; Bernabò et al. 2011; Rauh et al. 2012; Liendro et al. 2015). Toxicity results when CPF causes more than 70-80% inhibition of acetylcholinesterase (AChE) (Slotkin 2004). CPF is often applied in agricultural settings year round to kill insects through AChE inhibition (Giesy et al. 1999). Due to the conserved nature of biological mechanisms, inhibition of AChE can have the same effects in non-target animals and exposures in vertebrates that have been shown to cause 70-80% AChE produces a "cholinergic storm" with symptoms such as salivation, lachrymation, incontinence, unconsciousness, seizures, and even death (Schulze et al. 1997; Slotkin 2004). The exact dose that causes these effects is difficult to define as the effects of CPF on AChE inhibition varies depending on age and species (Carr and Chambers 1996; Kousba et al. 2007). For example in neonatal rats, the biomolecular inhibitory rate constant (Ki) was 0.95 nM/hr at 5 days, 0.50 nM/hr at 7 days, and 0.22 nM/hr at 17 days, which is similar to the adult rates (Kousba et al. 2007). Another study found the Ki in rats at 37°C was 7528 mM/min and in fish 958.8 mM/min (Carr and Chambers 1996). Further, the biomolecular effects have not been well studied in amphibians. Within amphibians, there is also a range of the inhibitory concentrations and lethal concentrations of CPF. For example, when Southern Leopard Frogs (Lithobates sphenoecephala) were exposed to 200 μg/L, there was 43% inhibition in AChE activity but in the Northern Cricket Frog (Acris crepitans) 200 μg/L exposures resulted in 60% inhibition of AChE activity (Widder and Bidwell 2008).

Less severe inhibition of AChE can also have negative impacts and has been shown to result in headaches, fatigue, blurred vision, effects on movement, among others (Schulze et al. 1997). While AChE inhibition has been the most well studied mechanism
of chlorpyrifos toxicity, there is evidence that CPF can act on neurodevelopmental processes independent of changes in acetylcholine. Impairments in neurodifferentiation, impacts on axonogenesis, and deficiencies in synapse formation and transmission are likely being impacted by CPF doses below the threshold for AChE inhibition (Slotkin 2004; Colborn 2006; Rosas and Eskenazi 2008). However, few studies have assessed the impacts of CPF at these low doses.

The goal of this dissertation is to further explore the effects of very low dose CPF exposures on a variety of biological parameters including body morphology, hormone concentrations, brain development, and behavior. Understanding how low dose CPF exposures are impacting animals is a point of concern because many pesticides, including chlorpyrifos, are found in surface waters at sublethal concentrations currently considered safe by the EPA (U.S.EPA 2015). This exposes humans and other non-target organisms to sublethal doses of CPF throughout the US. Further, the regulatory decisions on CPF are based, in part, on the exposure concentrations that do not result in overstimulation caused by prolonged acetylcholine signaling when AChE prevents breakdown of acetylcholine (U.S.EPA 2015). If CPF has effects below these concentrations, it is important that we understand what those effects are in order to make more sound regulatory and health decisions.

Developing organisms are more sensitive to low, sublethal doses of organophosphates than adults. These developmental exposures often result in long-term changes to brain anatomy, intelligence, and behavior (Ostrea Jr et al. 2002; Qiao et al. 2004; Roy et al. 2004; Roy et al. 2005; Rauh et al. 2012). Developmental exposures in fish, amphibians, and rodents have been shown to impact activity, learning, and memory
(Levin et al. 2002; Timofeeva et al. 2008; Khalil et al. 2013; Shuman-Goodier and Propper 2016). In developing humans, long term impairments in motor function, IQ, perceptual reasoning, and working memory have been observed (Bouchard et al. 2011; Engel et al. 2011; Rauh et al. 2011; Rauh et al. 2012).

To better understand the effects of low dose CPF exposures on neurodevelopment, Woodley et al. (2015) exposed tadpoles to low concentrations of CPF in mesocosm experiments to mimic natural pond settings. CPF caused both morphological and neurodevelopmental changes in the tadpoles (Woodley et al. 2015). These findings demonstrated that even these low doses can cause brain changes in tadpoles. However, it also opened the door to new questions, inspiring me to pursue three new avenues of research in the field. First, I wanted to determine if the insecticide-induced changes in amphibian brains were caused from direct CPF exposure or indirect disruptions of the food web caused by CPF (Aim 1) (reviewed in Hanazato 2001; Relyea and Diecks 2008; Bendis and Relyea 2016b). Second, I wanted to investigate the neuroanatomical and behavioral changes caused by CPF in a controlled laboratory setting (Aim 2). This would enable me to determine if the low dose CPF-induced brain changes also have functional consequences. Further, if these CPF effects are replicable in a laboratory study, it would provide more evidence that these changes are real and meaningful impacts of low dose CPF exposure. Third, it has been hypothesized that various mechanisms contribute to the effects of CPF exposure, inspiring me to begin analyzing physiological processes that might be modulating CPF-induced changes (Reviewed in Slotkin 2004). Anthropogenic contaminants, including CPF, have been shown to alter CORT concentrations, and previous studies have shown that CORT
modulates both phenotypic and behavioral responses of animals that are exposed to natural stressors (Hopkins et al. 1997; Hayes et al. 2006; Miller et al. 2009; McMahon et al. 2011; Acker and Nogueira 2012; Middlemis Maher et al. 2013; Mestre et al. 2019). Therefore, I also chose to evaluate the role of corticosterone in changes caused by sublethal CPF exposure (Aim 3).

**Model Organism**

I used Northern Leopard Frogs (*Lithobates pipiens*) as my model organism. Northern Leopard Frogs are medium sized anurans with an average adult body mass of 38g. They're widely distributed across North America from the Hudson Bay to Virginia on the east coast and from British Columbia to Arizona on the west coast (Kendrick 2014). However, populations have declined in the western United States with the species being protected in Arizona, Nevada, and New Mexico and is a species of special concern in California (NAC 2-18; Rogers and Peacock 2012; Thomson et al. 2016; AZGFD 2017-2018; NMDGF 2018).

Northern Leopard Frogs use grassy meadows, brush, and forests near permanent standing or slowly moving water as habitat, and they can travel from 45 m to 200 m from water sources during the summer, and potentially even farther for overwintering sites (Kendrick 2014). Breeding occurs in shallow water with vegetation during the spring (mid-March to early April), and females lay clutches with 2,000-6,500 eggs/clutch. While development is temperature-dependent, eggs generally hatch in 13-20 days and undergo metamorphosis 60-80 days after hatching (Kendrick 2014).

Larval amphibians (e.g., tadpoles) are a long-standing model of vertebrate development, providing us with multiple studies providing evidence that many
developmental mechanisms are conserved amongst vertebrates, enabling us to draw conclusions about vertebrate exposures to pesticides by using this model system. Further, anurans are amenable to both controlled laboratory experiments and field studies. Eggs are easy to obtain in the field or can be purchased, even outside of the breeding season. In the wild, frogs are also abundant and can be found in both pristine environments and environments which are exposed to pesticides at varying concentrations (Harris et al. 1998; Hua et al. 2013; U.S.EPA 2015). Understanding how pesticides impact frog populations is also important from a conservation aspect because populations of amphibians, including Northern Leopard Frogs in the western United States, are rapidly declining on a global scale and one of many causes in this decline is exposure to anthropogenic pollutants such as pesticides (Stuart et al. 2004; Hayes et al. 2010).

Specific Aims

**Aim 1: Determine if the insecticide-induced changes in amphibian brains are from direct CPF exposure or indirect disruptions of the food web caused by CPF.**

Pesticides can affect aquatic communities both directly and indirectly. Pesticides can directly interact with enzymes, receptors, and processes in the body to cause direct effects. They can also kill more sensitive members of a community, which can impact interactions among remaining members of the community, causing indirect effects.

Previous work showed exposure to trace amounts of CPF resulted in altered tadpole morphology and neurodevelopment in artificial ponds (mesocosms) (Woodley et al. 2015). CPF has been shown to directly affect neurodevelopment at higher doses (Slotkin 2004; Rauh et al. 2012). However, it's possible that CPF can also trigger a trophic cascade by killing a large fraction of zooplankton in pond communities and
ending with less food availability for tadpoles (reviewed in Hanazato 2001; Relyea and Diecks 2008; Bendis and Relyea 2016b). Therefore, it is unclear if the changes in relative body and brain dimensions of tadpoles documented by Woodley et al. (2015) resulted from direct CPF exposure on the amphibians or from the indirect effects of CPF on the aquatic community.

To determine whether effects resulted from direct CPF exposure or from disruption of the food web due to a pesticide-induced decline in zooplankton, I examined the impacts of CPF on amphibian development in mesocosms that had communities with either CPF-sensitive or CPF-resistant zooplankton. If CPF impacts neurodevelopment directly, I predicted that tadpoles raised in either community would have the same impacts on brain development. However, if the impacts of CPF are due to community changes caused from CPF, then brain changes would only be found in mesocosms with zooplankton sensitive to CPF.

**Aim 2: Investigate the neuroanatomical and behavioral changes of animals that are exposed to sublethal doses of the pesticide chlorpyrifos in a controlled laboratory setting.**

In order to decrease environmental impacts, more modern pesticides have been developed to be less persistent and have more stringent application requirements. These changes have resulted in aquatic habitats that have lower concentrations of pesticides contaminating these habitats. This has resulted in fewer acute poisonings, but low-dose chronic exposures are becoming more of a concern. Further, these low-dose exposures are less likely to cause notice because their effects are often sublethal, which may result in the effects not being observed or if they are observed may be difficult to link to
chemical exposures (Vyas 1999; Köhler and Triebskorn 2013). Understanding the biological changes that are caused by low-dose pesticide exposures, can help us monitor populations for sublethal effects of low-dose pesticide exposure, and provide insights into better treatments, management, and conservation strategies moving forward.

Further, few studies analyze the effects of low, ecologically relevant doses of CPF on neurodevelopment and behavior. Numerous studies have found evidence of the neurodevelopmental effects caused by exposure to unrealistically high doses of organophosphates. Further, these studies have tended to focus on either the anatomical effects or the behavioral effects caused by exposure, and most were limited to one life history stage. This limits our understanding of the link between neuroanatomical changes and functional behavior changes that occur over the lifetime of an organism in response to low dose organophosphate exposure.

With this aim, I wanted to determine how low, commonly encountered doses of CPF affect physiology, neurodevelopment, and behavior. I hypothesized that concentrations of CPF that result in changes in brain shape will also produce behavioral and hormonal alterations. Using controlled laboratory settings, tadpoles were exposed to controls or sublethal doses of CPF. I measured anatomical changes in the brain caused by CPF, conducted behavioral assays to measure animal activity and responsiveness to visual and olfactory stimuli, and measured waterborne corticosterone concentrations. In this way, I determined if the CPF-induced changes in brain anatomy were associated with functional outcomes related to behavior and physiology.

*Aim 3: Evaluate the role of corticosterone in changes caused by sublethal CPF exposure.*
CPF has been shown to cause changes in the brain through numerous mechanisms. The most well studied mechanism is AChE inhibition. However, CPF exposures impact neurodevelopment by mechanisms other than AChE inhibition. These other processes are still not well understood or defined.

Previous work has shown that CPF exposure results in elevated concentrations of CORT. These elevated concentrations of corticosterone (CORT) might be contributing to the effects seen in sublethal CPF exposures. CORT can modulate phenotypic changes and behavioral responses and is heavily involved in neurodevelopment.

To determine the role CORT plays in the biological impacts of CPF, I exposed animals to exogenous CORT to artificially elevate CORT concentrations, to CPF, or to CPF and metyrapone (MTP) simultaneously. MTP is a CORT biosynthesis blocker, which will enable me to see the effects of CPF in animals that do not have elevated CORT concentrations. I predicted that if CORT is contributing to the effects of low dose CPF exposures that tadpoles exposed to CORT and CPF would have similar phenotypes that would disappear in tadpoles exposed to both CPF and MTP.

Overall this research gives us a better understanding of how amphibians, which often live close to areas where pesticides are applied, are impacted by organophosphorous pesticides. Second, because amphibians are a common model for vertebrate development, outcomes of this work provide insight into how low dose CPF exposures are impacting vertebrate animals. This, in turn, will potentially provide insights into how we can better protect vertebrates through new conservation and management strategies.
Significance

My dissertation research will provide a better understanding of how low but still ecologically relevant concentrations of organophosphorous pesticides are impacting vertebrate development. This work will determine if the effects of CPF are direct or indirect, if there are functional consequences of CPF-induced brain changes, and finally will investigate the role of CORT in CPF-induced biological changes. The results of my dissertation will demonstrate impacts of organophosphate contamination and provide new insights into animal conservation.
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Chapter 2

Determine if the insecticide-induced changes in amphibian brains are from direct CPF exposure or indirect disruptions of the food web caused by CPF

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ABSTRACT

Widespread use of pesticides often contaminates natural habitats, exposing non-target organisms to pesticides that were designed to control pest populations. Even low concentrations of pesticides can affect aquatic communities both directly and indirectly. Previous work showed trace amounts of the pesticide chlorpyrifos (CPF) altered tadpole morphology and neurodevelopment in artificial ponds (mesocosms). To determine whether effects resulted from direct CPF exposure or from disruption of the food web due to a pesticide-induced decline in zooplankton, we examined the impacts of CPF on amphibian development in the presence of CPF-resistant zooplankton, a key component of the aquatic trophic community. Northern Leopard Frog (Lithobates pipiens) tadpoles were reared through metamorphosis in mesocosms containing either 0 or 1μg/L CPF and either CPF-resistant or CPF-sensitive Daphnia pulex zooplankton. Developmental exposure to CPF resulted in metamorphs with a relatively wider optic tectum, medulla, and diencephalon compared to controls, and this result was found regardless of the zooplankton population within the mesocosm. Thus, CPF directly impacted brain development, independent from the effects on the trophic community. With respect to
body shape, CPF had no effect on body shape of metamorphs reared in mesocosms with CPF-sensitive zooplankton, but body shape was sensitive to zooplankton population in the absence of CPF. To conclude, low, ecologically relevant doses of organophosphorous pesticides can directly impact neurodevelopment in a vertebrate model.

**INTRODUCTION**

In the United States, over 1.1 billion pounds of pesticides were applied in 2012 with an expenditure greater than $13.8 billion (Atwood and Paisley-Jones 2017). Pesticides are applied to kill or control target pest populations in agricultural uses, commercial settings, on government lands, and in residential areas by homeowners. Unfortunately, these pesticides can contaminate natural habitats exposing the species that live in these habitats to a wide range of pesticide types (Harris et al. 1998; U.S.EPA 2015).

Exposure to pesticides, either in controlled laboratory tests or during pesticide application to natural environments, can be lethal across a wide range of non-target taxa, depending on the concentration (Mayer and Ellersieck 1986). In an effort to reduce non-target effects, pesticides have been developed that breakdown quickly after application in response to natural environmental processes to ensure lower concentrations of pesticides in natural settings (Howard 1991; Giesy et al. 1999). These lower concentrations of pesticides typically do not have lethal effects in non-target organisms but may still have important sublethal effects. For example, low concentrations of pesticides can affect amphibian growth and development and the trophic communities of which amphibians are a part (Hanazato 2001; Relyea and Diecks 2008; Bendis and Relyea 2016b). Thus, it
is important to study how these low, putatively safe, concentrations may impact natural aquatic communities (Sparling and Fellers 2009; Hayes et al. 2010).

Chlorpyrifos (CPF) is an organophosphate insecticide that inhibits cholinesterase, which results in an accumulation of the neurotransmitter acetylcholine; this leads to toxic effects through the continued stimulation of the nervous system (Slotkin 2004; Bernabò et al. 2011). While this is applied to kill insects, this mechanism of toxicity also affects vertebrates by irreversibly inhibiting cholinesterases. In addition, lower concentrations of CPF, similar to concentrations that are often found in natural habitats, can directly act on developmental processes independent of changes in acetylcholine (Slotkin 2004; Colborn 2006). CPF affects neurodevelopment in humans and other animals (Slotkin 2004; Rauh et al. 2012; Mishra and Devi 2014; Woodley et al. 2015). Aquatic communities are particularly vulnerable due to agricultural use of pesticides. Understanding how exposure to CPF impacts neurodevelopment is important because many young animals, such as amphibian larvae, can be exposed to CPF during their development.

To determine the effects of low concentrations of CPF on amphibian development, Woodley et al. (2015) exposed tadpoles to CPF in mesocosms, which are semi-natural ponds that mimic the natural aquatic community. CPF caused morphological and neurodevelopmental changes in tadpoles (Woodley et al. 2015). However, other members of aquatic communities, like zooplankton, can also be affected by pesticides (reviewed in Hanazato 2001). Insecticides like CPF can trigger a trophic cascade by killing a large fraction of the zooplankton. With few zooplankton remaining, their algal food source (i.e., phytoplankton) can become very abundant, causing a decline in light transmission through the water column. The reduced light transmission causes a
reduction of attached algae (i.e., periphyton) at the bottom of the water column, which is the main food source for tadpoles (reviewed in Hanazato 2001; Relyea and Diecks 2008; Bendis and Relyea 2016b). Therefore, it is unclear if the changes in relative body and brain dimensions of tadpoles documented by Woodley et al. (2015) resulted from direct CPF exposure on the amphibians or from the indirect effects of CPF on the aquatic community. If the effects of CPF were caused by the CPF-induced trophic cascade, then the negative impacts of low-concentration exposures to CPF might be mitigated in communities containing pesticide-resistant zooplankton populations.

Bendis and Relyea (2016b) showed that community structure can buffer the effects of pesticide contamination of aquatic communities. They treated Northern Leopard Frog tadpoles (Lithobates pipiens) with CPF in mesocosms containing either pesticide-sensitive zooplankton or pesticide-resistant zooplankton. These pesticide-resistant zooplankton are specifically resistant to CPF and should be able to survive in pesticide contaminated waters, preventing trophic cascades and possibly protecting the aquatic community from trophic changes when exposed to insecticides (Bendis and Relyea 2016b; 2016a). As expected, mesocosms with CPF-resistant zooplankton had more stable food webs and greater food availability, and Leopard Frog metamorphs from these mesocosms had increased mass and survival compared to mesocosms with CPF-sensitive zooplankton. These results indicate that community structure can buffer the effects of pesticides on important traits like body mass and survival.

To determine if morphological and neurodevelopmental responses of amphibians to pesticides are driven by direct exposure to CPF or are the result of indirect CPF-induced trophic cascades, we examined the body and brain morphology of Northern
Leopard Frogs from a study by Bendis and Relyea (2016b). Ecological communities containing CPF-resistant or CPF-sensitive populations of zooplankton (Daphnia pulex) were exposed to either 0 or 1 μg/L CPF. In mesocosms with CPF-sensitive zooplankton, we predicted that when exposed to 1 μg/L CPF, there would be changes in brain and body morphology of Leopard Frog metamorphs due to the indirect effects of decreased food availability (Bendis and Relyea 2016b). In mesocosms with CPF-resistant zooplankton, we predicted that when exposed to 1 μg/L CPF, there would be no morphological changes in brain and body morphology possibly due to CPF-resistant zooplankton stabilizing the food web (Bendis and Relyea 2016b).

**METHODS**

Because the present study is an extension of a published study, we describe the key methods below and refer readers to Bendis and Relyea (2016b) for additional experimental details. The following methods were approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Pittsburgh.

**The mesocosm experiment**

A mesocosm experiment was designed with a full factorial combination of four populations of Daphnia pulex zooplankton (two populations that are sensitive to CPF and two populations that are resistant to CPF) and a range of CPF concentrations (0, 0.25, 0.50, and 1.0 μg/L; Sigma-Aldrich) from which we selected the concentrations of 0 or 1 μg/L (Figure 2.1). We decided to analyze animals from only two concentrations in the interest of time. The concentrations 0 and 1 μg/L were chosen before beginning any experimental analysis because they were most similar to those used in previous work showing neurological effects of CPF exposure in tadpoles and because the concentration
of 1 μg/L is similar to concentrations found in surface waters (U.S.EPA 2015; Woodley et al. 2015). The two populations of CPF-resistant *D. pulex* did not differ in their effects on the community and the two populations of CPF-sensitive *D. pulex* did not differ in their effect on the community (Bendis and Relyea 2016b), therefore we pooled them into a single treatment group of resistant *Daphnia* and a single treatment group of sensitive *Daphnia*. The 4 treatment combinations (0 μg/L CPF with sensitive-*Daphnia*, 0 μg/L CPF with resistant-*Daphnia*, 1 μg/L CPF with sensitive-*Daphnia*, 1 μg/L CPF with resistant-*Daphnia*) were replicated six times for a total of 24 mesocosms. The mesocosms were set up outside at the University of Pittsburgh’s Donald S. Wood Field Laboratory at the Pymatuning Laboratory of Ecology in 2012. Each mesocosm represented an experimental unit, containing well water, phytoplankton, periphyton, *Daphnia pulex* zooplankton, and 30 recently hatched Northern Leopard Frog (*Lithobates [Rana] pipiens*) tadpoles that were collected from a pond in northwestern Pennsylvania (Crawford County).

Four days after adding tadpoles to the mesocosm, CPF was first applied to the mesocosm. CPF was then applied to mesocosms every 2.5 weeks. Tadpoles developed in mesocosms until front legs emerged (Figure 2.1). They were then removed to containers without CPF to complete metamorphosis at which point they were euthanized using a 2% solution of MS-222. Carcasses were preserved and stored in 10% phosphate buffered formalin.

Although there were 6 mesocosms per treatment, a few preserved samples were lost due to drying, resulting in 5 mesocosms per treatment (except for the 1 μg/L CPF-sensitive zooplankton group where there were only 4 mesocosms). Within each
mesocosm, 10 metamorphs were measured for morphological dimensions and the measurements were averaged to obtain a mesocosm mean and standard error for each trait. In 5 of the mesocosms, fewer than 10 metamorphs survived to metamorphosis; from mesocosms containing 0 μg/L CPF with CPF-sensitive *D. pulex*, 3 of the mesocosm averages were based on 4, 8, and 8 metamorphs, from the mesocosms containing 1 μg/L CPF with CPF-sensitive *D. pulex* 1 mesocosm average was based on 5 metamorphs, and from mesocosms containing 1 μg/L CPF with CPF-resistant *D. pulex* 1 mesocosm average was based on 7 metamorphs.

**Metamorph body and brain morphology**

Preserved metamorphs were rinsed in water, blotted dry, and weighed. The dorsal surface of each metamorph was photographed. Fifteen linear dimensions of the metamorphs were measured (Figure 2.2) using Image J software (US National Institutes of Health). Brains were removed and trimmed of cranial nerves. The brains were then weighed, and the dorsal and ventral surfaces were each photographed 3 times independently to produce 3 dorsal images per brain and 3 ventral images per brain. Brains were moved and repositioned between capturing each image. Using Image J software, we measured 5 linear dimensions on each of the dorsal images and 4 linear dimensions each of the ventral images (Figure 2.2B). Each linear dimension was measured once from each of the 3 images and then averaged to get a single estimate for each brain dimension for each metamorph.

**Statistical analysis**

SPSS was used for all statistical analyses. Before analyzing differences in body or brain traits, the linear measurements were corrected for differences in body mass or brain
mass, respectively, because more massive metamorphs have larger body and brain traits. For each linear measurement, we conducted an ANCOVA with treatment as a fixed effect and either body mass or brain mass as a covariate. If necessary, values were log-transformed to achieve linearity and homogeneity of slopes. Data were adjusted for body mass or brain mass by adding the residual value for each animal to the overall estimated marginal means (EMM) generated by the ANCOVA (see Appendix 1). By adding residuals to the EMM (instead of just using residuals), we get values that are more biologically meaningful and thus easier to interpret. Finally, means of mass adjusted values were calculated for each mesocosm.

To reduce the number of linear dimensions describing brain or body mass, we did a principal component analysis (PCA) on the mass-adjusted mesocosm means. Before conducting PCA we confirmed that the assumptions of PCA (KMO>0.6 and Bartlett’s test ≤ 0.05) were met. We used commonly accepted PCA methods that converted the correlated morphological variables into uncorrelated principal components (PCs) using a varimax rotation and eigenvalues > 1. It is not statistically valid to force more PC than result from using these standard methods. The number of PCs that resulted from PCA are reported in the results. PCs were normally distributed with equal variances (data were log transformed when necessary to meet these requirements) were analyzed using ANOVAs with pesticide treatment and zooplankton type as factors.

RESULTS

Brain morphology

The PCA of the nine mass-adjusted brain measurements yielded three PCs with eigenvalues greater than 1 (Table 2.1). PC-1 loaded strongly (factor score above 0.5) with
optic tectum width, medulla width, and diencephalon width. PC-2 loaded strongly with telencephalon length, optic tectum length and medulla length. PC-3 loaded strongly with telencephalon width, olfactory bulb length, and diencephalon length.

CPF affected the relative brain dimension captured by PC-1 (p=0.015; Table 2.2). That is, metamorphs exposed to 1 μg/L CPF had wider optic tecta, wider diencephalons, and wider medullas than controls (Figure 2.3). Other brain dimensions were not impacted by CPF (PC-2 and PC-3, Table 2.2). This makes sense given that PC-1 accounted for over 45% of the variance in the data compared to <20% for PC-2 and PC-3. Zooplankton sensitivity did not affect any of the PCs describing brain shape, either as a main effect or as an interaction with CPF (Table 2.2).

**Body morphology**

For body morphology, we conducted PCA two ways: one with all mass-adjusted body measurements, and one using only the mass-adjusted measurements for the body and the left limbs to ensure that using the very highly correlated variables of right and left body parts did not skew the results. Conclusions from both of these analyses were the same, so only the data from the PCA using all of the mass-adjusted body measurements is presented here.

The PCA of the fifteen mass-adjusted body measurements identified four PCs with eigenvalues greater than 1 (Table 2.3). PC-1 loaded with right foot length, right and left leg length, right and left thigh length, and right forearm length. PC-2 loaded with right and left thigh width, right and left forearm width, and body width. PC-3 loaded with body length and head width. PC-4 loaded with left foot and left forearm length.

There was no main effect of CPF concentration or zooplankton on any of the PCs
describing body shape, but there was a significant interactive effect between CPF concentration and zooplankton population on PC-3 (p=0.010); in other words, the effects of CPF on PC-3 depended on the zooplankton population (Table 2.2). Specifically, metamorphs in mesocosms with CPF-sensitive zooplankton had longer bodies and wider heads than metamorphs in mesocosms with CPF-resistant zooplankton in the absence of CPF but not in the presence of CPF (Figure 2.4).

**DISCUSSION**

The present study represents an important extension of Bendis and Relyea (2016b) who documented important ecological effects of CPF in aquatic communities with either CPF-sensitive or CPF-resistant *Daphnia* zooplankton. We tested for neurodevelopmental and morphological effects of CPF in the amphibians in those aquatic communities. Bendis and Relyea (2016b) found that applying CPF to mesocosms with CPF-sensitive *Daphnia* triggered a trophic cascade: a decrease in zooplankton abundance, an increase in phytoplankton, and a decrease in periphyton compared to control mesocosms without CPF. These CPF-induced cascading trophic events were associated with lower metamorph survival, smaller metamorph mass, and longer time to metamorphosis. In contrast, application of CPF to mesocosms with CPF-resistant *Daphnia* had less of an effect on the trophic community and the amphibians therein (Bendis and Relyea 2016b). As detailed below, we found that CPF directly impacted amphibian brain development, independent of effects on the trophic community. That is, while CPF can impact the trophic community leading to survival effects on the tadpole our results suggest developmental effects on the tadpoles are driven primarily on the direct CPF effects on the tadpoles themselves. With respect to body shape, there was an
interaction between zooplankton population and CPF, partly due to an unexpected effect of zooplankton population on body shape in the absence of CPF.

**Brain morphology**

We found that Leopard Frog metamorphs exposed to as little as 1 μg/L CPF during development had brains that were wider in several dimensions compared to the controls, after adjusting for brain mass. These effects were present regardless of whether animals were reared with CPF-resistant or CPF-sensitive zooplankton, supporting the hypothesis that the effects of CPF on neurodevelopment are due to direct effects of CPF and are independent of pesticide effects on the aquatic community and food availability. Moreover, the concentration tested in the present study (1 μg/L CPF) was lower than previously tested amounts, and yet brain changes still occurred at this extremely low concentration. Ecological values in surface waters when CPF is applied appropriately are less than 10 μg/L and more often around 1 μg/L (Stone et al. 2014). This is relevant because CPF is often found in wetlands at concentrations near or even above 1 μg/L. It is uncertain whether CPF is acting through changes in cholinesterase activity or via other mechanisms. While the degree of cholinesterase inhibition caused by CPF seems to be species specific in anurans, in the Southern Leopard Frog (*L. sphencephala*) there was no cholinesterase inhibition found using the Ellman method when tadpoles were exposed to 10 μg/L CPF or less, and 25% cholinesterase inhibition doesn’t occur until tadpoles were exposed to 55 μg/L CPF (Widder and Bidwell 2008). Thus, the neurodevelopmental changes found in the present study could be caused by mechanisms unrelated to cholinesterase activity.
Our work is consistent with studies showing that amphibian brain development is remarkably sensitive to low concentrations of pesticides as well as biotic factors like conspecific densities and predators (Gonda et al. 2010; Liao et al. 2015; Woodley et al. 2015). We found that exposure to the pesticide CPF resulted in increased widths in the optic tectum (the main region of the brain responsible for vision), the medulla (involved in respiration and auditory function), and the diencephalon (controls homeostasis by regulating the endocrine system; involved in motor function control; acts as a relay center in the brain). In response to biotic factors, plastic brain development is argued to be beneficial, shaping animals to excel in a specific environment. If increased widths are due to neurogenesis, they may be beneficial to the animal, possibly by increasing visual perception or making the animal more adapted to maintaining homeostasis in adverse situations. However, in response to pesticides, it is usually assumed that impacts are maladaptive. If the increased widths found in this study are due to apoptosis and neural swelling, then these brain changes are likely harmful. Enlarged brain regions caused by CPF-exposures in rodents have been shown to be due to perikaryal swelling in the brain (Roy et al. 2004; Roy et al. 2005). In addition, CPF-induced brain changes in rodents include a decrease in cell numbers, neuritic projections and a deficit in synaptic communication (reviewed in Slotkin 2004). In humans with developmental exposure to CPF, MRIs show changes in the size of white matter in numerous brain gyri as well as cortical thinning (Rauh et al. 2012). Behaviorally, humans that were developmentally exposed to CPF had deficits in IQ and learning (Rauh et al. 2012; Butler-Dawson et al. 2016). This leads us to hypothesize that the changes found in this study are likely maladaptive. It is still unclear if the low dose pesticide-induced brain changes we found
impact other traits such as behavior, reproductive success, and fitness. This could be determined by a histological analysis looking for markers of apoptosis (for example using bromodeoxyuridine [BrdU]), or markers of necrosis (Bauer and Patterson 2005). If the changes we found are due to adverse brain changes, then animals developmentally exposed to CPF could have visual and auditory impairments, changes in their motor function, or trouble maintaining the proper homeostasis, especially in stressful situations. Other studies have shown that developmental exposures to CPF reduced tadpole swim speed and reduced fish swim speed, swim distance, and thigmotaxis (Widder and Bidwell 2008; Richendrfer et al. 2012; Khalil et al. 2013; Jin et al. 2015; Shuman-Goodier and Propper 2016).

Our findings in metamorphs result from larval exposure to CPF because animals were removed from the mesocosms prior to metamorphosis. Brain changes that span life history events are not a unique phenomenon. For example, brain changes caused by tadpole crowding affected both tadpole and juvenile Common Frogs (*Rana temporaria*) (Trokovic et al. 2011). In fact, embryonic and early life stage neurodevelopment is especially sensitive to environmental impacts with long-lasting effects across a range of animals (Whitney et al. 1995; Marco et al. 2011). However, this is not always the case. In Woodley et al. (2015), exposure to CPF during the tadpole stage resulted in altered tadpole brain shape but not an altered metamorph brain shape. The differences between the present student and Woodley et al. (2015) may be due to the time course of CPF exposure or the concentration of CPF. In the current study, amphibians were exposed repeatedly to 1 μg/L CPF while it was only a single exposure to 5 μg/L CPF in Woodley et al. (2015).
**Body morphology**

Body shape is sensitive to environmental cues during development in many taxa and can have both functional and fitness-related consequences (Losos 1990; Relyea and Hoverman 2003; Ficetola and De Bernardi 2006; Johansson et al. 2010). For example, exposure to the herbicide RoundUp® (active ingredient: glyphosate) resulted in shorter tadpole bodies and increased tail depth (Relyea 2012; Katzenberger et al. 2014). We found no evidence of an impact of CPF on metamorph body shape in mesocosms with CPF-sensitive zooplankton (Figure 2.4), despite the widespread food web changes caused by CPF. In contrast, metamorphs emerging from mesocosms with CPF-resistant zooplankton had relatively longer bodies and wider heads when exposed to CPF compared to controls that were not exposed to CPF, despite the lack of food-web changes when CPF was added.

The unexpected observation that zooplankton population altered amphibian body length and head width in the absence of CFP was echoed in community-level results reported by Bendis and Relyea (2016b); mesocosms with sensitive zooplankton (and no CPF) had lower periphyton abundance, smaller metamorphs, and longer times to metamorphosis compared to mesocosms with resistant zooplankton. Tanks had screen covers to prevent any odonate predators from entering tanks. Zooplankton abundance was similar across mesocosms unexposed to CPF, so other, more subtle, differences between the populations of zooplankton may account for the effects on the amphibians (Bendis and Relyea 2016b).

The differences in body shape due to zooplankton population may be related to differences in food availability during development, as has been shown elsewhere (Alford
and Harris 1988; Relyea 2001a; Relyea and Hoverman 2003). Wider heads may be a compensatory mechanism for small body mass or reduced food availability (Relyea and Hoverman 2003; Gomez-Mestre et al. 2010; Stoler and Relyea 2013). Wider heads and gapes could help small metamorphs consume a larger range of prey sizes and grow faster (Toft 1980; Emerson et al. 1994). This is supported by previous work showing that animals compensate for food deprivation with catch-up growth during the juvenile stage (Boone 2005).

Conclusions

The present study provides evidence of the unexpected impacts of exposure to low, ecologically relevant doses of organophosphorous pesticides on neurodevelopment in vertebrates. We demonstrated that ecologically relevant concentrations of an insecticide can have direct effects on brain development that can persist through metamorphosis and possibly impact organisms after they have left the contaminated aquatic habitat. To better understand the effects of insecticides on brain development, more work needs to be done to determine if these pesticide-induced brain changes are affecting behavior, reproductive success, and fitness.

ACKNOWLEDGEMENTS

All work was conducted by Sara J McClelland with the following exceptions. Rick A. Relyea, R.J. Bendis and their team conducted the live-animal component of the mesocosm experiments. This work was partially funded by University of Pittsburgh’s G. Murray McKinley Research Fund (RJB), the Society for Freshwater Science (RJB), and the National Science Foundation (RAR).
Table 2.1. Principal components analysis of 9 mass-adjusted brain dimensions of Northern Leopard Frog metamorphs

<table>
<thead>
<tr>
<th>Results of PCA</th>
<th>Principal Component</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PC-1</td>
</tr>
<tr>
<td>% of Variance</td>
<td>46.2</td>
</tr>
<tr>
<td>Eigenvalue</td>
<td>4.2</td>
</tr>
<tr>
<td>Factor Loading</td>
<td></td>
</tr>
<tr>
<td>Telencephalon width</td>
<td>0.439</td>
</tr>
<tr>
<td>Telencephalon length</td>
<td>0.266</td>
</tr>
<tr>
<td>Optic tectum width</td>
<td>0.902</td>
</tr>
<tr>
<td>Optic tectum length</td>
<td>-0.027</td>
</tr>
<tr>
<td>Medulla length</td>
<td>0.337</td>
</tr>
<tr>
<td>Diencephalon width</td>
<td>0.892</td>
</tr>
<tr>
<td>Diencephalon length</td>
<td>0.329</td>
</tr>
<tr>
<td>Olfactory bulb length</td>
<td>0.273</td>
</tr>
<tr>
<td>Medulla width</td>
<td>0.744</td>
</tr>
</tbody>
</table>

PCA = Principal Components Analysis; PC = Principal Component
Table 2.2. Results of univariate tests for each principal component\(^a\)

<table>
<thead>
<tr>
<th>Dependent variable</th>
<th>Factors</th>
<th>Pesticide (CPF)</th>
<th>Zooplankton</th>
<th>CPF x Zooplankton</th>
</tr>
</thead>
<tbody>
<tr>
<td>Brain Morphology</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PC-1</td>
<td>(F_{(1,15)}=7.515, p=0.015^*)</td>
<td>(F_{(1,15)}=0.168, p=0.688)</td>
<td>(F_{(1,15)}=1.763, p=0.204)</td>
<td></td>
</tr>
<tr>
<td>PC-2</td>
<td>(F_{(1,15)}=0.322, p=0.579)</td>
<td>(F_{(1,15)}=2.158, p=0.163)</td>
<td>(F_{(1,15)}=1.417, p=0.252)</td>
<td></td>
</tr>
<tr>
<td>PC-3</td>
<td>(F_{(1,15)}=0.718, p=0.410)</td>
<td>(F_{(1,15)}=0.005, p=0.947)</td>
<td>(F_{(1,15)}=0.868, p=0.366)</td>
<td></td>
</tr>
<tr>
<td>Body Morphology</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PC-1</td>
<td>(F_{(1,15)}=0.384, p=0.545)</td>
<td>(F_{(1,15)}=2.083, p=0.169)</td>
<td>(F_{(1,15)}=3.265, p=0.091)</td>
<td></td>
</tr>
<tr>
<td>PC-2</td>
<td>(F_{(1,15)}=0.475, p=0.501)</td>
<td>(F_{(1,15)}=0.018, p=0.896)</td>
<td>(F_{(1,15)}=0.123, p=0.731)</td>
<td></td>
</tr>
<tr>
<td>PC-3</td>
<td>(F_{(1,15)}=1.240, p=0.283)</td>
<td>(F_{(1,15)}=1.468, p=0.244)</td>
<td>(F_{(1,15)}=8.817, p=0.010^*)</td>
<td></td>
</tr>
<tr>
<td>PC-4</td>
<td>(F_{(1,15)}=0.170, p=0.686)</td>
<td>(F_{(1,15)}=1.573, p=0.229)</td>
<td>(F_{(1,15)}=0.369, p=0.553)</td>
<td></td>
</tr>
</tbody>
</table>

\(^a\) Principal components describe mass-adjusted body morphology or mass-adjusted brain morphology for Northern Leopard Frog metamorphs (n = 5 mesocosms per treatment combination except for 1 μg/L CPF, sensitive *Daphnia* where n = 4 mesocosms).

\^* \ p < 0.05

CPF = chlorpyrifos; PC = principal component
Table 2.3. Principal components analysis of 15 mass-adjusted body dimensions of Northern Leopard Frog metamorphs

<table>
<thead>
<tr>
<th>Results of PCA</th>
<th>Factors</th>
</tr>
</thead>
<tbody>
<tr>
<td>% of Variance</td>
<td>PC-1</td>
</tr>
<tr>
<td>Eigenvalue</td>
<td>49.5</td>
</tr>
<tr>
<td>Factor Loading</td>
<td>7.4</td>
</tr>
<tr>
<td>body length</td>
<td>0.297</td>
</tr>
<tr>
<td>head width</td>
<td>0.030</td>
</tr>
<tr>
<td>body width</td>
<td>-0.193</td>
</tr>
<tr>
<td>left forelimb length</td>
<td>0.034</td>
</tr>
<tr>
<td>left forelimb width</td>
<td>0.356</td>
</tr>
<tr>
<td>left thigh length</td>
<td>0.834</td>
</tr>
<tr>
<td>left thigh width</td>
<td>0.322</td>
</tr>
<tr>
<td>left leg length</td>
<td>0.790</td>
</tr>
<tr>
<td>left foot length</td>
<td>0.515</td>
</tr>
<tr>
<td>right forelimb length</td>
<td>0.696</td>
</tr>
<tr>
<td>right forelimb width</td>
<td>0.172</td>
</tr>
<tr>
<td>right thigh length</td>
<td>0.842</td>
</tr>
<tr>
<td>right thigh width</td>
<td>0.396</td>
</tr>
<tr>
<td>right leg length</td>
<td>0.769</td>
</tr>
<tr>
<td>right foot length</td>
<td>0.768</td>
</tr>
</tbody>
</table>

PCA = Principal Components Analysis; PC = Principal Component
Figure 2.1. Experimental Design for Chapter 2. Tadpoles were exposed to CPF during development in mesocosms with either CPF-sensitive *Daphnia pulex* or CPF-resistant *Daphnia pulex*. Once forelimbs emerged frogs were removed from treatments. Although there were 6 mesocosms per treatment, a few preserved samples were lost due to drying, resulting in 5 mesocosms per treatment (except for the 1 μg/L CPF-sensitive zooplankton group where there were only 4 mesocosms). Within each mesocosm, 10 metamorphs were measured. In 5 of the mesocosms, fewer than 10 metamorphs survived to metamorphosis; from mesocosms containing 0 μg/L CPF with CPF-sensitive *D. pulex*, 3 of the mesocosm averages were based on 4, 8, and 8 metamorphs, from the mesocosms containing 1 μg/L CPF with CPF-sensitive *D. pulex* 1 mesocosm average was based on 5 metamorphs, and from mesocosms containing 1 μg/L CPF with CPF-resistant *D. pulex* 1 mesocosm average was based on 7 metamorphs.

<table>
<thead>
<tr>
<th>CPF</th>
<th><em>Daphnia pulex</em></th>
<th># of Mesocosms</th>
<th># Tadpoles/ Mesocosms</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 μg/L</td>
<td>Sensitive</td>
<td>5</td>
<td>10, 10, 8, 8, 4</td>
</tr>
<tr>
<td>0 μg/L</td>
<td>Resistant</td>
<td>5</td>
<td>10, 10, 10, 10, 10</td>
</tr>
<tr>
<td>1 μg/L</td>
<td>Sensitive</td>
<td>4</td>
<td>10, 10, 10, 10, 5</td>
</tr>
<tr>
<td>1 μg/L</td>
<td>Resistant</td>
<td>5</td>
<td>10, 10, 10, 10, 7</td>
</tr>
</tbody>
</table>
Figure 2.2. Metamorph Body and Brain Morphology (A) Northern Leopard Frog metamorph showing the linear dimensions used to describe body morphology: 1 body length, 2 head width, 3 body width, 4 arm width, 5 arm length, 6 thigh width, 7 thigh length, 8 leg length, 9 foot length; (B) Dorsal and ventral view of a Northern Leopard Frog metamorph brain showing the linear dimensions used to describe brain morphology: 1 telencephalon length, 2 telencephalon width, 3 optic tectum length, 4 optic tectum width, 5 medulla length, 6 olfactory bulb length, 7 diencephalon length, 8 diencephalon width (which is more posterior than the end of the telencephalon), 9 medulla width.
Figure 2.3. Relative metamorph brain shape (PC-1) when exposed to 0 or 1μg/L chlorpyrifos (CPF) in mesocosms with zooplankton (*Daphnia pulex*) that were either sensitive or resistant to CPF. Mean +/- SEM are graphed. There was an effect of CPF concentration (p = 0.015) but no effect of zooplankton population (p = 0.688) or interaction between CPF and zooplankton population (p = 0.204) on relative brain shape. (n = 5 mesocosms except for the treatment combination of 1μg/L CPF plus sensitive zooplankton where n = 4 mesocosms)
Figure 2.4. Relative metamorph body shape (PC-3) when exposed to 0 or 1μg/L chlorpyrifos (CPF) in mesocosms with zooplankton (*Daphnia pulex*) that were either sensitive or resistant to CPF. Mean +/- SEM is graphed. There was an interactive effect between zooplankton and CPF concentration (*p* = 0.010), but no main effect of zooplankton population (*p* = 0.244) or CPF (*p* = 0.283) on relative body shape. (*n* = 5 mesocosms except for the treatment combination of 1μg/L CPF plus sensitive zooplankton where *n* = 4 mesocosms)
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Bendis RJ, Relyea RA. 2016a. If you see one, have you seen them all?: Community-wide effects of insecticide cross-resistance in zooplankton populations near and far from agriculture. Environ Pollut 215:234-246.


Chapter 3

Investigate the neuroanatomical and behavioral changes of animals that are exposed to sublethal doses of the pesticide chlorpyrifos in a controlled laboratory setting

ABSTRACT

Due to the ramifications of pesticide exposure, regulations are intended to keep concentrations of pesticides in nature low enough to have no observable effects on non-target organisms. However, the question remains whether these low concentrations of pesticides are safe for non-target organisms. To better understand how ecologically relevant concentrations of pesticides may be affecting vertebrate organisms, we exposed an amphibian model, the Northern Leopard Frog (*Lithobates pipiens*), to 0, 1, or 10 μg/L of the organophosphorous pesticide chlorpyrifos during development in a controlled laboratory study (chlorpyrifos most commonly contaminates natural habitats at concentrations less than 10 μg/L). We then measured standard body and brain morphometrics, behavior, and corticosterone concentration in both tadpoles and metamorphs. We found that Northern Leopard Frog tadpoles exposed to very low, putatively safe concentrations of the organophosphorous pesticide CPF had altered brain morphology and behavior compared to control animals. Tadpoles exposed to 1 μg/L CPF, but not 10 μg/L CPF, had changes in their relative brain mass, relative brain shape (wider and longer telencephala and longer olfactory bulbs), behavior, body length, and body condition compared to controls. Tadpoles exposed to 10 μg/L CPF had altered corticosterone concentrations, behavior, body length, and body condition compared to controls. There was no effect of CPF on tadpole developmental stage or body mass. After undergoing metamorphosis, only the effects on brain morphology persisted in tadpoles.
that had been exposed to 1 μg/L CPF during development. The present study provides evidence of neurodevelopmental effects of CPF that carry over to multiple life history changes. We also found behavioral and neuroendocrine effects during the larval stage of development. Both the neurodevelopmental and behavioral effects occurred in a nonmonotonic dose response adding to the growing number of nonmonotonic biological effects displayed in animals exposed to low doses of CPF. This work provides evidence that brain morphology, behavior, and corticosterone concentrations could make useful endpoints when studying animal responses to low dose organophosphate contamination. However, due to the nonmonotonic manner in which CPF has the potential to impact animals, scientists and regulatory agencies need to act with caution when making conclusions about lowest observed adverse effect levels (LOAEL) and no observed adverse effect levels (NOAEL).

INTRODUCTION

Over one billion pounds of pesticides are applied every year in the United States (Atwood and Paisley-Jones 2017). This extensive use of pesticides in human societies has enabled us to increase crop yields and prevent vector-borne diseases. In the coming years, humanity will require increased farm productivity to feed a growing population and the increase in disease vectors resulting from climate change will require an intensification of our efforts to control these populations (Smith et al. 2010; Field 2014). These factors will likely increase humanity’s reliance on pesticides (Delcour et al. 2015). While there are certain benefits to using pesticides, these chemicals function by interfering with biological systems to cause lethality. Due to the conservation of biological pathways, the effects of pesticides are not constrained to only pests. With our increased reliance on
pesticides, it is imperative that we determine how pesticides are impacting the health of humans, wildlife, and ecosystems.

For decades, the medical and scientific communities have been trying to address this issue by studying the effects of acute pesticide exposures in non-target organisms, which can cause extreme toxicity and lethal effects in a range of taxa (Fleischli et al. 2004; Liendro et al. 2015; Jayaraj et al. 2016). Due to the knowledge gained from these studies, companies have developed pesticides that are less persistent and regulations that require a more cautious approach to pesticide application. However, these changes have resulted in low-dose chronic exposures becoming more of a concern. These low-dose exposures are less likely to cause notice because their effects are often sublethal, which may result in the effects not being observed or if they are observed may be difficult to link to chemical exposures (Vyas 1999; Köhler and Triebeskorn 2013). Understanding the biological changes that are caused by low-dose pesticide exposures, can help us monitor populations for sublethal effects of low dose pesticide exposure, and provide insights into better treatments, management, and conservation strategies moving forward.

There are multiple classes of pesticides currently being used that are categorized based on their mode of action. Insecticides are heavily used to target both agricultural pests and insect vectors. Organophosphates are the most commonly used insecticides in the United States, and have replaced the more environmentally persistent organochlorines (Atwood and Paisley-Jones 2017). However, even though organophosphates break down more quickly than organochlorines, they are still detected in numerous water samples in North America and are found on produce sold for human consumption (Canada 2011; EPA 2011; Stone et al. 2014).
Organophosphates function mainly through the irreversible inactivation of acetylcholinesterase. Upon exposure to high concentrations, there is a buildup of the neurotransmitter acetylcholine that causes continued activation of the nervous system across a variety of taxa, which can result in mild symptoms like salivation and muscle twitching to more extreme consequences such as convulsion and death (Slotkin 2004). At lower doses, organophosphates can cause neurological deficits through a multitude of other mechanisms that are less well understood (Slotkin 2004).

Developing organisms are particularly sensitive to organophosphates due to the brain's sensitivity to neuroactive chemicals, especially when the neurotransmitter acetylcholine is affected, as it plays many developmental roles in proper brain formation (Yanai et al. 2002). Developmental exposure to low, sublethal concentrations can cause neural abnormalities and changes in brain morphology, making organophosphate exposures especially dangerous for larval and fetal life stages (Ostrea Jr et al. 2002; Qiao et al. 2004; Roy et al. 2004; Roy et al. 2005; Rauh et al. 2012). In addition to causing changes in brain development, organophosphate exposure during development also affects intellectual capacity and behavior. Impacts on activity, learning, and memory have been observed in fish, amphibians, and rodents (Levin et al. 2002; Timofeeva et al. 2008; Khalil et al. 2013; Shuman-Goodier and Propper 2016). In children that were exposed prenatally to the organophosphate chlorpyrifos, impairments in motor function, IQ, perceptual reasoning, and working memory have all been documented (Bouchard et al. 2011; Engel et al. 2011; Rauh et al. 2011; Rauh et al. 2012). Some have even hypothesized that one of the reasons for the increased number of children born with neurobehavioral impairments, such as autism spectrum disorder, may be due to pesticide
exposures during development (Rauh et al. 2006; Bouchard et al. 2010; Visser et al. 2010).

Numerous studies have found evidence of the neurodevelopmental effects caused by exposure to organophosphates, but most focused on either the anatomical effects or the behavioral effects caused by exposure, and most were limited to one life history stage. This limits our understanding of the link between neuroanatomical changes and functional behavior changes that occur over the lifetime of an organism in response to organophosphate exposure. In a seminal paper in children, exposures of at least 4.39 pg/g of organophosphates in utero were correlated with both morphological brain effects and behavioral effects at the ages of 5.9-11.2 years (Rauh et al. 2012). Human studies, while exceedingly important, suffer from the inability to conduct controlled causal experiments. In addition, this study, and many of the others testing the effects of organophosphates, analyzed the effects of doses that, while realistic, are still uncommonly high. More work is needed to determine how the lowest, most commonly encountered doses affect neurodevelopment and behavior. The most commonly encountered concentrations in surface waters are around 1 ug/L. It is critical to know whether being exposed to these concentrations is safe.

To further explore the impact of how these extremely low, and more common, doses of organophosphates impact organismal development and physiology, we used the Northern Leopard Frog (Lithobates [formerly Rana] pipiens) as a model organism. Amphibians are an ideal model for testing the neurodevelopmental effects of pesticides because they have often been used as a model for vertebrate development providing an abundance of background information on their developmental processes, they are easy to
obtain and maintain in laboratory conditions, it is easy to control the timing of exposure during development, and maternal effects are minimal. Further, in the wild, amphibians are often exposed to contaminated water sources during development, which encompasses the life stages that are most sensitive to organophosphate exposures (Slotkin 2004). Since amphibians are experiencing massive population declines, understanding their vulnerability to pesticides is especially pressing (List 2010).

In this study, we wanted to determine if the neurodevelopmental effects caused by organophosphates had any functional impacts on the behavior of the animals and on their stress levels throughout multiple life stages. To meet these aims, we exposed tadpoles to either a vehicle control or to one of two ecologically relevant doses of an organophosphate and analyzed their effects on tadpoles and metamorphs (animals that had recently finished undergoing metamorphosis). We hypothesized that animals exposed to the common organophosphate chlorpyrifos would have neurodevelopmental impacts and predicted that evidence of these impacts would be revealed through changes in brain morphology, behavior, and increased stress levels, with the animals exposed to the higher dose showing more exaggerated effects.

METHODS

Animal Care

Eggs were obtained from Nasco (Fort Atkinson, WI). According to the technicians at Nasco, four females and eight males were given pituitary hormone injections to produce the fertilized eggs (personal communication). These eggs were received in masses of 100 and were distributed evenly among the treatments. Once received, eggs were kept at 9°C for 13 days, the temperature was then raised
approximately 1°C daily for 9 days, until hatching began. Hatched tadpoles were moved to room temperature and kept at 22-24°C with a 14hr light:10hr dark cycle throughout the remainder of the experiment. Aquaria contained carbon and UV-filtered well water, and a bubble stone within a rudimentary filter to aerate the water. Tadpoles were fed a gel food mix (made with 2.25 g agar 12 g of TetraMin tropical tablets (Spectrum Brands Pet, LLC, Blacksburg, VA) ground up and boiled with 90-100 mL water) ad libitum (adjusted based on personal communication with Michael Benard). Partial water changes were done twice a week and particulates were removed from the bin as needed.

Once forearms emerged (Gosner stage 42) animals were placed into terrariums that were tilted with water covering approximately half the bin and wet, unbleached paper towels covering the ground with additional wet, crumpled paper towels forming a refuge for the animals (Gosner 1960). Once metamorphosis was complete, metamorphs were fed waxworms until the tail was completely gone.

The Duquesne University and University of Pittsburgh Institutional Animal Care and Use Committee approved this experiment (Duquesne permit # 1602-02; University of Pittsburgh permit # 16037940).

**Treatments**

Tadpoles at Gosner stage 24 were haphazardly assigned to aquaria (Gosner 1960). After 24 hours, survival was 100%, and all aquaria were treated (day 1; see Figure 3.1). Tadpoles were exposed to vehicle controls, a low dose of 1 μg/L CPF the organophosphate chlorpyrifos (CPF; Chem Service, Inc., West Chester, PA), and a higher, but still relatively low, dose of 10 μg/L CPF. Vehicle controls consisted of a 0.04% ethanol solution; the same concentration of ethanol used to dilute CPF. These
doses of CPF are ecologically relevant, sublethal, and did not cause changes in acetylcholinesterase activity using the Ellman method in Southern Leopard Frogs (the closest species analyzed for acetylcholinesterase activity) (Widder and Bidwell 2008). A water sample from each treatment was taken from bins at the start of the experiment and sent to an outside laboratory (AG Services Lab, University of Georgia, Athens, GA) for confirmation of CPF concentration, which found that the actual concentrations were: 0 μg/L CPF, 0.25 μg/L CPF, 10.9 μg/L CPF (detection level 0.05 μg/L). In the rest of this chapter, nominal concentrations will be referred to. All water was treated on day one of the experiment. Clean, treated water was kept in covered cattle tanks that blocked all light and was used for all tadpole water changes for approximately 7 weeks (Figure 3.1).

**Tadpole water borne hormone sample collection and behavior**

At days 40-43, one tadpole from each bin was randomly selected (selector visually assigned numbers to each tadpole in the bin, assistant chose a number using a random number generator, and selector used a soft net to capture specific tadpole), was rinsed with untreated water, and placed into one liter of untreated water by itself. The tadpole was held in this container for 12 hours then transferred to the center of the behavior arena, which was identical to their home container. Fifty milliliters of water from their holding container was collected and frozen at -20°C for later analysis of water borne corticosterone (Gabor et al. 2013). Tadpoles had one hour to acclimate to the behavior arena. They were then recorded for twenty minutes to allow analysis of baseline behaviors (Figure 3.3). Recordings were analyzed using ToxTrac behavior software for time inactive, distance travelled, time that tadpoles spent in the center of the arena, speed while tadpoles were active, acceleration, and exploration of the arena, which was
quantified by determining how many areas of the arena were visited (Rodriguez et al. 2018).

Immediately after the baseline behavior recording cameras were turned off and visual experiments were set up (Figure 3.3). Recordings were stopped between each assay, during which the visual cues were added or changed. To set up the visual assays, a clean, sealed, glass container was placed on one side of the arena; the container had one of the following: water, water and an Aeshnidae dragonfly larvae (a natural predator that is about twice as long as the tadpoles, though narrower than the tadpoles in body width), or water and a tadpole. Tadpoles were recorded for twenty minutes. Recordings were analyzed using ToxTrac behavior software for time spent in each quadrant of the arena, time inactive, distance travelled, speed while tadpoles were active, acceleration, and exploration of the arena.

The same day as the behavior experiments, olfactory cues were prepared. To prepare the olfactory cues tadpoles that were not a part of this experiment were fed to aeshnidae dragonfly larvae, a natural predator of Northern Leopard Frog tadpoles in the wild. Tadpoles respond more readily to olfactory cues when predators are fed conspecifics (Wilson and Lefcort 1993). After feeding, the dragonfly larvae were removed, and the water was filtered through grade 1 Whatman filters to remove any particulate matter from the water. One hundred mL of this water, containing both predatory cues (kairomones) and conspecific alarm pheromones, was placed in disposable cups for quick deliverance during the olfactory behavior assay. Water with olfactory cues was steadily added to one side of each arena over a 5-10 second time span. The side that the cue was added to alternated, and there was no difference in the side that
the cue was added to among treatments. Immediately after the water was added to the arena, a video recording was started and recorded for 10 minutes to analyze acute tadpole responses to olfactory cues. Recordings were analyzed using ToxTrac behavior software for time inactive, distance travelled, time that tadpoles spent in the center of the arena, speed while tadpoles were active, acceleration, and exploration of the arena (Rodriguez et al. 2018).

Metamorph water borne hormone sample collection and behavior

Once metamorphosis was complete, days 69-83, each animal was individually assessed for water borne corticosterone concentrations and behavior. Metamorphs were quickly and gently picked up in a gloved hand and placed into a beaker containing 45 milliliters of water for one hour, and then transferred to the center of the behavior arena. Water was then transferred to a conical tube and frozen at -20°C for later analysis of water borne corticosterone. Behavior arenas were the same type of container as the home terrarium but were flat and did not contain water. Metamorphs had one hour to acclimate to the behavior arena. They were then recorded for twenty minutes to allow analysis of baseline behaviors (Figure 3.3). Recordings were analyzed using ToxTrac behavior software for time inactive, distance travelled, time that metamorphs spent in the center of the arena, speed while metamorphs were active, acceleration, and exploration of the arena (Rodriguez et al. 2018).

Immediately after the baseline behavior recording cameras were turned off and visual experiments were set up (Figure 3.3). Recordings were stopped between each assay, during which the visual cues were added or changed. To set up the visual assays, a clean, sealed, glass container that was either empty or contained four waxworms (prey
that the metamorphs had previously been fed on) was placed on one side of the arena. Metamorphs were recorded for twenty minutes. Recordings were analyzed manually for the time it took animals to face the jar and the number of lunges the metamorphs made towards the jar.

The same day as the behavior experiments, the olfactory cues were prepared. Two grams of ground waxworms were mixed with 40mL of this water and filtered through grade 1 Whatman filters. Unbleached paper towels were then saturated with 10mL of either the olfactory cue or water only. One paper towel was added to each side of the arena, with one side containing the paper towel saturated with water and the other side containing the paper towel saturated with the food olfactory cue (Figure 3.3). The side of the arena (left vs. right) that the cue was added to alternated, and there was no difference in the side that the cue was added to among treatments. Immediately after the paper towels were added, a video recording was started and recorded for 20 minutes to analyze responses to food cues. Recordings were analyzed manually for the time they spent in each zone of the arena (paper towel with food cue, center, or paper towel with water), the amount of time they spent in the zone they were in when the recording was started (starting position), and the number of times they travelled between these zones.

**Tadpole and Metamorph Body and Brain Morphology**

Either immediately after (metamorphs) or the day following (tadpoles) behavioral assays, animals were euthanized by an overdose of 0.2% MS222, weighed, and fixed in 10% phosphate-buffered formalin for later analysis of developmental stage (tadpoles), body and brain morphology.

To assess changes in body morphology, animals were photographed and body
dimensions were measured using Image J (Figure 3.2; US National Institutes of Health, Bethesda, MD). Brains were dissected out, cranial nerves were trimmed, brains were weighed, and the dorsal and ventral surfaces were each photographed 3 times with moving and repositioning the brain independently each time to produce 3 dorsal images per brain and 3 ventral images per brain. Brain dimensions were then measured (Figure 3.2; US National Institutes of Health, Bethesda, MD). Each linear dimension was measured once from each of the 3 images and averaged resulting in 3 measures being averaged to get a single estimate for each brain dimension for each individual animal.

**Statistical analysis: Corticosterone**

SPSS was used for all statistical analyses in this study. Water samples were sent to an outside lab for solid-phase extraction and radio-immuno assay analysis to determine the concentration of corticosterone (Oregon National Primate Research Center Endocrine Lab, Beavertown, OR; CORT detection levels of 1 pg/mL). Control samples had low to non-detectable concentrations of corticosterone. Corticosterone concentrations were linearly related to body mass, which might impact the rate of hormones passing through the body to the water (Gabor et al. 2013). This was done by conducting an ANCOVA with CORT concentration as a fixed effect and either body mass as a covariate. Data were adjusted for body mass by adding the residual value for each animal to the overall estimated marginal means (EMM) generated by the ANCOVA (see Appendix 1). By adding residuals to the EMM (instead of just using residuals), we get values that are more biologically meaningful and thus easier to interpret. For tadpoles, these values were log transformed to achieve homoscedasticity, and then analyzed with analysis of variance. For metamorphs, these values were log transformed, but transformations did not solve the
problem of heteroscedasticity; untransformed data were analyzed using both analysis of variance and Kruskal-Wallis tests. There was no difference in conclusions derived from the two different statistical analyses. As analysis of variance is robust against violations of assumptions, results from the analysis of variance are reported in the text. Untransformed data are plotted in the figures to aid visualization.

**Statistical analysis: Behavior**

Due to the relatively large number of behavior variables, and their relatedness, a principal component analysis (PCA) was conducted (Icenogle et al. 2004). Three PCA were conducted: 1) tadpole baseline and tadpole olfactory data, 2) tadpole visual data, and 3) all metamorph behavior data. Tadpole baseline and olfactory data were conducted as one PCA because the arenas were the same in each of these assays and behavioral responses were highly correlated. Tadpole visual assays were conducted as a separate PCA because the addition of the glass jar changed the dimensions of the arena. Behavior data for each analysis satisfied the requirements of PCA (KMO>0.5 and Bartlett’s test ≤ 0.05). The PCA converted the correlated behavioral variables into uncorrelated principal components (PCs) using a varimax rotation. PCs with eigenvalue > 1, normal distributions, and equal variances were analyzed using ANOVAs.

**Statistical analysis: Morphology**

Animals varied naturally in body mass. Thus, it was necessary to correct the linear body and brain measurements for differences in body mass before analyzing for treatment effects. For each linear measurement, we conducted an ANCOVA with treatment as a fixed effect and either body mass as a covariate. If necessary, values were log-transformed to achieve linearity and homogeneity of slopes. Data were adjusted for
body mass by adding the residual value obtained from regressing the linear measurement on body mass) for each animal to the overall estimated marginal means (EMM) generated by the ANCOVA. By adding residuals to the EMM (instead of just using residuals), we get values that are more intuitive and thus easier to interpret.

Next, we did a PCA on the linear dimensions describing brain or body morphology to reduce the number of correlated variables. Before conducting PCA we confirmed that the assumptions of PCA (KMO > 0.5 and Bartlett’s test ≤ 0.05) were met. The PCA converted correlated morphological variables into uncorrelated principal components (PCs) using a varimax rotation.

Finally, we tested for treatment effects using ANOVAs. Dependent variables were PCs with eigenvalue > 0.97 for tadpoles or > 1 for metamorphs that were normally distributed with equal variances (data were transformed when necessary to meet these requirements).

**Statistical analysis: Body Condition**

We estimated body condition factor (CF) using the regression line of body mass versus body length in both tadpoles and metamorphs (Brodeur et al. 2011; Hegde and Krishnamurthy 2014). Body length was used as a measure of body size because it can be consistently and accurately measured. If the relationship between body mass and body length is linear, then the equation of the line can be used to calculate the average body mass for a specific body length. Using the residuals between the observed body mass and the calculated body mass is a common method for determining the body condition of each individual. If an individual has a positive residual (their observed body mass is greater than the calculated body mass), then they are considered to have a good body
condition (Schulte-Hostedde et al. 2005). If an individual has a negative residual (their observed body mass is less than the calculated body mass), then they are considered to have a poor body condition (Schulte-Hostedde et al. 2005). In this study, untransformed data was normally distributed with equal variances, and the residuals that were obtained from this method remained consistent with increasing body mass (i.e. the residuals did not tend to increase with body size), therefore untransformed data was used in determining body condition (Schulte-Hostedde et al. 2005).

Some previous studies that have analyzed body condition between different treatment groups have used only the data from the control treatment to determine a “healthy condition” regression line (Brodeur et al. 2011; Hegde and Krishnamurthy 2014). Other studies use all the data to determine the regression line. We analyzed body condition in two ways: 1) using the data from the control treatment to determine the regression line for all treatments, and 2) using all data from all of the treatments to determine the regression line. The conclusions from these analyses were the same. Here we present the findings that were obtained from using the data from all of the treatments to create the regression line. Body condition factor scores were then analyzed using ANOVAs and Student-Newman-Keuls post-hoc tests when appropriate.

RESULTS

Tadpole Morphology and Body Condition

Relative brain mass differed among treatments in tadpoles, with tadpoles that had been exposed to 1 μg/L CPF having heavier brains than other treatments (Figure 3.2; F(2, 41) = 4.89 p = 0.013).
As shown by the PCA, the nine different body mass-adjusted brain dimensions reduced to three principal components (PCs) (Supplementary Table 3.1). Of the three principal components, only PC-2 differed among treatments (Table 3.1; Figure 3.8). PC-2 encompassed telencephalon length, telencephalon width, and olfactory bulb width. Tadpoles that developed in the presence of 1 μg/L CPF had an increase in the relative dimensions represented by PC-2 (Figure 3.4; F(2, 41) = 4.23, p = 0.021) when compared to controls. There was no difference in PC-2 between controls and tadpoles exposed to 10 μg/L CPF.

Exposure to CPF did not affect tadpole developmental stage (Figure 3.7; 33.49 ± 0.23, mean ± SEM Gosner stage; F(2, 42) = 0.14; p = 0.867) or body mass (Figure 3.7; 1.39g ± 0.06 mean ± SEM body mass; F(2, 42) = 1.67; p = 0.201). However, a multivariate analysis of body shape analyzing the seven tadpole relative body dimensions found an effect of treatment (Table 3.1; F(14, 72) = 2.02; p = 0.028). Further analysis found that tadpoles exposed to either 1 μg/L CPF or 10 μg/L CPF had longer bodies than controls (Figure 3.4; F(2, 41) = 9.67; p < 0.001).

Tadpoles exposed to either 1 μg/L CPF or 10 μg/L CPF had poorer body conditions than controls (Figure 3.6; F(2, 41) = 8.05; p = 0.001).

**Tadpole Corticosterone**

Exposure to CPF affected waterborne corticosterone concentrations; tadpole exposed to 10 μg/L CPF had increased corticosterone concentrations (Figure 3.4; F(2, 41) = 3.72; p = 0.033).

**Tadpole Behavior**
The 14 variables pertaining to baseline and olfactory behaviors reduced to four principal components (Table 3.2). The baseline variables loaded on to either PC-1 or PC-3, while the variables measured after exposure to the olfactory cues loaded on to either PC-2 or PC-4 (Table 3.2). PC-4 differed among treatments (Figure 3.5; $F_{(2,42)} = 3.49; p = 0.040$); upon exposure to the olfactory cue, tadpoles exposed to 10 μg/L CPF spent more time in the center and explored more than controls or tadpoles exposed to 1 μg/L CPF.

Eighteen behavioral variables from three separate visual assays reduced to five PCs (Table 3.2). The time tadpoles spent near the novel stimulus of an empty jar in the behavior arena loaded on to PC-5 (Table 3.7). PC-5 differed among treatments (Figure 3.5; $F_{(2,42)} = 3.97; p = 0.026$); tadpoles exposed to 1 μg/L CPF spent more time near the novel stimulus than other treatment groups.

**Metamorph Morphology and Corticosterone**

CPF exposure had no effect on body mass ($F_{(2,40)} = 0.12; p = 0.888$), relative brain mass ($F_{(2,40)} = 0.64; p = 0.532$), or time to metamorphosis ($F_{(2,40)} = 1.03; p = 0.365$) (Figure 3.10).

A PCA of metamorph brain shape reduced nine body mass-adjusted brain dimensions to four principal components (Table 3.8). Of the four principal components, only PC-1 differed among treatments (Table 3.2; $F_{(2,40)} = 3.31, p = 0.047$). PC-1 loaded strongly for optic tectum length, optic tectum width, and medulla length (Table 3.8). Metamorphs that developed in the presence of 1 μg/L CPF had changes in the relative dimensions represented by PC-1, with increases in optic tectum length and width and decreases in medulla length when compared to controls; there was no difference in PC-1 between controls and tadpoles exposed to 10 μg/L CPF (Figure 3.6).
A PCA of metamorph body shape reduced fifteen body mass-adjusted dimensions to four principal components (Table 3.9). Of the four principal components, only PC-4 differed among treatments (Table 3.2). PC-4 loaded strongly for arm width. Metamorphs that developed in the presence of 10 μg/L CPF had wider arms than metamorphs that developed exposed to 1 μg/L CPF (Figure 3.6; \( F_{(2, 40)} = 3.72, p = 0.033 \)); there was no difference in PC-4 between controls and tadpoles exposed to CPF.

CPF exposure had no effect on metamorph body condition (Figure 3.6; \( F_{(2, 41)} = 1.53, p = 0.230 \)) or waterborne corticosterone concentrations (Figure 3.6; \( F_{(2, 12)} = 1.35, p = 0.296 \)).

**Metamorph Behavior**

Eleven variables from four metamorph behavioral assays reduced to four principal components (Table 3.10). There was no effect of treatment on any of the principal components describing metamorph behavior (Table 3.4; Figure 3.12).

**DISCUSSION**

We found that Northern Leopard Frog tadpoles exposed to very low, putatively safe concentrations (1 or 10 μg/L) of the organophosphorous pesticide CPF during larval development had altered brain morphology and behavior compared to control animals. Tadpoles exposed to 1 μg/L CPF, but not 10 μg/L CPF, had changes in their relative brain mass (after adjusting for body mass), relative brain shape (after adjusting for body mass), behavior, body length, and body condition compared to controls. Tadpoles exposed to 10 μg/L CPF during larval development had altered corticosterone concentrations, behavior, body length, and body condition compared to controls. There was no effect of CPF on tadpole developmental stage or body mass. After undergoing metamorphosis, only the
effects on brain morphology persisted in tadpoles that had been exposed to 1 μg/L CPF during development. These results are discussed in more detail below.

**Tadpole Brain Morphology**

Because larger animals have larger brains, and we were interested in the relative brain changes that occurred due to pesticide exposures, we controlled all brain measurements for body size. After controlling for body mass, we found that tadpoles exposed to 1 μg/L CPF had brains that were relatively heavier than controls. These changes in brain mass were associated with changes in tadpole brain shape. Tadpoles exposed to 1 μg/L CPF had brains that were relatively larger in several dimensions (telencephalon width, telencephalon length, and olfactory bulb length). The increase in the relative size of the telencephalon and olfactory bulb are likely the cause of the increased brain mass in tadpoles exposed to 1 μg/L CPF.

Vertebrates are often exposed to CPF concentrations near 1 μg/L in nature (Canada 2011; Stone et al. 2014). Our finding that exposure to these very low, commonly encountered doses of CPF causes neurodevelopmental changes provides a realistic endpoint that should be considered in toxicological analysis of the effects of low dose organophosphate exposure. This recommendation is also supported by previous research that showed tadpoles in mesocosm settings exposed to 5 μg/L CPF had changes in brain shape (Woodley et al. 2015). The evidence from these two studies affirms that the neurodevelopmental effects of CPF can be replicated in different settings and occur at even lower doses than previously tested.

Unlike tadpoles that were exposed to 1μg/L CPF during development, tadpoles exposed to the still relatively common dose of 10 μg/L CPF did not have any changes in
relative brain mass or relative brain shape. This is similar to previous findings that showed tadpoles in mesocosm settings exposed to 5 µg/L CPF had changes in relative brain shape, but animals exposed to 20 µg/L CPF did not (Woodley et al. 2015). The differences in neuroanatomical impacts of CPF exposure at these two different doses provides evidence that the effects of CPF exposure occur in a non-monotonic dose response. Other non-monotonic effects of CPF have been seen in neurobehavioral abnormalities in zebrafish and rodents (Levin et al. 2002; Levin et al. 2003). Levin et al. (2002) speculated that small increases in acetylcholine caused by low-dose CPF exposure were acting beneficially in neurodevelopment by offsetting non-cholinergic effects of CPF, and at higher doses this effect is lost. However, it is controversial to consider these low-dose effects to be beneficial. Others have hypothesized that low-dose effects are more likely caused by the toxins getting through the body's self-defense mechanism and interrupting endocrine processes (Vandenberg et al. 2012; Slotkin et al. 2013).

Ecological toxins that cause low-dose biological effects in a non-monotonic manner are not unusual and are often associated with endocrine disruption (Vandenberg et al. 2012). While organophosphates are not usually thought of as endocrine disrupters, CPF has been shown to be weakly estrogenic and it also causes non-monotonic changes in thyroid hormone (thyroxine, T4) (Andersen et al. 2002; Slotkin et al. 2013). Non-monotonic effects have also been seen in both lipid peroxidation and antioxidant enzyme levels, indicators of oxidative stress (Wu et al. 2011). It’s possible that either the endocrine effects or the oxidative stress effects could be contributing to the neurodevelopmental changes found in this study. More work is needed to determine if either the hormonal or oxidative stress effects are mechanistically involved in the impacts
of CPF on brain shape and size.

While this study shows that CPF impacts neurodevelopment, the lack of histological analysis of the brains in this study limits our ability to determine the cause of the increased telencephalon and olfactory bulb size seen in tadpoles exposed to 1 μg/L CPF. Previous studies in rodents and humans exposed to chlorpyrifos found that increases in brain size were associated with increased numbers of astrocytes and perikaryal swelling (Garcia et al. 2002; Roy et al. 2004; Roy et al. 2005). CPF exposures also resulted in apoptosis and decreased neuronal cell numbers, decreased neuritic projections, and decreased white matter (Slotkin 2004; Rauh et al. 2012). If negative cellular effects also caused the changes in tadpole brains found in this study, then tadpoles might show neurological deficits. The telencephalon is involved in sensory processing, motor output, avoidance learning, and social behavior (Altig and McDiarmid 1999). Neurological deficits caused by damage to the telencephalon might affect an animal’s ability to process and respond to environmental stimuli, predators, and interactions with conspecifics. The olfactory bulb, responsible for olfaction, is especially important in tadpoles as it is used to locate food and identify conspecific and predatory chemical cues in the water (Kiesecker et al. 1996; Veeranagoudar et al. 2004). Any damage to this region could have impacts on a tadpole’s ability to survive in an environment with limited food access or predator exposures.

**Tadpole Behavior**

To explore whether the impacts of CPF on neurodevelopment had functional consequences, we tested to see if CPF exposure resulted in behavioral changes. Tadpoles exposed to 1μg/L spent more time near a novel stimulus item (a sealed jar filled with
water) than tadpoles in other treatment groups. This change in behavior could be the result of changes in how animals process or respond to stimuli in their environment. If this behavior causes animals to spend more time in novel situations it could be beneficial if these situations are associated with food sources. However, if the novel situation is dangerous, it could have negative effects on the animal.

Unlike other studies, we did not find an effect of CPF on activity and thigmotaxis (Shuman-Goodier and Propper 2016). It is possible that we did not observe any differences on activity levels because of the differences in concentration that were used among studies or because of the time we gave tadpoles to acclimate to the behavior arena. Rodents injected with 1 or 5 mg/L CPF had hyperactivity when first placed in an arena when compared to controls, but normal activity resumed after approximately an hour of adjustment (Levin et al. 2002; Icenogle et al. 2004). In our study, animals were given an hour to acclimate to the arena potentially preventing us from determining if animals exposed to CPF have different activity levels in new environments.

Interestingly, even though no brain changes were found in tadpoles exposed to 10 μg/L CPF, animals in this treatment group showed behavioral changes in response to the olfactory cues. In tests with kairomone/conspecific alarm cues, tadpoles exposed to 10 μg/L CPF during development spent more time in the center of the arena and more time exploring the arena. Acute exposure to kairomone/conspecific alarm cues usually causes tadpoles to decrease their activity and increase time spent hiding (Sharma et al. 2008; Schoeppner and Relyea 2009). If exposure to certain concentrations of CPF causes tadpoles to be more active instead of hiding in the presence of predators, this could increase their risk of predation.
These behavioral changes correspond to differences in the concentration of corticosterone, which is a hormone involved with metabolism and stress responses. Tadpoles exposed to 10 μg/L CPF, but not 1 μg/L CPF, had elevated corticosterone concentrations. Animals that have chronically elevated corticosterone respond differently to acute stressors, such as predatory cues, than animals with no history of elevated stress levels (Middlemis Maher et al. 2013). Elevated corticosterone caused by CPF exposure could be the cause of the different behavioral response to kairomones found in this study, but more research should be done to confirm this.

**Metamorph Brain Morphology**

Metamorphs (note that exposure occurred during the tadpole stage) exposed to 1 μg/L CPF had similar brain masses (relative to body mass) but altered brain shapes (after adjustment for body mass) compared to controls or metamorphs that had been exposed to 10 μg/L CPF. After developmental exposure to 1 μg/L CPF, metamorphs had brains with relatively larger optic tecta (length and width) and relatively smaller medulla (length). The change observed in the relative shape of brains in these metamorphs were carry-over effects from larval exposure to CPF, as they were not exposed to treatments during the metamorph stage.

Our study suggests that changes in relative brain morphology represent useful end points for toxicological analysis of prolonged, low dose effects in vertebrates. Like our study, other studies have also found that environmental impacts on vertebrate neurodevelopment can be long-lasting and persist through major life history changes like metamorphosis (Marco et al. 2011; Trokovic et al. 2011). Further, carry-over effects of CPF were also found in more natural (mesocosm) environmental conditions, where
tadpoles that were exposed to 1 μg/L CPF developed into metamorphs with altered brain shape (changes in the optic tecta, medulla, and diencephalon) (McClelland et al. 2018). However, Woodley et al. (2015) found that tadpoles reared in mesocosms exposed to 5 μg/L CPF impacted tadpole, but not metamorph, brain structure; this may be due to the length or developmental timing of exposures. The current study and McClelland et al. (2018) exposed tadpoles to CPF for several weeks, whereas the Woodley et al. (2015) study used a one-time exposure at the start of the experiment.

The altered relative brain morphology in metamorphs could have effects on the function of the optic tecta, responsible for vision, or the medulla, which is involved in respiration and auditory function. While we cannot judge the cellular causes for the changes in relative brain shape, if these changes are adverse, like in other animals exposed to CPF, then metamorphs that developed in the presence of CPF could have visual and auditory impairments (Garcia et al. 2002; Roy et al. 2004; Slotkin 2004; Roy et al. 2005; Rauh et al. 2012).

**Metamorph Behavior**

After undergoing metamorphosis, no behavioral changes were seen in response to CPF exposure during development. This result was surprising for a number of reasons. First, the neuroanatomical changes observed in this study were present in both tadpoles and metamorphs. Second, longitudinal studies in children that were exposed to high, but sublethal doses of CPF prenatally had long-term behavioral effects during childhood, providing evidence that neurobehavioral effects of CPF are long lasting (Rauh et al. 2006; Bouchard et al. 2011; Engel et al. 2011; Rauh et al. 2011). It is possible that the neurobehavioral effects of such low dose exposures disappear by juvenile life stages.
However, a more likely explanation is that the behavioral assays used for testing metamorph behavior in this study were not sensitive enough to detect neurobehavioral impacts of CPF exposure. In the visual cue assays, all metamorphs responded so strongly to the prey cue that it likely overwhelmed any potential effects of CPF. The olfactory cue had the opposite effect, in that there were no responses in any treatment group to the olfactory cue. Therefore, we do not feel we can make a conclusion as to whether CPF exposure resulted in neurobehavioral effects on metamorphs.

**Body Condition**

Body condition is commonly used as a means to estimate health in animals (Schulte-Hostedde et al. 2005; Brodeur et al. 2011). Animals that have good body condition likely have more metabolizable tissue (fat, protein, lean mass) than those in poor body condition (Schulte-Hostedde et al. 2005; MacCracken and Stebbings 2012). This has been assumed to increase animal fitness, because in times of poor resources, animals can metabolize this tissue in order to survive. Further, it is likely that these increased energy reserves permit animals to devote more energy to energetically high demand activities such as reproduction or immune function.

In this study, tadpoles exposed to CPF, regardless of the concentration, were relatively longer with poorer body condition than animals in the control group. This could potentially impact tadpole survival thereby impacting the fitness of animals living in contaminated environments. The differences in body condition were not due to any difference in access to food, as all animals had constant access to food. Nor was it due to developmental stage, as there was no difference in developmental stage among treatment groups.
Other studies examining the relationship between body condition and pesticides have found that frogs living in contaminated habitats have poorer body condition than those from controls sites (Brodeur et al. 2011; Hegde and Krishnamurthy 2014). These studies assessed the body condition of frogs living in wild populations, where the application of pesticides may have affected the amount of food the animals had access to. However, in our study, all animals had constant access to food. Therefore, if the difference in treatment groups was due to an increased energy demand caused by CPF exposure, animals could have potentially made up for this deficit by consuming more. It is possible that their growth (tadpoles exposed to CPF were also longer than controls) and energy demand outpaced their ability to consume enough energy to match their energy output and maintain a good body condition, but more work is needed to analyze this question. Future studies should assess the amount of protein, lean mass, and fat in each of these groups in an attempt to determine how CPF exposure is influencing body condition in tadpoles.

After metamorphosing, there was no longer a difference in the body condition of animals among treatments. The time between the developmental stages when tadpole body condition was assessed and when animals underwent metamorphosis, may have given the animals exposed to CPF enough time to “catch up” in gaining weight. Compensatory growth is a common strategy employed by tadpoles and metamorphs after periods of stressful or low resource environmental conditions that cause a period of slowed growth (Dahl et al. 2012; Orizaola et al. 2014; Hsu et al. 2018). This could suggest that the potential hazard in regard to having fewer energy reserves can be overcome if tadpoles can survive beyond a certain developmental stage. However, there
could be other trade-offs that occur, such as reduced burst speed, in order to enable compensatory growth (Arendt 2003). Further, the ability for animals to have periods of faster growth may be limited based on latitude (Orizaola et al. 2014). More research is needed to more closely investigate if compensatory growth is occurring, and if so, how that could be impacting other parameters of animal fitness.

**Conclusion**

The present study provides evidence of neurodevelopmental, neurobehavioral, and neuroendocrine effects of exposure to very low, and commonly encountered doses of the organophosphate CPF in vertebrates. Both the neurodevelopmental and neurobehavioral effects occurred in a nonmonotonic dose response adding to the growing number of nonmonotonic biological effects displayed in animals exposed to low doses of CPF. Such responses require both scientists and regulatory agencies to act with caution when making conclusions about lowest observed adverse effect levels (LOAEL) and no observed adverse effect levels (NOAEL).

The neurodevelopmental effects found in this study were present at two life history stages, showing that early life exposures to CPF can be long lasting, persist through metamorphosis, and possibly impact animals even after they are no longer being exposed to the pesticide. Further, these effects have now been seen in both controlled laboratory studies (this study) and in mesocosm studies (Woodley et al. 2015; McClelland et al. 2018). More work analyzing these effects in natural populations are still needed. However, to date, the evidence suggests that the endpoints used in this study could be relevant for monitoring sublethal, low dose impacts of organophosphate exposures.
ACKNOWLEDGEMENTS

The work described in Chapter 3 was partially funded by grants from the G. Murray McKinley Research Fund at the Pymatuning Lab of Ecology, Society of Wetland Scientists Mid-Atlantic Chapter Research Grant, and the American Society of Ichthyologists and Herpetologists Gaige Award (all awarded to SJM).
Table 3.1. Results of univariate tests for tadpole brain principal components and tadpole body variables\textsuperscript{b}.

<table>
<thead>
<tr>
<th>Dependent variable</th>
<th>Pesticide (CPF)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Brain Morphology</strong></td>
<td></td>
</tr>
<tr>
<td>PC-1</td>
<td>$F(2, 41) = 0.250$, $p = 0.780$</td>
</tr>
<tr>
<td>PC-2</td>
<td>$F(2, 41) = 4.234$, $p = 0.021^*$</td>
</tr>
<tr>
<td>PC-3</td>
<td>$F(2, 41) = 1.401$, $p = 0.258$</td>
</tr>
<tr>
<td><strong>Body Morphology</strong></td>
<td></td>
</tr>
<tr>
<td>Multivariate analysis</td>
<td>$F(14, 72) = 2.018$, $p = 0.028^*$</td>
</tr>
<tr>
<td>Body length</td>
<td>$F(2, 41) = 9.665$, $p &lt; 0.001^*$</td>
</tr>
<tr>
<td>Body width</td>
<td>$F(2, 41) = 1.301$, $p = 0.283$</td>
</tr>
<tr>
<td>Body depth</td>
<td>$F(2, 41) = 2.679$, $p = 0.081$</td>
</tr>
<tr>
<td>Tail length</td>
<td>$F(2, 41) = 0.820$, $p = 0.448$</td>
</tr>
<tr>
<td>Tail width</td>
<td>$F(2, 41) = 0.155$, $p = 0.857$</td>
</tr>
<tr>
<td>Tail depth</td>
<td>$F(2, 41) = 1.651$, $p = 0.204$</td>
</tr>
<tr>
<td>Muscle depth</td>
<td>$F(2, 41) = 1.025$, $p = 0.368$</td>
</tr>
</tbody>
</table>

\textsuperscript{a} Principal components describe mass-adjusted brain morphology for Northern Leopard Frog tadpoles: PC-1 represents optic tectum width, optic tectum length, medulla length, diencephalon width, medulla width; PC-2 represents telencephalon length, telencephalon width, olfactory bulb length; PC-3 represents diencephalon length

\textsuperscript{b} Northern Leopard Frog body variables did not meet the requirements of principal component analysis therefore a multivariate analysis on mass-adjusted body variables was conducted

* $p < 0.05$

CPF = chlorpyrifos; PC = principal component
Table 3.2. Results of tadpole behavior principal component (PC) analyses.

<table>
<thead>
<tr>
<th>PC</th>
<th>Factors</th>
<th>Univariate Results</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><strong>Tadpole baseline and olfactory behavior</strong></td>
<td></td>
</tr>
<tr>
<td>PC-1</td>
<td><strong>Baseline:</strong></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Time inactive</td>
<td>( F_{(2,42)} = 1.178, \ p = 0.318 )</td>
</tr>
<tr>
<td></td>
<td>Distance travelled</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Speed</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Acceleration</td>
<td></td>
</tr>
<tr>
<td>PC-2</td>
<td><strong>Olfactory:</strong></td>
<td>( F_{(2,42)} = 0.896, \ p = 0.416 )</td>
</tr>
<tr>
<td></td>
<td>Time inactive</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Distance travelled</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Speed</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Acceleration</td>
<td></td>
</tr>
<tr>
<td>PC-3</td>
<td><strong>Baseline:</strong></td>
<td>( F_{(2,42)} = 0.036, \ p = 0.965 )</td>
</tr>
<tr>
<td></td>
<td>Time in center</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Exploration</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Exploration of center</td>
<td></td>
</tr>
<tr>
<td>PC-4*</td>
<td><strong>Olfactory:</strong></td>
<td>( F_{(2,42)} = 3.487, \ p = 0.040^* )</td>
</tr>
<tr>
<td></td>
<td>Time in center</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Exploration</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Exploration of center</td>
<td></td>
</tr>
<tr>
<td></td>
<td><strong>Tadpole visual behavior</strong></td>
<td></td>
</tr>
<tr>
<td>PC-1</td>
<td><strong>All Visual Assays:</strong></td>
<td>( F_{(2,42)} = 1.810, \ p = 0.176 )</td>
</tr>
<tr>
<td></td>
<td>Distance travelled</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Speed</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Acceleration</td>
<td></td>
</tr>
<tr>
<td>PC-2</td>
<td><strong>All Visual Assays:</strong></td>
<td>( F_{(2,42)} = 0.191, \ p = 0.827 )</td>
</tr>
<tr>
<td></td>
<td>Time inactive</td>
<td></td>
</tr>
<tr>
<td>PC-4</td>
<td><strong>Predator, Conspecific Assays:</strong></td>
<td>( F_{(2,42)} = 1.230, \ p = 0.303 )</td>
</tr>
<tr>
<td></td>
<td>Exploration</td>
<td></td>
</tr>
<tr>
<td>PC-3</td>
<td><strong>Predator, Conspecific Assays:</strong></td>
<td>( F_{(2,42)} = 0.393, \ p = 0.677 )</td>
</tr>
<tr>
<td></td>
<td>Time near object</td>
<td></td>
</tr>
<tr>
<td>PC-5*</td>
<td><strong>Empty Jar Assay:</strong></td>
<td>( F_{(2,42)} = 3.971, \ p = 0.026^* )</td>
</tr>
<tr>
<td></td>
<td>Time near object</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Exploration</td>
<td></td>
</tr>
</tbody>
</table>

*p < 0.05

PC = principal component
Table 3.3. Results of univariate tests for metamorph brain\textsuperscript{a} and body\textsuperscript{b} principal components.

<table>
<thead>
<tr>
<th>Dependent variable</th>
<th>Pesticide (CPF)</th>
</tr>
</thead>
</table>

**Brain Morphology**

- **PC-1**: $F_{(2, 40)} = 3.312$, $p = 0.047^*$
- **PC-2**: $F_{(2, 40)} = 0.083$, $p = 0.920$
- **PC-3**: $F_{(2, 40)} = 0.536$, $p = 0.589$
- **PC-4**: $F_{(2, 40)} = 2.556$, $p = 0.090$

**Body Morphology**

- **PC-1**: $F_{(2, 40)} = 2.501$, $p = 0.095$
- **PC-2**: $F_{(2, 40)} = 0.325$, $p = 0.724$
- **PC-3**: $F_{(2, 40)} = 0.123$, $p = 0.885$
- **PC-4**: $F_{(2, 40)} = 3.720$, $p = 0.033^*$

\textsuperscript{a} Principal components describe mass-adjusted brain morphology: PC-1 represents optic tectum length, width, medulla length; PC-2 represents telencephalon length, olfactory bulb length; PC-3 represents diencephalon length, medulla width; PC-4 represents telencephalon width, diencephalon width.

\textsuperscript{b} Principal components describe mass-adjusted body morphology: PC-1 represents right and left foot length, right and left thigh length, left leg length, right arm length; PC-2 represents right and left thigh width, right leg length, left arm length; PC-3 represents body length, body width, and head width; PC-4 represents right and left arm width.

* $p < 0.05$

CPF = chlorpyrifos; PC = principal component
Table 3.4. Results of metamorph behavior principal component analysis*.

<table>
<thead>
<tr>
<th>PC</th>
<th>Factors</th>
<th>Univariate Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>PC-1</td>
<td><strong>Baseline:</strong>&lt;br&gt;Distance travelled&lt;br&gt;Speed&lt;br&gt;Acceleration&lt;br&gt;Exploration</td>
<td>( F_{(2, 38)} = 0.745, p=0.481 )</td>
</tr>
<tr>
<td>PC-2</td>
<td><strong>Visual:</strong>&lt;br&gt;Time to notice&lt;br&gt;jar with worms&lt;br&gt;Number of lunges at jar&lt;br&gt;with worms&lt;br&gt;<strong>Baseline:</strong>&lt;br&gt;Time inactive</td>
<td>( F_{(2, 38)} = 0.240, p=0.788 )</td>
</tr>
<tr>
<td>PC-3</td>
<td><strong>Visual:</strong>&lt;br&gt;Time to notice&lt;br&gt;empty jar&lt;br&gt;Number of lunges at&lt;br&gt;empty jar</td>
<td>( F_{(2, 38)} = 0.135, p=0.874 )</td>
</tr>
<tr>
<td>PC-4</td>
<td><strong>Olfactory:</strong>&lt;br&gt;Number of zone&lt;br&gt;changes&lt;br&gt;Time in starting&lt;br&gt;position</td>
<td>( F_{(2, 38)} = 0.913, p=0.410 )</td>
</tr>
</tbody>
</table>

*Note: Baseline time spent in center was also included in the principal component analysis but did not load strongly on any PC.
PC = principal component
Table 3.5. Principal components analysis of 9 mass-adjusted brain dimensions of Northern Leopard Frog tadpoles.

<table>
<thead>
<tr>
<th>Results of PCA</th>
<th>Principal Component</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PC-1</td>
</tr>
<tr>
<td>% of Variance</td>
<td>39.7</td>
</tr>
<tr>
<td>Eigenvalue</td>
<td>3.6</td>
</tr>
<tr>
<td>Factor Loading</td>
<td></td>
</tr>
<tr>
<td>Telencephalon width</td>
<td>0.553</td>
</tr>
<tr>
<td>Telencephalon length</td>
<td>0.265</td>
</tr>
<tr>
<td>Optic tectum width</td>
<td>0.707</td>
</tr>
<tr>
<td>Optic tectum length</td>
<td>0.759</td>
</tr>
<tr>
<td>Medulla length</td>
<td>-0.652</td>
</tr>
<tr>
<td>Diencephalon width</td>
<td>0.690</td>
</tr>
<tr>
<td>Diencephalon length</td>
<td>0.031</td>
</tr>
<tr>
<td>Olfactory bulb length</td>
<td>0.067</td>
</tr>
<tr>
<td>Medulla width</td>
<td>0.601</td>
</tr>
</tbody>
</table>

PCA = Principal Components Analysis; PC = Principal Component
Table 3.6. Principal components analysis of 14 baseline and olfactory behavior measurements for Northern Leopard Frog tadpoles.

<table>
<thead>
<tr>
<th>Results of PCA</th>
<th>PC-1</th>
<th>PC-2</th>
<th>PC-3</th>
<th>PC-4</th>
</tr>
</thead>
<tbody>
<tr>
<td>% of Variance</td>
<td>36.8</td>
<td>25.5</td>
<td>13.3</td>
<td>9.1</td>
</tr>
<tr>
<td>Eigenvalue</td>
<td>5.2</td>
<td>3.6</td>
<td>1.9</td>
<td>1.3</td>
</tr>
<tr>
<td>Factor Loading</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline time inactive</td>
<td>-0.832</td>
<td>-0.184</td>
<td>-0.105</td>
<td>-0.103</td>
</tr>
<tr>
<td>Baseline distance travelled</td>
<td>0.965</td>
<td>0.184</td>
<td>0.063</td>
<td>0.086</td>
</tr>
<tr>
<td>Baseline speed</td>
<td>0.919</td>
<td>0.206</td>
<td>0.058</td>
<td>0.151</td>
</tr>
<tr>
<td>Baseline acceleration</td>
<td>0.945</td>
<td>0.177</td>
<td>0.109</td>
<td>0.126</td>
</tr>
<tr>
<td>Olfactory time inactive</td>
<td>-0.073</td>
<td>-0.785</td>
<td>0.105</td>
<td>0.150</td>
</tr>
<tr>
<td>Olfactory distance travelled</td>
<td>0.211</td>
<td>0.956</td>
<td>-0.035</td>
<td>0.016</td>
</tr>
<tr>
<td>Olfactory speed</td>
<td>0.237</td>
<td>0.912</td>
<td>-0.040</td>
<td>0.146</td>
</tr>
<tr>
<td>Olfactory acceleration</td>
<td>0.229</td>
<td>0.929</td>
<td>-0.053</td>
<td>0.044</td>
</tr>
<tr>
<td>Baseline time in center</td>
<td>-0.026</td>
<td>-0.077</td>
<td>0.864</td>
<td>0.058</td>
</tr>
<tr>
<td>Baseline exploration</td>
<td>0.333</td>
<td>-0.018</td>
<td>0.855</td>
<td>0.172</td>
</tr>
<tr>
<td>Baseline exploration of center</td>
<td>0.096</td>
<td>-0.068</td>
<td>0.913</td>
<td>0.261</td>
</tr>
<tr>
<td>Olfactory time in center</td>
<td>-0.057</td>
<td>-0.185</td>
<td>0.452</td>
<td>0.568</td>
</tr>
<tr>
<td>Olfactory exploration</td>
<td>0.192</td>
<td>0.220</td>
<td>0.218</td>
<td>0.836</td>
</tr>
<tr>
<td>Olfactory exploration of center</td>
<td>0.053</td>
<td>-0.053</td>
<td>0.094</td>
<td>0.935</td>
</tr>
</tbody>
</table>

PCA = Principal Components Analysis; PC = Principal Component
Table 3.7. Principal components analysis of 18 visual behavior measurements for Northern Leopard Frog tadpoles.

<table>
<thead>
<tr>
<th>Results of PCA</th>
<th>PC-1</th>
<th>PC-2</th>
<th>PC-3</th>
<th>PC-4</th>
<th>PC-5</th>
</tr>
</thead>
<tbody>
<tr>
<td>% of Variance</td>
<td>44.4</td>
<td>12.3</td>
<td>9.1</td>
<td>7.9</td>
<td>6.0</td>
</tr>
<tr>
<td>Eigenvalue</td>
<td>8.0</td>
<td>2.2</td>
<td>1.6</td>
<td>1.4</td>
<td>1.1</td>
</tr>
</tbody>
</table>

Factor Loading

| Control distance travelled | 0.864 | -0.067 | -0.269 | -0.036 | 0.183 |
| Control speed             | 0.878 | 0.149  | -0.140 | 0.069  | 0.052 |
| Control acceleration      | 0.853 | -0.158 | -0.302 | -0.061 | 0.199 |
| Control time inactive     | 0.062 | 0.865  | 0.036  | 0.075  | 0.026 |
| Control exploration       | -0.068| -0.119 | -0.254 | 0.498  | 0.611 |
| Control time near object  | 0.082 | 0.035  | 0.190  | -0.091 | 0.877 |
| Conspecific distance travelled | 0.935 | -0.073 | -0.029 | 0.112  | 0.008 |
| Conspecific speed         | 0.927 | -0.033 | -0.104 | 0.119  | 0.040 |
| Conspecific acceleration  | 0.901 | -0.056 | 0.055  | 0.131  | -0.083 |
| Conspecific time inactive  | 0.025 | 0.721  | -0.175 | -0.201 | 0.011 |
| Conspecific exploration   | 0.221 | -0.039 | -0.199 | 0.853  | -0.108 |
| Conspecific time near object | -0.123| 0.068  | 0.803  | -0.161 | -0.110 |
| Predator distance travelled | 0.943 | 0.036  | 0.010  | 0.118  | -0.051 |
| Predator speed            | 0.938 | 0.113  | 0.017  | 0.102  | -0.058 |
| Predator acceleration     | 0.917 | -0.039 | 0.005  | 0.190  | -0.057 |
| Predator time inactive    | -0.121| 0.806  | 0.097  | -0.017 | -0.084 |
| Predator exploration      | 0.409 | -0.123 | 0.310  | 0.583  | 0.304 |
| Predator time near object  | -0.167| -0.134 | 0.701  | 0.035  | 0.353 |

PCA = Principal Components Analysis; PC = Principal Component
Table 3.8. Principal components analysis of 9 mass-adjusted brain dimensions of Northern Leopard Frog metamorphs.

<table>
<thead>
<tr>
<th>Results of PCA</th>
<th>Principal Component</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PC-1</td>
</tr>
<tr>
<td>% of Variance</td>
<td>27.9</td>
</tr>
<tr>
<td>Eigenvalue</td>
<td>2.5</td>
</tr>
<tr>
<td>Factor Loading</td>
<td></td>
</tr>
<tr>
<td>Telencephalon width</td>
<td>-0.207</td>
</tr>
<tr>
<td>Telencephalon length</td>
<td>-0.097</td>
</tr>
<tr>
<td>Optic tectum width</td>
<td>0.855</td>
</tr>
<tr>
<td>Optic tectum length</td>
<td>0.614</td>
</tr>
<tr>
<td>Medulla length</td>
<td>-0.760</td>
</tr>
<tr>
<td>Diencephalon width</td>
<td>0.585</td>
</tr>
<tr>
<td>Diencephalon length</td>
<td>-0.022</td>
</tr>
<tr>
<td>Olfactory bulb length</td>
<td>-0.062</td>
</tr>
<tr>
<td>Medulla width</td>
<td>0.156</td>
</tr>
</tbody>
</table>

PCA = Principal Components Analysis; PC = Principal Component
Table 3.9. Principal components analysis of 15 mass-adjusted body dimensions of northern leopard frog metamorphs.

<table>
<thead>
<tr>
<th>Results of PCA</th>
<th>Principal Component</th>
</tr>
</thead>
<tbody>
<tr>
<td>% of Variance</td>
<td>PC-1</td>
</tr>
<tr>
<td>Eigenvalue</td>
<td>29.8</td>
</tr>
<tr>
<td>Factor Loading</td>
<td></td>
</tr>
<tr>
<td>body length</td>
<td>0.368</td>
</tr>
<tr>
<td>body width</td>
<td>0.024</td>
</tr>
<tr>
<td>head width</td>
<td>0.202</td>
</tr>
<tr>
<td>left forelimb length</td>
<td>0.346</td>
</tr>
<tr>
<td>left forelimb width</td>
<td>-0.082</td>
</tr>
<tr>
<td>left thigh length</td>
<td>0.733</td>
</tr>
<tr>
<td>left thigh width</td>
<td>-0.157</td>
</tr>
<tr>
<td>left leg length</td>
<td>0.596</td>
</tr>
<tr>
<td>left foot length</td>
<td>0.745</td>
</tr>
<tr>
<td>right forelimb length</td>
<td>0.535</td>
</tr>
<tr>
<td>right forelimb width</td>
<td>0.064</td>
</tr>
<tr>
<td>right thigh length</td>
<td>0.814</td>
</tr>
<tr>
<td>right thigh width</td>
<td>0.089</td>
</tr>
<tr>
<td>right leg length</td>
<td>0.476</td>
</tr>
<tr>
<td>right foot length</td>
<td>0.836</td>
</tr>
</tbody>
</table>

PCA = Principal Components Analysis; PC = Principal Component
Table 3.10. Principal components analysis of 11 baseline, olfactory, and visual behavioral measurements for Northern Leopard Frog metamorphs.

<table>
<thead>
<tr>
<th>Results of PCA</th>
<th>Principal Component</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PC-1</td>
</tr>
<tr>
<td>% of Variance</td>
<td>32.1</td>
</tr>
<tr>
<td>Eigenvalue</td>
<td>3.9</td>
</tr>
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</tr>
<tr>
<td>Olfactory time in starting position</td>
<td>0.211</td>
</tr>
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</table>

PCA = Principal Components Analysis; PC = Principal Component
Figure 3.1. Methods used for testing how low, ecologically relevant concentrations of chlorpyrifos (CPF) impact animal physiology. Tadpoles were exposed to ethanol (EtOH), 1 μg/L CPF, or 10 μg/L CPF on day 1. Tadpoles were randomly selected and removed from the tank for each assay: water-borne corticosterone concentrations (WB Cort), tadpole behavior, tadpole body and brain morphology (the same tadpole was used for WB Cort, behavior, and then body and brain morphology). Remaining tadpoles underwent metamorphosis, were immediately removed from tanks, and allowed to finish metamorphosing. After metamorphosis was complete, juveniles were immediately tested for WB Cort, juvenile behavior, juvenile body and brain morphology (the same juvenile was used for WB Cort, behavior, and then body and brain morphology). Numbers represent final sample sizes (for starting number, the numbers are: number of individuals per tank and the total number of tanks).
Figure 3.2. Body and Brain Morphology (A) Leopard frog tadpole showing the linear dimensions used to describe tadpole body morphology: 1 body length, 2 body depth, 3 muscle depth, 4 tail depth, 5 tail length, 6 body width, 7 tail width; (B) Leopard frog metamorph showing the linear dimensions used to describe metamorph body morphology: 1 body length, 2 head width, 3 body width, 4 arm width, 5 arm length, 6 thigh width, 7 thigh length, 8 leg length, 9 foot length; (C) Dorsal and ventral view of a Leopard frog tadpole brain and (D) Dorsal and ventral view of a Leopard frog metamorph brain: tadpole and metamorph brains show the linear dimensions used to describe brain morphology: 1 telencephalon length, 2 telencephalon width, 3 optic tectum length, 4 optic tectum width, 5 medulla length, 6 olfactory bulb length, 7 diencephalon length, 8 diencephalon width, 9 medulla width.
Figure 3.3. Behavioral Set-up and Design. A) Experimental set up with computers and webcams overhead to record the behavioral arenas; B) Design for tadpole visual assays; C) Screenshot of tadpole baseline behavior video; D) Design for metamorph visual assays; E) Design for metamorph olfactory assays; F) Screenshot of metamorph visual behavior video.
Figure 3.4. Effects of Chlorpyrifos (CPF) on Tadpole Biology (A) Relative brain mass was heavier when tadpoles were exposed to 1 μg/L CPF; (B) Tadpoles exposed to 1 μg/L CPF had increased telencephalon widths, lengths, and olfactory bulb lengths; (C) Tadpoles exposed to 10 μg/L CPF had increased corticosterone concentrations (D) Bodies were longer in tadpoles exposed to 1 or 10 μg/L; (E) Tadpoles exposed to 1 or 10 μg/L had poorer body condition than controls. Mean +/- SEM is graphed. Points labeled with different letters are significantly different; p<0.05, Student-Newman-Keuls post-hoc tests, n=14-15 (see Figure 3.1).
Figure 3.5. Effects of Chlorpyrifos (CPF) on Tadpole Behavior (A) After tadpoles were exposed to an olfactory cue consisting of kairomones and conspecific alarm cues, tadpoles that had developed exposed to 10 μg/L CPF spent more time in the center of the arena and explored the arena more than the tadpoles from other treatments. (B) Tadpoles that had developed exposed to 1 μg/L CPF spent more time near the novel stimulus of an empty glass jar than tadpoles from other treatments. Mean +/- SEM is graphed. Points labeled with different letters are significantly different; p<0.05, Student-Newman-Keuls post-hoc tests, n=15.
Figure 3.6. Developmental Effects of Chlorpyrifos (CPF) on Metamorphs. Metamorphs were exposed to CPF during development but removed from the treatments as soon as forelimbs emerged. (A) Metamorphs exposed to 1 μg/L CPF had increased optic tectum widths, lengths, and decreased medulla lengths; (B) There was no effect of chlorpyrifos on metamorph body morphology when compared to controls, however, tadpoles exposed to 10 μg/L CPF had wider arms as metamorphs than tadpoles exposed to 1 μg/L CPF; (C) There was no effect of CPF on corticosterone concentrations in metamorphs; (D) There was no effect of CPF on metamorph body condition. Mean +/- SEM is graphed. Points labeled with different letters are significantly different; p<0.05, Student-Newman-Keuls post-hoc tests, n=14 (0 μg/L); 15 (1 μg/L); 14 (10 μg/L).
Figure 3.7. Tadpole biological and body morphological variables that were not affected by exposure to chlorpyrifos. (A) Gosner stage, (B) body mass, (C) body width, (D) body depth, (E) tail length, (F) tail width, (G) tail depth, (H) muscle depth. Mean +/- SEM is graphed; p>0.05. Tadpole body variables did not meet the assumptions of principal component analysis; therefore, body dimensions were first assessed with a multivariate analysis of variance (MANOVA) that showed significant effects. These effects were then
explored with analysis of variance and further significant effects were analyzed with a Student-Newman-Keuls post-hoc test, n=15.
Figure 3.8. Tadpole brain mass and brain morphological variables that were not affected by exposure to chlorpyrifos. (A) brain mass before adjusting for body mass, (B) PC-1, (C) PC-2. Mean +/- SEM is graphed. p>0.05, n=14 (0 μg/L); 14 (1 μg/L); 15 (10μg/L).
Figure 3.9. Tadpole behavioral variables that were not affected by exposure to chlorpyrifos. (A) PC-1 of the Principal Component Analysis (PCA) of baseline and olfactory behavior data, (B) PC-2 of the PCA of baseline and olfactory behavior data, (C) PC-3 of the PCA of baseline and olfactory behavior data, (D) PC-1 of the PCA of all
visual behavior data, (E) PC-2 of the PCA of all visual behavior data, (F) PC-3 of the PCA of all visual behavior data, (G) PC-4 of the PCA of all visual behavior data. Mean +/- SEM is graphed. p>0.05, n=15.
Figure 3.10. Metamorph biological and body morphological variables that were not affected by exposure to chlorpyrifos. (A) time to metamorphosis, (B) body mass, (C) PC-1, (D) PC-2, (E) PC-3. Mean +/- SEM is graphed. p>0.05, n=14 (0 μg/L); 15 (1 μg/L); 14 (10 μg/L).
Figure 3.11. Metamorph brain mass and brain morphological variables that were not affected by exposure to chlorpyrifos. (A) brain mass before adjusting for body mass, (B) brain mass after adjusting for body mass, (C) PC-2, (D) PC-3, (E) PC-4. Mean +/- SEM is graphed. p>0.05, n=14 (0 μg/L); 15 (1 μg/L); 14 (10μg/L).
Figure 3.12. Metamorph behavioral variables that were not affected by exposure to chlorpyrifos. Results of the Principal Component Analysis (PCA) of all metamorph behavior data (baseline, olfactory, and visual behavior data), (A) PC-1 (B) PC-2, (C) PC-3, (D) PC-4. Mean +/- SEM is graphed. p>0.05, n=14 (0 μg/L); 15 (1 μg/L); 14 (10μg/L).
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Chapter 4

Evaluate the role of corticosterone in changes caused by sublethal CPF exposure

ABSTRACT

Organophosphorous pesticides like chlorpyrifos (CPF) are neurotoxicants that can cause changes in the brain through numerous mechanisms. The most well studied mechanism is inhibition of the enzyme acetylcholinesterase (AChE). However, CPF exposures impact neurodevelopment by mechanisms other than AChE inhibition. These other processes are still not well understood or defined. We hypothesized that elevated concentrations of corticosterone (CORT) contribute to the effects seen in sublethal CPF exposures. CORT modulates many phenotypic changes and behavioral responses and is heavily involved in neurodevelopment. We exposed tadpoles to either a vehicle control, a negative control (metyrapone [MTP], a CORT biosynthesis blocker), CPF, CORT, or CPF+MTP to investigate the role of CORT in neurological changes caused by low-dose CPF exposures. The CORT treatment resulted in physiological concentrations of CORT. However, results did not support our hypothesis; while CPF and CORT both impacted relative brain shape, they did so in different ways. In addition, pigmentation, and relative body morphology of tadpoles exposed to CPF or CORT differed. However, there was a trend for animals exposed to both CPF and MTP to have reduced neurological effects compared to CPF, suggesting a potential means of mitigating the neurological effects of CPF. More research is needed to determine how the neurological impacts of CPF are modulated, as well as investigating whether MTP ameliorates the effects of low-dose CPF exposures.
INTRODUCTION

The pesticide industry makes billions of dollars every year in order to produce toxic chemicals that people apply in virtually every sector of American life. While these chemicals help us eliminate a variety of pests, and can protect crops from destruction, they also contaminate water ways and expose non-target organisms to harmful chemicals (Stone et al. 2014; U.S.EPA 2015). As the world faces increasing concentrations of animal extinctions, it's more important than ever to understand how pesticide contamination is impacting animals so that we can attempt to devise more precise ways to protect threatened species that are exposed to these chemicals. It is also important to better understand the impacts of pesticide contamination because environmental, animal, and human health are closely linked (http://www.onehealthinitiative.com/mission.php).

Organophosphorous pesticides, a specific class of insecticides, were designed as neurotoxicants to kill insects. They work through competitively binding and irreversibly inhibiting acetylcholinesterase, the enzyme that degrades the neurotransmitter acetylcholine. Due to biological conservation of proteins and processes in the nervous system, these chemicals have been proven to be neurotoxicants to a wide variety of taxa (Mayer and Ellersieck 1986). Toxicity occurs mainly by irreversibly inhibiting acetylcholinesterase (AChE) preventing the breakdown of the neurotransmitter acetylcholine and causing continual stimulation (Slotkin 2004; Rauh et al. 2012). Overt toxicity occurs when the dose of CPF produces a 70-80% or greater inhibition of acetylcholinesterase (AChE) (Slotkin 2004). The does that causes this level of inhibition is different depending on species and age of the animal in question (Carr and Chambers 1996; Kousba et al. 2007). However, in the Southern Leopard Frog (Lithobates
sphenocephala), the closest species to our model organism that has been tested, it was found that at the highest exposure tested (200 μg/L CPF) there was 43% inhibition in AChE activity and (Widder and Bidwell 2008). Further, there was no cholinesterase inhibition when tadpoles were exposed to 10mg/L chlorpyrifos or less, and 25% cholinesterase inhibition did not occur until tadpoles were exposed to 55 mg/L chlorpyrifos (Widder and Bidwell 2008).

In addition to being a neurotransmitter, acetylcholine is also involved in many neuronal processes that occur during development. It is involved in neural cell proliferation, growth, differentiation, apoptosis, and localization of cells during neurodevelopment (Reviewed in Slotkin 2004). If these processes are disrupted, it can impact neurodevelopment, learning, and memory processes across a wide range of animal taxa that have been exposed either maternally, through oral consumption, dermal absorption, or inhalation (Reviewed in Slotkin 2004). Humans that were exposed to the organophosphate chlorpyrifos (CPF) during development had detectable size changes in different brain regions and had deficits in intelligence quotient (IQ) and learning (Rauh et al. 2012; Butler-Dawson et al. 2016). Changes in brain size have also been found in rodents (Roy et al. 2004; Roy et al. 2005). While these exposures may have resulted in AChE inhibition, it is likely that organophosphates impact neurodevelopment through various mechanisms other than just AChE inhibition alone (Reviewed in Slotkin 2004).

The hypothesis that organophosphates are acting through more mechanisms than just AChE inhibition to impart brain changes is supported by our previous work that expose Northern Leopard Frog (Lithobates pipiens, formerly Rana pipiens) tadpoles to very low doses of CPF (0.25-5 μg/L). We found that even at these low doses, changes
still occurred in relative brain size and relative brain morphology (Woodley et al. 2015; McClelland and Woodley in preparation). While we didn't test for AChE activity, other studies have found that in Southern Leopard Frog (*Lithobates sphenoecephalus*, formerly *Rana sphenoecephala*) tadpoles there was no evidence of AChE inhibition when exposed to less than 10 mg/L CPF, and there was only 25% AChE inhibition in tadpoles exposed to 55 mg/L CPF (Widder and Bidwell 2008). It's not clear what the level of detection was in this study. It's possible that a more sensitive assay could detect some inhibition, these levels would likely be a small percentage. Thus, it is unlikely that AChE inhibition was the cause of the brain changes found in the Northern Leopard Frog tadpoles, and it is likely that CPF is impacting neurodevelopment through one or more other mechanisms.

One possible physiological mechanism that could be contributing to the changes that result from sublethal CPF exposure is through the stress response and activation of the hypothalamic-pituitary-adrenal/interrenal (HPA/I) axis. Multiple studies link exposure to anthropogenic contaminants, including numerous pesticides, to CORT concentrations (Hopkins et al. 1997; Hayes et al. 2006; Miller et al. 2009; McMahon et al. 2011). This includes exposure to CPF in rats and lizards (Acker and Nogueira 2012; Mestre et al. 2019). Additionally, low, sublethal CPF exposures increased corticosterone (CORT) concentrations in tadpoles (see above Chapter 3 Aim 2 research McClelland 2020). This is important because previous studies have shown that CORT can modulate both phenotypic and behavioral responses of animals (including tadpoles) that are exposed to natural stressors, such as exposure to predator cues (Middlemis Maher et al. 2013). In addition, tadpoles that are raised in the presence of ecologically relevant concentrations of CPF have changes in the size of specific brain regions, which are
similar to those seen in tadpoles reared in the presence of predator cues (Woodley et al. 2015). It’s possible that the CPF-induced CORT concentration increases could be the cause of the neurodevelopmental effects that occur when animals are exposed to sublethal CPF.

The HPA/I axis (frogs have interrenal glands instead of adrenal glands) functions similarly in all vertebrates to mediate responses to environmental cues and plays an important role in development (including neural-development), physiology, and behavior (Denver 2009). The HPA/I axis is also stimulated in stressful situations, which results in the hypothalamus releasing corticotropin releasing factor (CRF). CRF binds to the CRF1 receptor of the corticotrope cells in the anterior pituitary, causing a signal cascade that results in the release of adrenocorticotropic hormone (ACTH) (Charmandari et al. 2005; Denver 2009). ACTH travels through the blood stream to the adrenal/interrenal glands to activate steroid biosynthetic pathways. As part of the process, the enzyme 11β-hydroxylase converts an inactive precursor cortisone into cortisol or corticosterone) (Charmandari et al. 2005). In some vertebrates, cortisol is produced as the primary glucocorticoid while others, including frogs, produce corticosterone (CORT). Corticosterone/cortisol then travels through the body to target tissues where it then binds to mineralocorticoid or glucocorticoid receptors that are either membrane bound or found intracellularly. When CORT is bound to membrane receptors, it initiates signal cascades causing a range of effects; intracellular receptors bound with CORT can form homodimers and heterodimers that act as transcription factors to regulate gene expression for CORT response elements (Sapolsky et al. 2000; Charmandari et al. 2005; Harris 2019).
One way of blocking CORT effects is to inhibit CORT biosynthesis using metyrapone (MTP) (Glennemeier and Denver 2002; Middlemis Maher et al. 2013). MTP inhibits 11β-hydroxylase, which blocks the inactive cortisone from being converted to CORT in the adrenal glands thereby preventing increases in hormone concentrations (Jahn et al. 2003). If elevated CORT contributes to neurodevelopmental changes produced by low-dose CPF exposure, then preventing CORT increases in animals exposed to sublethal CPF will also prevent the neurodevelopmental effects caused by sublethal CPF exposure.

We hypothesized that CORT contributes to the phenotypic changes induced by sublethal pesticide exposure. To test this hypothesis, we exposed animals to a vehicle control, a negative control (MTP), CORT, CPF, and CPF+MTP. We used concentrations of CORT that were physiologically relevant. We predicted similar hormonal and brain changes in animals exposed to CPF and CORT when compared to controls. Further, by exposing animals to CPF and MTP simultaneously, we could see the effects of CPF without elevated CORT concentrations. We predicted that in animals exposed to CPF+MTP, the effects of CPF would be diminished or disappear. Understanding how CPF is impacting neurodevelopment can help us to find better ways of protecting animals exposed to CPF and increase aid our conservation efforts worldwide.

METHODS

Animal Care

This research was done with approval from the Duquesne University Institutional Animal Care and Use Committee (IACUC # 1701-3) and scientific collecting permits from the Pennsylvania Fish and Boat Commission (permit # 2017-01-0040). Three partial
egg masses were collected on April 4, 2017 from a pond in Linesville, PA. Eggs were transported to Duquesne University on April 9 and placed into an incubator at 12.5 °C from April 10 until April 28 (to slow development for logistical reasons). For the remainder of the experiment, tadpoles were kept at 22-24°C with a 14hr light:10hr dark cycle.

Aquaria (bins) were 15L Sterilite™ plastic bins (42.5 cm x 30.2 cm x 17.8 cm) containing 5 L of water. While I did not test for chemical leaching, Sterilite bins are made of high density polyethylene plastic, which should not leach chemical components into the water (Lithner 2011). All water was sediment-, carbon- and UV-filtered tap water, with a bubble stone to aerate the water. Tadpoles were fed ad libitum a gel food mix (made with 2.25 g agar 12 g of TetraMin tropical tablets (Spectrum Brands Pet, LLC, Blacksburg, VA) ground up and boiled with 90-100 mL water) ad libitum (adjusted based on personal communication with Michael Benard). Partial water changes were done twice a week during which time treatments were renewed.

There were 10 tadpoles per bin and 10 bins per treatment. Bins were housed on 3 different shelving units in the room. Some bins were at the back of the shelving units and others were at the front of the unit. Also, the middle unit was occasionally moved. Depending on their location, bins were exposed to slight variations in illumination and disturbance. To attempt to control for room effects, bins of the different treatments were distributed evenly on the shelving units.

**Treatments**

Tadpoles at Gosner Stage 25 were haphazardly assigned to treatments on May 2. On May 4, tadpoles were exposed to one of five treatments: a vehicle control (0.009%
ethanol), 5 μg/L CPF, 5 μg/L CPF plus 110 μM MTP, 110 μM MTP, or 125 nM CORT (Middlemis Maher et al. 2013). Five μg/L CPF was chosen because it is commonly found in surface waters, has been shown to cause neurological effects, and is too low to robustly decrease AChE activity (Widder and Bidwell 2008; Stone et al. 2014; Woodley et al. 2015). The concentrations of MTP and CORT were chosen because they have been shown to reduce (MTP) or increase (CORT) endogenous CORT concentrations in a physiologically realistic manner in Northern Leopard Frog tadpoles (Glennemeier and Denver 2002).

Water samples were collected and sent to the University of Georgia (Athens, GA) for determination of the actual exposure concentrations of CPF. The analysis determined that the actual concentrations in the CPF alone exposure group were 3.6 μg/L and in the CPF + MTP exposure group were 5.3 μg/L. In this chapter, I refer to the nominal concentrations.

Treatment exposures ended on May 24 by placing tadpoles into a new bin with clean (untreated) water. To ensure that the treatments were rinsed off of their bodies, tadpoles were removed from the treatment using a clean net, gently blotted dry, and placed into clean untreated water. This was repeated a second time. After the second rinse, tadpoles were put in their final bin with clean water. After exactly 24 hours, 100 mL of water was collected from the new tadpole bin and frozen at -20°C for later analysis of water borne CORT. Tadpoles were then euthanized with an overdose of 0.2% tricaine methanesulfonate (MS222), dabbed dry with paper towels, weighed, and fixed in 10% phosphate buffered formalin for later analysis of developmental stage, and body and
brain morphology (Woodley et al. 2015; McClelland et al. 2018). We also collected water from bins that did not contain tadpoles as controls for the waterborne hormone assays.

*Corticosterone Concentration, Body Position, Pigmentation*

Water samples collected at the end of the experiment were filtered using Whatman filter paper (Grade 1), frozen at -20°C and sent to an commercial lab for solid-phase extraction and radio-immuno assay analysis to determine the concentration of CORT (Oregon National Primate Research Center Endocrine Lab, Beavertown, OR; detection limit of 1 pg/mL). The extraction procedure removed the conjugated forms of CORT so only free CORT was measured.

During this experiment we noticed that some bins had tadpoles that were maintaining their bodies in a vertical position in the water column, with their heads pointing up. We also noticed that some bins had tadpoles that were very light, almost transparent, in color. On May 23, the day before ending treatments, we recorded the number of tadpoles in each bin that were in a vertical position. On May 24, as tadpoles were rinsed and placed into bins with untreated water, their coloration was observed and recorded.

*Body and Brain Morphology*

To assess changes in body morphology, dorsal and lateral views of tadpoles were photographed using a digital camera, and body dimensions were measured using Image J (Figure 4.1; US National Institutes of Health, Bethesda, MD). To assess changes in brain morphology, brains were dissected out, cranial nerves were trimmed, brains were weighed, and the dorsal and ventral surfaces were each photographed 3 times independently to produce 3 dorsal images per brain and 3 ventral images per brain. Brain
dimensions were then measured using Image J (Figure 4.1; US National Institutes of Health, Bethesda, MD). Each linear dimension was measured once from each of the 3 images and averaged resulting in 3 measures being averaged to get a single estimate for each brain dimension for each individual animal.

**Statistical Analysis**

*Corticosterone*

Many studies that use water borne CORT correct values for differences in body mass. However, there was no correlation between body mass and CORT concentration in this study. Therefore, we did not mass adjust the CORT values to prevent overcorrecting the data for variables that should not be in the model.

CORT concentrations were heteroscedastic; log transformations did not solve the problem of heteroscedasticity. Untransformed CORT concentration data were analyzed using both analysis of variance and Kruskal-Wallis tests. There was no difference in conclusions derived from the two different statistical analyses, and results from both analyses are presented.

*Body and Brain Morphology*

Animals that are larger in body size have larger body parts and brains. As we were interested in relative differences in body and brain shape in this study, we used mass adjustments to control for body size. Body morphological variables were adjusted for differences in body mass. Brain morphological variables were adjusted for differences in brain mass, which more closely aligns with brain shape than body mass. For each linear measurement, we conducted an ANCOVA with treatment as a fixed effect and either body mass or brain mass as a covariate. If necessary, values were log-transformed to
achieve linearity and homogeneity of slopes. Data were adjusted for body mass or brain mass by adding the residual value for each animal to the overall estimated marginal means (EMM) generated by the ANCOVA (see Appendix 1). By adding residuals to the EMM (instead of just using residuals), we get values that are more intuitive and thus easier to interpret.

After completing mass adjustments, we averaged the values all of the animals that were in the same bin. This provided one value for each variable for each bin and avoids the problem of pseudoreplication.

We then conducted a Principal Component Analysis (PCA) on the linear dimensions describing body or brain morphology to reduce the number of correlated variables we were analyzing. Before conducting the PCA we confirmed that the assumptions of PCA (KMO>0.5 and Bartlett’s test ≤ 0.05) were met. The PCA converted correlated morphological variables into uncorrelated principal components (PCs) using a varimax rotation.

**MANCOVA**

A multivariate analysis of covariance (MANCOVA) was used to determine if there were overall effects of the treatments on the dependent variables. Treatment was the fixed factor and room position was the covariate. Room position of the bins was used as a covariate in the model because there were slight differences in survival based on room position (Figure 4.1). While bins exposed to different treatments were spread equally throughout the room, we wanted to ensure that the positional effects were accounted for in our analyses.
Further, as there were statistically significant effects of the treatments on developmental stage, we also reran the analyses using stage in the model. We were concerned about including stage in the model as the differences in stage were subtle (a difference in Gosner stage of 31.9 to 32.9) and not biologically meaningful. After rerunning the analyses and including stage in the model, we did not find any changes in the outcomes of the analyses. To avoid over correcting the data, we chose not to include stage in the model.

Analyses of variance (ANOVA) were then used to follow up the MANCOVA to determine which variables were affected by the treatments. When appropriate, the Least Significant Difference (LSD) post-hoc analysis with a False Discovery Rate (FDR) adjustment to p-values was used. A 10% false discovery rate was used for the adjustment. All statistical analysis was conducted in SPSS.

RESULTS

Effects of Room Position

While I attempted to control for positional effects of where the tadpole bins were placed in the aquatic animal room, position had an effect on survivorship in the bins (Table 4.1, Figure 4.2). Because there was this effect, I chose to add room position as a covariate to the statistical models to account for any other potential effects it may have had. In addition to affecting survivorship levels, it also had an effect on brain mass and brain morphology (Table 4.2).

Effects of Chlorpyrifos
There was no effect of CPF on survivorship (Figure 4.3), body mass (Figure 4.5), brain mass (Figure 4.6), CORT concentration (Figure 4.7), pigmentation (Figure 4.8), or body position in the water column (Figure 4.9) when compared to controls (Table 4.2).

Tadpoles exposed to CPF were slightly more developed and weighed slightly more than tadpoles exposed to MTP during development (Figure 4.4, Figure 4.5, Table 4.2). However, the developmental level and mass of tadpoles exposed to CPF or MTP were not different than tadpoles exposed to the vehicle controls or to CORT (Figure 4.4, Figure 4.5, Table 4.2).

A PCA of tadpole body shape reduced seven body mass-adjusted dimensions to two principal components (Table 4.3). PC-1 loaded strongly for body length, tail length, tail depth, tail width, and muscle depth. PC-2 loaded strongly for body depth and body width (Table 4.3). There was no effect of CPF on tadpole body morphology (Figure 4.10, Table 4.2).

A PCA of tadpole brain shape reduced nine different body mass-adjusted brain dimensions to two principal components (Table 4.4). PC-1 loaded strongly for telencephalon length, telencephalon width, optic tectum length, optic tectum width, diencephalon length, diencephalon width, and medulla width. PC-2 loaded strongly for medulla length and olfactory bulb length (Table 4.4). Tadpoles exposed to CPF had an increase in the relative dimensions represented by PC-1 (Figure 4.11A, Table 4.2). There was no effect of CPF on PC-2 (Figure 4.11B, Table 4.2).

**Combined Effects of Chlorpyrifos and Metyrapone**

Treating tadpoles with both MTP and CPF was no different than CPF alone on tadpole survivorship (Figure 4.3), Gosner stage (Figure 4.4), body mass (Figure 4.5),
brain mass (Figure 4.6), pigmentation (Figure 4.8), body position in the water column (Figure 4.9), body morphology (Figure 4.10), or brain morphology (Figure 4.11).

Tadpoles exposed to both CPF+MTP had slightly higher corticosterone concentrations when compared to tadpoles exposed to CPF alone (Figure 4.7). However, neither treatment group differed from animals in the vehicle control group or MTP group (Figure 4.7).

Tadpoles exposed to CPF were slightly more developed and weighed slightly more than tadpoles exposed to MTP during development (Figure 4.4). Tadpole stage and mass when exposed to both CPF+MTP simultaneously was no different than when tadpoles were exposed to either CPF or MTP alone, or than the vehicle controls (Figure 4.4).

Tadpoles exposed to both CPF+MTP decreased the effects of CPF on the relative dimensions represented by PC-1 (Figure 4.11A).

**Effects of Corticosterone**

There was no effect of CORT on survivorship (Figure 4.3), developmental stage (Figure 4.4) or brain mass (Figure 4.6) on tadpoles exposed to CORT during development (Table 4.2).

Tadpoles exposed to CORT had decreased body mass (Figure 4.5), increased concentrations of CORT (Figure 4.7), reduced pigmentation (Figure 4.8), and were found more often with their bodies held vertically in the water column (Figure 4.9) than tadpoles in other treatment groups (Table 4.2).

Increased CORT concentrations during development had an effect on body morphology (Table 4.2), resulting in tadpoles with shorter bodies, shorter, shallower, and
wider tails, and deeper muscles than other treatment groups (Figure 4.10A, Table 4.2).

There was no effect of CORT on PC-2 (Figure 4.10B, Table 4.2), which loaded strongly for body depth and body width (Table 4.3).

Increased CORT concentrations during development also had an effect on brain morphology (Table 4.2). CORT did not affect PC-1. However, tadpoles with increased CORT during development had shorter medullas and shorter olfactory bulbs when compared to controls (Figure 4.11B, Table 4.2).

**Effects of Metyrapone**

MTP alone was used to determine if MTP had any biological effects to help us interpret the results on our CPF+MTP treatment group. In this group, there was no effect of MTP on survivorship (Figure 4.3), Gosner stage (Figure 4.4), body mass (Figure 4.5), brain mass (Figure 4.6), CORT concentration (Figure 4.7), pigmentation (Figure 4.8), body position in the water column (Figure 4.9), tadpole body morphology (Figure 4.10), or tadpole brain morphology (Figure 4.11) when compared to controls (Table 4.2).

Tadpoles exposed to MTP were slightly less developed and weighed slightly less than tadpoles exposed to CPF during development (Figure 4.4; Table 4.2). However, the developmental level and mass of tadpoles exposed to CPF or MTP were not different than tadpoles exposed to the vehicle controls or to CORT (Figure 4.4; Table 4.2). Tadpoles exposed to MTP also had relatively shorter and narrower telencephala, optic tecta, diencephala, and narrower medullas than tadpoles exposed to CORT (Figure 4.11), but there was no difference between tadpoles exposed to MTP and controls (Figure 4.11).

**DISCUSSION**
Northern Leopard Frog tadpoles in this study were exposed to either CPF, exogenous CORT, or CPF+MTP to determine if the physiological effects of sublethal CPF exposure are mediated by the HPA/I axis. Tadpoles that were exposed to exogenous CORT had physiologically realistic increases in CORT concentrations. Animals exposed to CPF did not have increased concentrations of CORT. Further, we found that tadpoles exposed to both CORT and CPF had changes in their relative brain shape (after adjusting for body mass). However, the changes in relative brain shape were different between tadpoles with increased CORT and tadpoles exposed to CPF. The effects of CPF also did not match the effects of CORT on the body mass, body morphology, pigmentation, or body position in this study. Although we hypothesized that CPF exerts some of its effects through changes in CORT, this hypothesis was not supported. We also hypothesized that if the effects of sublethal CPF exposure were due to changes in CORT concentrations, that MTP (a corticosteroid biosynthesis blocker), might be able to mitigate the effects of CPF. Interestingly, tadpoles exposed to both CPF+MTP showed a potential trend of decreased effects on relative brain morphology caused by CPF exposure. This is the first piece of evidence that neurodevelopmental effects of CPF are not caused by elevations in CORT. These results are discussed in more detail below.

**CORT concentrations**

It is important to point out that the concentrations of CORT achieved by the CORT treatment were physiologically relevant. Exposing animals to unrealistically high concentrations (pharmacological) of CORT can alter phenotypes in ways that are not relevant to normal physiology. In previous studies from our lab, physiologically realistic concentrations of water-borne CORT in Northern Leopard Frogs ranged from 1-17
pg/ml (unpublished). The CORT concentrations of tadpoles in this study fall within this range; tadpoles in the non-CORT exposed treatment groups had CORT values around 2.3 pg/ml. Tadpoles in the CORT exposure groups had CORT concentrations that were double the other treatments (5.7 pg/ml ± 0.42 pg/ml), but that fell within the physiologically realistic range of CORT concentration. These values confirm that our treatments increased CORT concentrations within physiological levels. Furthermore, the elevated concentrations of CORT found in the CORT treatment group had values that were similar to those induced by CPF in my other study (McClelland and Woodley in preparation; see Chapter 3 Aim 2 above). However, in the current study, exposure to CPF did not increase CORT concentrations. This could be due to the concentrations of CPF that tadpoles were exposed to. In my other project, exposure to 10 μg/L CPF resulted in increased CORT (McClelland and Woodley in preparation; see Chapter 3 Aim 2 above). Here, tadpoles were exposed to the nominal concentration of 5 μg/L CPF.

Effects of Chlorpyrifos, CORT, and Metyrapone on Brain Morphology

To determine the relative brain changes caused by pesticide exposures, we controlled for brain mass when analyzing brain shape. Tadpoles exposed to CPF had brains that were relatively larger in several dimensions (telencephalon length and width, optic tectum length and width, diencephalon length and width, and medulla width). Relative changes in brain morphology were also seen in previous research where tadpoles were exposed to 5 μg/L or 1 μg/L CPF had changes in brain shape (Woodley et al. 2015; McClelland and Woodley in preparation).

While CORT also affected relative brain morphology, tadpoles exposed to CORT had no differences in relative telencephalon length and width, optic tectum length and
width, diencephalon length and width, and medulla width from controls. Instead, tadpoles exposed to CORT had relatively shorter medulla and olfactory bulb lengths. CPF had no impacts on the shape of these brain regions in tadpoles. Thus, it is unlikely that CORT is involved in the neurodevelopmental effects of CPF.

Even though the relative brain changes found in animals exposed to sublethal CPF did not match the effects of tadpoles with elevated CORT, we do see a potential effect of MTP. The main effects of CPF on relative brain shape were decreased in tadpoles that were exposed to both CPF and MTP. However, it is important to note that there was no significant difference between the two groups as indicated by pairwise comparison tests.

MTP is often used by researchers to block CORT in studies analyzing the physiological effects of elevated CORT (Glennemeier and Denver 2002; Middlemis Maher et al. 2013). It is also used as a medical treatment for patients with Cushing's syndrome (hypercortisolism) (Verhelst et al. 1991). MTP functions by inhibiting the HPA/I axis from creating increased concentrations of CORT by inhibiting the enzyme 11β-hydroxylase (Glennemeier and Denver 2002; Jahn et al. 2003; Middlemis Maher et al. 2013). In this study, we did not see any correlation between the effects of CPF and elevated CORT, suggesting that the effects of CPF are not due to elevated CORT concentrations. Therefore, the potential mitigating effects must be acting though a mechanism other than CORT.

In human studies, MTP also affects the hypothalamus-pituitary-thyroid (HPT) axis, with MTP causing an increase in thyroid stimulating hormone (Samuels 2000). Further, it has been shown in rats that pre-natal CPF exposures results in a decrease of brain thyroxine concentrations in juveniles and adults (Slotkin et al. 2013).
together, this suggests that sublethal CPF exposures may be impacting the HPT axis in tadpoles causing the relative brain morphology changes found in this study. These effects may be able to be mitigated by MTP treatments counteracting the HPT axis effects. Future work should analyze the effects of CPF and MTP on the HPT axis in tadpoles to determine if MTP can reduce the impacts of low, dose organophosphate exposures in tadpoles.

**Effects of Chlorpyrifos and Metyrapone on Development**

Tadpoles exposed to CPF were also slightly more developed than tadpoles exposed to MTP during development. Further, when CPF+MTP were given simultaneously, the effect of CPF disappeared. However, the effects on Gosner stage were small when assessing developmental difference (CPF mean Gosner stage 32.9 ± 0.14, MTP mean Gosner stage 31.9 ± 0.26). These stages are differentiated by tadpoles having either one indentation in the tip of their hind limb (stage 32) or two indentations (stage 33) that will eventually form toes; other body parts and "key traits" remain stable during these stages (Gosner 1960). So, while a statistically significant difference was found, we caution the readers not to over interpret this finding, as it is unlikely that these differences are biologically meaningful.

**Effects of CORT**

While CORT is unlikely to be mediating the effects of low dose CPF exposures on neurodevelopment, there were numerous effects of elevated CORT concentrations in this study that can provide a better understanding of how chronically elevated concentrations of CORT can impact animal development. CORT plays an important role in mediating organismal responses to environmental changes (Reviewed in Dickens and
Romero 2013; Harris 2019). In the current study, CORT affected relative brain morphology, body size, relative body morphology, body pigmentation, and even body positioning.

*Body Size and Morphology*

Chronically elevated CORT concentrations impacted both body mass and body shape. Tadpoles with elevated CORT concentrations had decreased body mass. Further, even when controlling for body mass, increased concentrations of CORT also resulted in tadpoles having relatively smaller bodies with decreased tail lengths, decreased tail depths, increased tails widths, and increased tail muscle depths. In agreement with our findings, other studies have also found that elevated CORT concentrations in tadpoles resulted in decreased body mass and/or smaller bodies (Glennemeier and Denver 2002; Middlemis Maher et al. 2013). These studies also found that CORT affected tail morphology. Like the current study, previous work showed that increased CORT concentrations resulted in deeper tail muscles, but unlike this study, tail fins were also deeper (Glennemeier and Denver 2002; Middlemis Maher et al. 2013). Similar to the current study, Gabor et al. found that tadpoles with artificially elevated levels of CORT had decreased tail depths (Gabor et al. 2019).

CORT has also been shown to mediate phenotypic plasticity when tadpoles are exposed to predators or predatory cues (Middlemis Maher et al. 2013). Predator-induced changes in body morphology are variable and depend on the species of both the predator and the tadpoles, and may even be affected by environmental variables (Relyea 2001b; Benard 2004). Many studies have found that tadpoles have smaller bodies in the presence of predators (Reviewed in Benard 2004). Predator-induced changes are also usually
associated with increased tail depths, which may increase tadpole survival in the presence of predators by serving as a target to keep predatory strikes away from the body (Reviewed in Benard 2004). However, Relyea (2001b) found that while there was an effect on tail height in Wood Frogs, there was no effect on the tail height of Northern Leopard Frogs (the model used in the current study) exposed to predatory insects (Relyea 2001b). Increased tail muscle depth has also been seen in studies analyzing predator-induced morphology and, like other predator-induced traits, is also believed to be involved with a tadpoles ability to escape predation (Van Buskirk et al. 1997).

The fitness effects that we found of CORT on tadpole morphology are likely highly dependent on the environment that the tadpoles are in. If predators exist, these morphological changes may give the tadpoles an advantage. However, if no predators exist in their environment, and some other stressor has resulted in these morphological changes, the fitness consequences are more nebulous. It's possible there are costs associated with these CORT-induced changes.

**Pigmentation**

Tadpoles with higher CORT concentrations were much more likely to have lighter pigmentation than tadpoles in other treatment groups. The reduced pigmentation that is caused by increased CORT concentrations is not unique to tadpoles; it has been found in numerous other taxa. Previous work has shown that elevated CORT concentrations decreased melanin production in birds, lizards, frogs and fish, but to the best of our knowledge has not been reported in tadpoles (Nielsen 1978; Van der Salm et al. 2006; Roulin et al. 2008; Kindermann et al. 2013; San-Jose and Fitze 2013). There are also a number of studies that mention that tadpoles reared in the presence of predators or
a predator signal have color changes (McCollum and Van Buskirk 1996; Touchon and Warkentin 2008). While these are often black spots on the tail or increased red tail pigmentation, some studies have also reported achromatic predator morphs (Touchon and Warkentin 2008). San-Jose and Fitze (2013) suggested that the duration of the stress response may be responsible for the differences in color changes.

A simple explanation for our results on pigmentation relates to the physiological mechanism that connects the stress response and melanogenesis. CRF causes expression of the gene proopiomelanocortin (POMC) in the pituitary. POMC is cleaved to produce both ACTH and MSH (melanocyte stimulating hormone). MSH binds to melanocortin receptors to induce the synthesis of eumelanin (Ducrest et al. 2008). Thus, treatment with CORT would activate negative feedback to inhibit production of POMC and its products including MSH, thereby decreasing melanin production. Decreased melanin production results in lighter pigmentation (Arnold et al. 1975; Ermak and Slominski 1997; Slominski et al. 2004; Ducrest et al. 2008). Future work could be done to test whether these pigmentary changes are due to decreased melanin production or if it is solely a physiological change where pigments are concentrated in the chromatophores making animals appear lighter (Ligon and McCartney 2016). MS-222 causes melanin molecules to disperse within chromatophores (Bolker et al. 2005). Therefore, if the color differentiation exists before and after euthanasia with MS-222, then it is likely a change in the amount of melanosome pigments within the melanophores. However, if the color differentiation that exists disappears after euthanasia with MS-222, then the pigmentation differences were likely a physiological difference.

Body Position
Tadpoles that had high CORT concentrations were more likely to have their bodies vertically positioned with heads pointed up. While it was still a small percentage of animals (approximately 12% of animals), it was a noticeable phenomenon. Tadpoles in environments lacking dissolved oxygen are sometimes found in a vertical position with their heads at the surface (frogsafe.org.au). In this study, the tadpoles were not at the surface of the water, but instead found throughout the water column. Further, bins contained bubble stones making it unlikely that there were would be a lack of dissolved oxygen in the water. This position may be associated with stress in tadpoles and could be another noninvasive indicator of tadpole physiological state. Future studies should explore such behavioral responses to CORT treatment as this is the first report of such a CORT-induced positional effect.

Conclusion

This is the first study to assess the role of CORT when animals are exposed to low, commonly encountered concentrations of the organophosphate CPF. Exposure to exogenous CORT and exposure to CPF had different outcomes on tadpole development. Tadpoles exposed to CPF and CORT differed in their hormone profiles, changes in mass, pigmentation, relative body shape, and relative brain shape. This leads me to conclude that CORT is not a major transducer of the developmental changes seen in tadpoles exposed to low doses of CPF. Interestingly, there was a possible trend of MTP reducing the neurodevelopmental impacts of CPF exposure. More research is needed to determine how the neurological impacts of CPF are modulated, as well as investigating whether MTP ameliorates the effects of low-dose CPF exposures.
ACKNOWLEDGEMENTS

Laura Brannelly, Veronica Saenz, and Michel Ohmer assisted with collecting frog eggs for this research. Patrice Clemenza worked as a paid laboratory assistant for staging development and dissecting brain; Madison Durbin worked as a paid laboratory assistant for doing image analysis. This work was partially funded by the Society for the Study of Amphibians and Reptiles Roger Conant Grants in Herpetology and the PEO Scholar's Award (both SJM).
Table 4.1. Sample sizes for each treatment were 10 bins with each bin value the average of the surviving tadpoles in that bin (up to 10 tadpoles).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Number of Bins</th>
<th>Survival/Bin</th>
<th>% Survivorship (Mean)</th>
<th>% Survivorship (SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ethanol</td>
<td>10</td>
<td>10, 10, 10, 10, 9, 9, 8, 8, 7</td>
<td>90</td>
<td>3.33</td>
</tr>
<tr>
<td>CORT</td>
<td>10</td>
<td>10, 10, 9, 9, 9, 8, 7, 6, 6, 4</td>
<td>78</td>
<td>6.29</td>
</tr>
<tr>
<td>CPF</td>
<td>10</td>
<td>10, 10, 9, 9, 9, 8, 8, 7, 7, 5</td>
<td>82</td>
<td>4.90</td>
</tr>
<tr>
<td>CPF+MTP</td>
<td>10</td>
<td>10, 10, 10, 9, 9, 9, 8, 8, 7, 5</td>
<td>85</td>
<td>5.00</td>
</tr>
<tr>
<td>MTP</td>
<td>10</td>
<td>10, 10, 10, 9, 9, 9, 8, 7, 7, 7</td>
<td>86</td>
<td>4.00</td>
</tr>
</tbody>
</table>
Table 4.2. Results of analysis of variance for all dependent variables.

<table>
<thead>
<tr>
<th>Dependent variable</th>
<th>Effect of Treatment</th>
<th>Effects of Room Position (Covariate)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Multivariate Analysis</td>
<td>$F_{(48,144)} = 2.903, p &lt; 0.001^*$</td>
<td>$F_{(12,33)} = 4.136, p = 0.001^*$</td>
</tr>
<tr>
<td>Univariate Analyses</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Survivorship</td>
<td>$F_{(4,44)} = 0.854, p = 0.449$</td>
<td>$F_{(1,44)} = 0.015, p = 0.903$</td>
</tr>
<tr>
<td>Gosner Stage</td>
<td>$F_{(4,44)} = 2.828, p = 0.036^*$</td>
<td>$F_{(1,44)} = 1.507, p = 0.226$</td>
</tr>
<tr>
<td>Body Mass</td>
<td>$F_{(4,44)} = 9.245, p &lt; 0.001^*$</td>
<td>$F_{(1,44)} = 2.437, p = 0.126$</td>
</tr>
<tr>
<td>Brain Mass</td>
<td>$F_{(4,44)} = 1.062, p = 0.386$</td>
<td>$F_{(1,44)} = 4.310, p = 0.044^*$</td>
</tr>
<tr>
<td>Mass-adjusted Brain Mass</td>
<td>$F_{(4,44)} = 0.218, p = 0.927$</td>
<td>$F_{(1,44)} = 13.258, p = 0.001^*$</td>
</tr>
<tr>
<td>CORT Concentration</td>
<td>$F_{(4,44)} = 28.632, p &lt; 0.001^*$</td>
<td>$F_{(1,44)} = 0.023, p = 0.881$</td>
</tr>
<tr>
<td>Pigmentation</td>
<td>$F_{(4,44)} = 53.302, p &lt; 0.001^*$</td>
<td>$F_{(1,44)} = 1.969, p = 0.168$</td>
</tr>
<tr>
<td>Vertical Body Position</td>
<td>$F_{(4,44)} = 3.483, p = 0.015^*$</td>
<td>$F_{(1,44)} = 1.045, p = 0.312$</td>
</tr>
<tr>
<td>Body Morphology</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PC-1</td>
<td>$F_{(4,44)} = 143.922, p &lt; 0.001^*$</td>
<td>$F_{(1,44)} = 0.100, p = 0.753$</td>
</tr>
<tr>
<td>PC-2</td>
<td>$F_{(4,44)} = 0.088, p = 0.986$</td>
<td>$F_{(1,44)} = 0.374, p = 0.544$</td>
</tr>
<tr>
<td>Brain Morphology</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PC1</td>
<td>$F_{(4,44)} = 3.070, p = 0.026^*$</td>
<td>$F_{(1,44)} = 41.124, p &lt; 0.001^*$</td>
</tr>
<tr>
<td>PC2</td>
<td>$F_{(4,44)} = 4.391, p = 0.004^*$</td>
<td>$F_{(1,44)} = 0.085, p = 0.772$</td>
</tr>
</tbody>
</table>

a Principal components describe body mass-adjusted body morphology for Northern Leopard Frog tadpoles: PC-1 represents body length, tail length, tail depth, muscle depth, and tail width; PC-2 represents body depth and body width

a Principal components describe brain mass-adjusted brain morphology for Northern Leopard Frog tadpoles: PC-1 represents telencephalon, optic tectum, and diencephalon length and width, medulla width; PC-2 represents medulla length and olfactory bulb length

* $p < 0.05$

CPF = chlorpyrifos; PC = principal component
Table 4.3. Principal components analysis of 7 mass-adjusted body dimensions of Northern Leopard Frog tadpoles

<table>
<thead>
<tr>
<th></th>
<th>Principal Component</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PC-1</td>
</tr>
<tr>
<td>% of Variance</td>
<td>52.0</td>
</tr>
<tr>
<td>Eigenvalue</td>
<td>3.6</td>
</tr>
<tr>
<td>Factor Loading</td>
<td></td>
</tr>
<tr>
<td>body length</td>
<td>0.722</td>
</tr>
<tr>
<td>body width</td>
<td>-0.582</td>
</tr>
<tr>
<td>body depth</td>
<td>0.089</td>
</tr>
<tr>
<td>muscle depth</td>
<td>-0.711</td>
</tr>
<tr>
<td>tail length</td>
<td>0.831</td>
</tr>
<tr>
<td>tail width</td>
<td>-0.762</td>
</tr>
<tr>
<td>tail depth</td>
<td>0.874</td>
</tr>
</tbody>
</table>
Table 4.4. Principal components analysis of 9 mass-adjusted brain dimensions of Northern Leopard Frog tadpoles.

<table>
<thead>
<tr>
<th>Results of PCA</th>
<th>Principal Component</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PC-1</td>
</tr>
<tr>
<td>% of Variance</td>
<td>68.1</td>
</tr>
<tr>
<td>Eigenvalue</td>
<td>6.1</td>
</tr>
<tr>
<td>Factor Loading</td>
<td></td>
</tr>
<tr>
<td>Telencephalon width</td>
<td>0.748</td>
</tr>
<tr>
<td>Telencephalon length</td>
<td>0.923</td>
</tr>
<tr>
<td>Optic tectum width</td>
<td>0.882</td>
</tr>
<tr>
<td>Optic tectum length</td>
<td>0.893</td>
</tr>
<tr>
<td>Medulla length</td>
<td>0.300</td>
</tr>
<tr>
<td>Diencephalon width</td>
<td>0.839</td>
</tr>
<tr>
<td>Diencephalon length</td>
<td>0.843</td>
</tr>
<tr>
<td>Olfactory bulb length</td>
<td>0.103</td>
</tr>
<tr>
<td>Medulla width</td>
<td>0.899</td>
</tr>
</tbody>
</table>
Figure 4.1. Body and brain morphology. A. Northern Leopard Frog tadpole showing the linear dimensions used to describe tadpole body morphology: 1 body length, 2 body depth, 3 muscle depth, 4 tail depth, 5 tail length, 6 body width, 7 tail width; B. Dorsal and ventral view of a Northern Leopard Frog tadpole brain showing the linear dimensions used to describe brain morphology: 1 telencephalon length, 2 telencephalon width, 3 optic tectum length, 4 optic tectum width, 5 medulla length, 6 olfactory bulb length, 7 diencephalon length, 8 diencephalon width, 9 medulla width.
Figure 4.2. Effect of room position on survivorship. There was an effect of the position of tadpole bins on the shelving unit where animal bins were held on tadpole survivorship. To account for these effects, room position was used as a covariate for all statistical analyses. Mean +/- SEM is graphed, p>0.05, n=10 bins (see Table 4.1).
Figure 4.3. Effect of treatment on survivorship. There was no effect of treatment on tadpole survivorship. Mean +/- SEM is graphed, p>0.05, n=10 bins (see Table 4.1).
Figure 4.4. Gosner stage. Tadpoles exposed to CPF were slightly more developed than those exposed to MTP, however, neither were different than the controls. While there was a statistically significant effect on stage, this effect should not be over interpreted as stages ranged from 31.9 to 32.9, which are identified by very subtle changes in toe morphology (Gosner, 1960). Mean +/- SEM is graphed. Points labeled with different letters are significantly different, $p<0.05$, $n=10$ bins (see Table 4.1).
Figure 4.5. Body mass. Tadpoles exposed to CORT weighed less than tadpoles in all other treatments. Tadpoles exposed to CPF weighed slightly more than those exposed to MTP, however, neither were different than the controls. Mean +/- SEM is graphed. Points labeled with different letters are significantly different, p<0.05, n=10 bins (see Table 4.1).
Figure 4.6. Brain mass. A. While the brains from tadpoles exposed to corticosterone appear to have weighed less than those in other treatment groups, this effect was not statistically significant. (B) After correcting brain mass for body mass to correct for the overall size of the tadpoles (larger tadpoles have larger brains), the brains of tadpoles
from all treatment groups weight relatively the same. Mean +/- SEM is graphed, p>0.05, 
n=10 bins (see Table 4.1).
Figure 4.7. Corticosterone concentration. Tadpoles exposed to CORT had higher CORT than all other treatments. Tadpoles exposed to CPF+MTP had slightly higher CORT concentrations than tadpoles exposed to CPF alone, however, neither were different than the controls. Mean +/- SEM is graphed. Points labeled with different letters are significantly different, p<0.05, n=10 bins (see Table 4.1).
Figure 4.8. Pigmentation. Tadpoles exposed to CORT had less skin pigmentation than other treatment groups. Mean +/- SEM is graphed. Points labeled with different letters are significantly different, p<0.05, n=10 bins (see Table 4.1).
Figure 4.9. Body orientation. Tadpoles that were exposed to CORT during development were found more often with their bodies held vertically in the water column compared to the other treatments. Mean +/- SEM is graphed. Points labeled with different letters are significantly different, p<0.05, n=10 bins (see Table 4.1).
Figure 4.10. Body mass-adjusted body morphology. (A) Principal Component (PC) 1. Tadpoles that were exposed to CORT during development had relatively longer bodies, relatively shorter, thinner, and wider tails, and thicker tail muscles. Tadpoles that were exposed to CPF+MTP during development had relatively longer bodies, relatively shorter, thinner, and wider tails, and thicker tail muscles than controls, but were not different than tadpoles exposed to CPF or MTP alone.; (B) PC 2. There was no effect of treatment on tadpole body depths or width. Mean +/- SEM is graphed. Points labeled with different letters are significantly different, p<0.05, n=10 bins (see Table 4.1).
Figure 4.11. Brain mass-adjusted brain morphology (A) Principal Component (PC) 1. Tadpoles that were exposed to CPF during development had relatively longer and wider telencephala, optic tecta, and diencephala, and wider medullas. (B) PC 2. Tadpoles that were exposed to CORT during development had relatively longer medullas and olfactory bulbs. +/- SEM is graphed. Points labeled with different letters are significantly different, p<0.05, n=10 bins (see Table 4.1).
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McClelland SJ, Woodley SK. in preparation. Changes in brain development and behavior in amphibians exposed to putatively safe doses of the common organophosphorous pesticide chlorpyrifos


Chapter 5
Conclusions

Summary

The goal of this work was to help us better understand how low, ecologically relevant doses of organophosphorous pesticides are impacting vertebrate development. To do this, I analyzed the effects of commonly encountered doses of the organophosphate chlorpyrifos (CPF) in mesocosm (artificial pond) and laboratory studies using an anuran model. Anurans make an excellent model for this system because it is easy to control the timing and duration of exposures, they exhibit quick developmental processes, and they exhibit complex behaviors. Altogether, anurans give us insights into how vertebrates are impacted by environmental insults.

My first aim was to test the hypothesis that phenotypic changes observed in tadpoles exposed to CPF are caused indirectly by trophic cascades in the food chain when CPF kills pond zooplankton. By using mesocosms with either Daphnia pulex (zooplankton) that would be resistant to CPF and survive exposure, or mesocosms with Daphnia pulex (zooplankton) that were sensitive and would be killed by CPF, I was able to determine how CPF affects vertebrates in two different aquatic communities. I found that, regardless of the aquatic community, exposure to low, ecologically relevant doses of organophosphorous pesticides has direct effects on brain development. These effects persisted through metamorphosis, possibly impacting organisms after they have left the contaminated aquatic habitat. There was an interactive effect between CPF and zooplankton type on body shape. This was partially due to an unexpected effect of zooplankton type. Interestingly, in the control mesocosms (i.e. no CPF exposure), the
type of zooplankton in the community had an effect of body shape suggesting that these types may not be equivalent. While assessing the impacts of different zooplankton populations on vertebrate development are beyond the scope of this dissertation, this definitely brings up a line of ecological questioning that should be pursued and caution should be used when considering different zooplankton types in future studies.

After determining that the lowest, most commonly encountered doses of CPF directly impact neurodevelopment, I then aimed to test the hypothesis that concentrations of CPF that result in changes in brain shape will also produce behavioral and hormonal alterations. Using controlled laboratory settings, I exposed tadpoles to either 1 µg/L CPF or 10 µg/L CPF. I found that tadpoles exposed to very low, putatively safe concentrations of organophosphates during larval development had altered brain morphology, behavior, and hormone concentrations compared to control animals. Both the neurodevelopmental and neurobehavioral effects occurred in a nonmonotonic dose response. The neurodevelopmental effects found in this study were present at two life history stages, showing that early life exposures to CPF can be long lasting, persist though metamorphosis, and possibly impact animals even after they are no longer being exposed to the pesticide. However, even though there were nonmonotonic effects and exposure to 1 µg/L CPF resulted in neurological effects in both tadpoles and metamorphs, the exact same regions of the brain were not necessarily impacted in the same ways (Tables 5.1 and 5.2). During metamorphosis, the body and brain are rearranged to enable tadpoles to move from an aquatic environment to living as juveniles and adults in a terrestrial environment so we might not expect to see the same brain regions impacted as in tadpoles. Furthermore, neurological effects of CPF were also seen in both mesocosm and
controlled laboratory setting, though these effects were not necessarily the same (Tables 5.1 and 5.2).

In my third aim, I tested the hypothesis that the neurological changes found in animals exposed to low doses of organophosphates would be partially mediated by activation of the hypothalamus pituitary adrenal/interrenal (HPA/I) axis and elevated concentrations of corticosterone (CORT). Tadpoles that were exposed to CPF and tadpoles with artificially elevated CORT concentrations had changes in their brain shape. However, while CPF and CORT both impacted brain shape, they did so in different ways. Further, tadpoles exposed to CPF and CORT had differing hormone profiles, pigmentation, and relative body shape. This led me to conclude that CORT is not a major transducer of the developmental changes seen in tadpoles exposed to low doses of CPF. However, there was a trend that tadpoles that were exposed to CPF and metyrapone (MTP, a corticosterone biosynthesis blocker) had reduced neurological effects. It's possible that the effects of MTP on the body's natural HPA/I is different than when animals are exposed to exogenous CORT to artificially elevate their CORT levels. It's possible that it could also be that MTP may have other impacts beyond inhibiting CORT synthesis. In either case, MTP could represent a potential means of mitigating the neurological effects of CPF.

**Implications and Future Directions**

**Low Dose Effects of Organophosphate Exposures**

Each of these studies provided evidence that exposure to low, commonly encountered, putatively safe doses of organophosphorous pesticides during development directly affects animal development. These changes consisted of altered brain...
morphology, behavior, and CORT concentration. This was true even at concentrations that were lower than previously tested (1 μg/L), and which are found in surface waters throughout the US (Stone et al. 2014; EPA 2016). Changes in brain morphology, behavior, and CORT concentrations could be relevant endpoints for monitoring sublethal, low dose impacts of organophosphate exposures in future studies.

In every study that I conducted, there were changes in tadpole brain shape when animals were exposed to CPF. The brain is a very dynamic and plastic organ. By using PCA, I attempted to measure overall, gross morphological changes. While similar CPF concentrations continually caused changes in relative brain morphology, the changes were not always the same (see Table 5.1 and 5.2). Each study that was conducted had some environmental changes (laboratory vs mesocosm, number and timing of pesticide applications, etc.) that could affect how CPF is impacting neurodevelopment. However, it’s important to point out that regardless of the differences in how the brains changed, the same concentrations of CPF resulted in morphological changes in the brain in a replicable manner, regardless of the differences in the experimental design. This work is consistent with studies showing that amphibian brain development is remarkably sensitive to low levels of pesticides as well as biotic factors like conspecific densities and predators (Gonda et al. 2010; Liao et al. 2015; Woodley et al. 2015). My research found that CPF impacted brain development resulting in morphological changes in lab environments, mesocosm environments, and were even found in tadpoles that came from different community structures in mesocosms. This suggests that animals in a variety of environments would likely experience neurological alterations if their habitat is, or becomes, contaminated with low concentrations of organophosphates.
The brain changes seen in larval amphibians exposed to low doses of CPF persisted through different developmental stages carrying over through metamorphosis into the juvenile frogs. Our findings in metamorphs result from larval exposure to CPF because animals were removed from treatments prior to metamorphosis. Brain changes that span life history events are not a unique phenomenon. For example, brain changes caused by tadpole crowding affected both tadpole and juvenile Common Frogs (Rana temporaria) (Trokovic et al. 2011). In fact, embryonic and early life stage neurodevelopment is especially sensitive to environmental impacts with long-lasting effects across a range of animals (Whitney et al. 1995; Marco et al. 2011). These results show that developmental CPF exposures can have long lasting effects, even when animals have stopped being exposed to the toxin.

There were also functional consequences of these low dose exposures. While I only analyzed behavior in one of my studies it showed that low doses of organophosphates can cause behavioral changes. I observed that tadpoles exposed to 1 μg/L CPF spent more time near a novel object (an empty jar), one measure often used to identify boldness. This change could be the result of changes in how animals process or respond to stimuli in their environment. It is not clear if this behavior would be advantageous or detrimental, and would likely depend on the environment the animals is living in. For example, if the novel situation is associated with food it could be beneficial, but if it is associated with predators, it could adversely affect survival.

This dissertation found important low dose effects on multiple variables, but more work is still needed to help elucidate these findings. I showed that CPF impacts neurodevelopment, but the lack of histological analysis limits our ability to determine the
cause of these changes. Studies analyzing the neurocellular structures that result in gross morphological changes would help us determine if these changes are maladaptive or not. Furthermore, I found that developmental CPF exposure affected larval and juvenile life history stages. However, it is still unclear how these exposures are impacting physiological traits in adults, and whether or not these changes impact survival and fitness. Finally, animals in these studies were exposed to CPF in laboratory and mesocosm studies but did not analyze the impacts of CPF contamination in natural ponds. More work analyzing these effects in natural populations are still needed.

**Non-Monotonic Effects of Organophosphates at Low Doses**

The brain and behavioral effects of exposure to low doses of the organophosphate CPF occurred in a non-monotonic dose response manner. Animals exposed to the concentration of 1-5 μg/L CPF had brain changes and animals exposed to 1 μg/L CPF (5 μg/L CPF was not tested for behavior) had behavioral changes. These effects were not seen in animals exposed to the slightly higher, but still ecologically relevant concentration of 10 μg/L CPF. This matched previous work that showed when tadpoles are exposed to 5 μg/L CPF there neurological effects that were not seen in tadpoles exposed to 20 μg/L CPF (Woodley et al. 2015).

My research provides additional evidence that the impacts of CPF at low doses are not linear. Other studies have also found that the behavioral effects of CPF exposure occurred in a nonmonotonic manner (Levin et al. 2002; Levin et al. 2003). CPF exposures have also been shown to cause changes in thyroid concentrations, lipid peroxidation, and antioxidant enzymes that are occurring non-linearly (Wu et al. 2011; Slotkin et al. 2013).
Both low dose effects and nonmonotonic dose responses to toxins are key hallmarks of endocrine disrupting chemicals (Reviewed in Vandenberg et al. 2012). Endocrine disrupting chemicals mimic or interfere with endogenous hormones, and have been shown to have adverse effects on health and animal survival (Hayes et al. 2006; Heindel 2007; Myers et al. 2009) Organophosphates are not usually thought of as endocrine disrupters, but in addition to my work here, there are other studies showing CPF may be weakly estrogenic and impact thyroid hormones concentrations (Andersen et al. 2002; Slotkin et al. 2013).

Interestingly, animals exposed to the slightly higher, but still common concentration of 10 μg/L CPF did not have brain changes, but they did have increased corticosterone concentrations, and behavioral differences from controls. This suggests that even at doses where brain changes do not occur, there may still be consequences of low dose CPF exposure.

These nonmonotonic results demonstrate that exposure to different concentrations of low, sublethal concentrations of organophosphates can have complex impacts on animals that are not straightforward. This requires both scientists and regulatory agencies to act with caution when testing different doses of organophosphates for neurodevelopmental, behavioral, and endocrine endpoints.

Future work should test how other doses of CPF impact animal development. This could help us adjust our expectations of which endpoints will be affected and may have important consequences when it comes to mitigation strategies. In addition to testing different doses of CPF, this work should be repeated using other species and other types of organophosphates to elucidate how widespread these effects are.
Physiological Processes That Might Be Contributing to the Neurological Impacts of Low Dose Organophosphate Exposures

Finally, based on these studies, the brain changes caused by low dose organophosphate exposure are not mediated by corticosterone concentrations. However, there is some potential that metyrapone, potentially acting in the body beyond inhibiting CORT synthesis, could mitigate the neurological effects of CPF. More research is needed to determine how the neurological impacts of CPF are modulated, as well as investigating whether MTP might ameliorate the effects of low dose CPF exposures.

One potential pathway that might be modulating the neurological impacts of CPF is the hypothalamus-pituitary-thyroid (HPT) axis. Pre-natal CPF exposure in rats decreases brain thyroxine concentrations in juveniles and adults (Slotkin et al. 2013). These changes occurred in a nonmonotonic dose response, similar to the results of my dissertation research (Slotkin et al. 2013). Further, MTP has also been shown to increase thyroid stimulating hormone (Samuels 2000). Taken together, this suggests that sublethal CPF exposures may be impacting the HPT axis in tadpoles causing the relative brain morphology changes found in this study.

Conclusion

Overall, my dissertation research provides a better understanding of how low, ecologically relevant concentrations of organophosphorous pesticides are impacting vertebrate development. Larval exposures to the organophosphate CPF caused direct changes in brain shape, behavior, and hormone levels in both mesocosm and laboratory-based studies. Changes in brain shape also persisted through development. These changes occurred in a non-monotonic dose response and were not mediated by CORT
concentrations. Interestingly, when we tested a slightly higher, but still ecologically relevant dose of CPF, animals did not have changes in brain shape. Rather, these animals had elevated concentrations of CORT that also had effects on behavior. This suggests that low, putatively safe CPF exposures can be impacting animals in different, and complex ways, requiring both scientists and regulatory agencies to act with caution when making conclusions about safe concentrations of exposure. More work analyzing these effects in natural populations are still needed. However, the evidence from this work suggests that brain morphology, corticosterone concentrations, and behavior can be useful endpoints for monitoring sublethal, low dose impacts of organophosphate exposures. This work provides new insights for conservation and management strategies of animals living in habitats with organophosphate contamination.
Table 5.1. Effects of CPF on tadpole brain mass and morphology across all chapters in this dissertation and Woodley et al. (2015).

<table>
<thead>
<tr>
<th>Study</th>
<th>Chapter 3</th>
<th>Chapter 4</th>
<th>Woodley et al. (2015)</th>
<th>Chapter 3</th>
<th>Woodley et al. (2015)</th>
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<td>Laboratory</td>
<td>Mesocosm</td>
<td>Laboratory</td>
<td>Mesocosm</td>
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<td>Nominal Concentrations</td>
<td>1 μg/L</td>
<td>5 μg/L</td>
<td>5 μg/L</td>
<td>10 μg/L</td>
<td>20 μg/L</td>
</tr>
<tr>
<td>Actual Concentrations</td>
<td>0.25 μg/L</td>
<td>3.6 μg/L</td>
<td>4.4 μg/L</td>
<td>10.9 μg/L</td>
<td>16.7 μg/L</td>
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<table>
<thead>
<tr>
<th>Variable</th>
<th>Brain Changes when Compared to Vehicle Control</th>
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<tr>
<td>Brain mass</td>
<td>-</td>
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<tr>
<td>Mass-adjusted brain mass</td>
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</tr>
<tr>
<td>Telencephalon width</td>
<td>↑</td>
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<tr>
<td>Telencephalon length</td>
<td>↑</td>
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<tr>
<td>Optic tectum width</td>
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<td>Optic tectum length</td>
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<tr>
<td>Medulla width</td>
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<tr>
<td>Medulla length</td>
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<tr>
<td>Diencephalon width</td>
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<tr>
<td>Diencephalon length</td>
<td>↑</td>
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<tr>
<td>Olfactory bulb length</td>
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</table>

Woodley et al.: Mesocosm, One CPF application June 7, Tadpoles and Metamorphs
Chapter 2: Mesocosm, Multiple CPF applications, Metamorphs Only
Chapter 3: Laboratory, One CPF application at start, but treated water used for water changes (water covered with dark covers in cattle tanks), Tadpoles and Metamorphs
Chapter 4: Laboratory, CPF reapplied twice a week for three weeks, Tadpoles only
Table 5.2. Effects of CPF on metamorph brain mass and morphology across all chapters in this dissertation and Woodley et al. (2015).

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<tbody>
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<td>Mesocosm</td>
<td>Laboratory</td>
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<td>Nominal Concentrations</td>
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<td>5 µg/L</td>
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<tr>
<td>Actual Concentrations</td>
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<td>10.9 µg/L</td>
<td>16.7 µg/L</td>
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<td>Variable</td>
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<tr>
<td>Brain mass</td>
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<tr>
<td>Mass-adjusted brain mass</td>
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<td>Telencephalon width</td>
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<td>Telencephalon length</td>
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<td>Optic tectum width</td>
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<tr>
<td>Optic tectum length</td>
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<tr>
<td>Medulla width</td>
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<tr>
<td>Medulla length</td>
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<tr>
<td>Diencephalon width</td>
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<tr>
<td>Diencephalon length</td>
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<tr>
<td>Olfactory bulb length</td>
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Chapter 4: Laboratory, CPF reapplied twice a week for three weeks, Tadpoles only
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Appendix 1: Principal Component Analysis (PCA) Method Guide for SPSS

*Explore
- look at: descriptives, outliers
  Make sure all data looks good/was entered properly
  normality - p > 0.05 means data is normal
  variance - p > 0.05 means data is homoscedastic (equal variance)

*Linearity of slopes
- split file by trt
- use legacy plots -> scatter -> y-axis: body (or brain) variable
  x-axis: mass
- OR-
  - you can just use the legacy plot without splitting the file & then use – set markers by: trt

*Homogeneity of slopes, residuals, & EMM (estimated marginal mean)
MANCOVA
Analyze
-> general linear model
  -> multivariate

DV: all body (or brain) variables
Fixed factor: trt
Covariate: mass

Model: trt, mass, trt*mass
Save: Residuals, unstandardized
Options: EMM for trt

Check results:
- all trt*mass must not be significant (p > 0.05)
  - if this is not the case, try to transform data.
  - If they do all meet this:
    -> take residuals + EMM to get MA_variable (mass adjusted variable)
    (note: do this step in excel)

*PCA
Analyze
-> Dimension Reduction
  -> Factor

Variables: All MA_variables
Descriptives: univariate
Initial solution

Coefficient

Significance levels

KMO and Bartlett’s
  (anti-image optional)

Extractions: *Correlational
  *Based on Eigenvalues > 1
  *Max iterations 25
  * Unrotated factor
  * Scree plot

Rotation: *Varimax, Rotated Solution, Max iterations 25

Scores: Save as variables, * Regression, Display factor scores

Options: leave default

Check results:
  KMO should be >0.6 (needs to be >0.5 to do analysis)
  Bartlett’s should be p<0.05
  Variance explained (want this high; should be >60% variance explained)

Pause looking at results to check correlations: Analyze -> Correlate -> Bivariate
  (all p> 0.05)

Back to results:
  Rotates Matrix: Use to figure out what factors load onto which PC (>0.7)

*MANOVA on Factor Scores/Principal Components

Analyze
  -> General Linear Model
    -> Multivariate
      DV: all PC (REGR factor scores)
      Fixed factor: trt
      Post Hoc: Tests for: trt, choose your post hoc tests

*Graph Principal Components:
  If 1 ind variable:
    Legacy Dialogue
      -> Error Bars
      *Simple
      *Summaries for groups
        Variable: y-axis (PC), category axis: x-axis -> ind variable

(Trt)
  Bars represent: SEM, multiplier 1
IF multiple ind variables:
  Legacy Dialogue
  -> Error Bars
  *Clustered
  *Summaries for groups
    Variable: y-axis (PC), category axis: x-axis – 1st ind

variable (Trt),
  define clusters by – 2nd ind variable
Bars represent: SEM, multiplier 1