TIME-DEPENDENT EFFECTS OF PREDATION RISK ON STRESSOR REACTIVITY AND GROWTH IN DEVELOPING LARVAL ANURANS (*Lithobates pipiens*)

A Thesis Submitted to the Committee on Graduate Studies in Partial Fulfillment of the Requirements for the Degree of Master of Science in the Faculty of Arts and Science

TRENT UNIVERSITY

Peterborough, Ontario, Canada

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Environmental & Life Sciences M.Sc. Graduate Program

January 2017
ABSTRACT

Time-Dependent Effects of Predation Risk on Stressor Reactivity and Growth in Developing Larval Anurans (*Lithobates pipiens*)

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The predator vs. prey dynamic is an omnipresent factor in ecological systems that may drive changes in life history patterns in prey animals through behavioural, morphological, and physiological changes. Predation risk can have profound effects on the life history events of an animal, and is influenced by the neuroendocrine stress response. Activation of the hypothalamic-pituitary-adrenal/interrenal axis, and the induction of stress hormones (e.g., corticosterone (CORT)) have been shown to mediate the onset of inducible anti-predator defensive traits including increased tail-depth, and reduced activity. The predator-prey relationship between dragonfly nymphs and tadpoles can be a powerful model system for understanding mechanisms that facilitate changes in the stress response in accordance with altered severity of risk. It has been well demonstrated early in tadpole ontogeny that increased corticosterone (CORT) levels, observed within three weeks of predator exposure, are correlated with increased tail depth morphology. However, the reactivity of the stress response in relation to the growth modulation in developing prey has yet to be fully explored. Accordingly, this thesis assessed the stress and growth response processes in tadpoles that were continuously exposed to perceived predation risk later in ontogeny. Continuous exposure of prey to predation risk for three weeks significantly increased CORT levels, and tail depth. However, tadpoles exposed to six weeks of predation risk acclimated to the presence of the predator, which was observed as a significant reduction of stressor-induced CORT levels. In addition, although increased tail depth has been attributed to predator defense, predator-naïve tadpoles began to display similar tail depth
morphology as treated tadpoles at the six week time point. Thus, this thesis suggests that the stress response in lower vertebrate systems (e.g., tadpoles) may operate in a similarly complex manner to that observed in higher vertebrates (e.g., rats), for which severity of risk associated with the stressor aids in defining activity of the stress response. Moreover, the lack of morphological difference between treatments among tadpoles exposed later in ontogeny suggests that the mechanisms for inducing defenses are normal morphological traits in the development of the animal. This thesis paves the way for future research to elucidate the relationship between the neuroendocrine stress response and hormonal pathways involved in growth modulation in the presence of environmental pressures.

Keywords: Corticosterone, R. pипiens, Tadpole, Acclimation, Habituation, Predation Risk, HPA/HPI axis, Growth Modulation, Inducible Defenses
Acknowledgements

I would first like to thank my thesis advisors Dr. Leslie Kerr, and Dr. Dennis Murray for giving me the opportunity to be involved in such thrilling and innovative research. I entered into the research world not knowing what to expect, and although the path to completion was unpredictable and challenging, the continuing support and encouragement from my advisors made it possible to reach the end point with the feeling of great success and accomplishment. The guidance I received helped me build the confidence I have in myself as a researcher, and has made me a more well-rounded individual. I am gratefully indebted to their belief in my abilities to succeed and carry on with my aspirations and dreams.

I would also like to thank the people who generously helped me along the way with my research, and who opened their doors to offer a helping hand whenever I needed it: Debbie Lietz, Dr. Gary Burness, Dr. Hugo Lehmann, Dr. Neil Emery, Dr. Holly Bates, Dr. Amanda Bennett, and Dr. Thomas Hossie. It is truly appreciated. I am also grateful to belong to such a supportive lab group, the Murray lab has always provided thoughtful discussion and feedback to help with the progression of my thesis.

Lastly, but with great importance, I would like to express my extreme gratitude to my friends and family for their unconditional love and support throughout my academic years. I cannot express how grateful I am for their unfailing effort to always lend an ear whenever I needed one to talk about the research that became a central part of my life for the past three years.

I can’t thank these people enough, without their support I wouldn’t be in the position I am today, and for that I will forever be grateful. Thank you.
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Chapter 1: General Introduction

Vertebrate Neuroendocrine Stress Axis

The vertebrate neuroendocrine stress axis plays a fundamental and multifaceted role in influencing the coping responses of an animal to challenges or threats in its environment. Coping responses include physiological (e.g., hormone or morphological), and behavioural (e.g., freezing, fleeing, fighting) adaptations/changes that have been formed through evolution to increase survival, and ultimately, fitness (Denver, 2009a; Leung and Woo, 2010; Hegab and Wei, 2014). Upon perception of a stressor (defined broadly as a challenge within an animal’s environment that upsets homeostasis) energy allocation is altered with the activation of the stress axis, specifically the hypothalamic-pituitary-adrenal (HPA) axis in mammals or the hypothalamic-pituitary-interrenal (HPI) axis in non-mammals. The HPA/HPI axis modifies physiological and behavioural systems (i.e., coping mechanisms), which work in concert to re-establish homeostasis (McEwen and Wingfield, 2003). Responses to environmental challenges are influenced by both internal processes (e.g., metabolic, hormonal, immunological) and the type of stressors (e.g., food restriction, perceived predation risk). The amount of energy allocated towards maintaining homeostasis is known as allostatic load (McEwen and Wingfield, 2003), and is influenced by the interaction between genetic and environmental factors. Thus, although the general structure and function of the stress response is thought to be similar among vertebrate taxa, the perception of the stressor and the life history of an animal (e.g., developmental rate, reproductive success, body condition) are species specific, and can influence the corresponding physiological and behavioural changes in response to environmental challenges (stressors).

As noted, the perception of environmental stressors activates the HPA axis in mammals, or the HPI axis in nonmammalian species (Denver, 2009a). The hormonal cascade that ensues
begins with the production and secretion of corticotropin-releasing hormone (CRH) from a cluster of neurons in the hypothalamus, which in turn act on the anterior pituitary gland to promote the synthesis and release of adrenocorticotropic hormone (ACTH), which is released into the peripheral circulation. It is the binding of ACTH to its cognate receptors within the adrenal cortex that promotes increased synthesis and release of corticosterone (CORT). Stressor-induced CORT levels (i.e., higher than non-stressed or base-line levels of CORT) will ultimately act in a negative feedback manner on the hypothalamus and pituitary to reduce the synthesis and secretion of CRH and ACTH. This negative feedback loop is essential for re-establishing basal levels of CORT once the stressor no longer poses a threat. The actions of CORT are mediated by mineralocorticoid (MR) and glucocorticoid (GR) receptors. MRs have a higher affinity for CORT, whereas GRs have a lower affinity for CORT (Denver, 2009a). Thus, MRs regulate the actions of basal CORT whereas GRs are responsible for the actions of stress-induced CORT (Romero, 2004; Denver, 2009b). As such, distinguishing the differences between the MR- and GR-mediated responses can aid in understanding how an animal is responding to changes in environmental conditions.

The structure and function of the stress axis (i.e., HPA/HPI axis) has been studied extensively across vertebrate taxa, showing similarities in the distribution pattern of stress-related neurohormones (e.g., CRH) in the brain between mammalian and non-mammalian species (Lovejoy and Balment, 1999; Yao et al., 2004; Yao et al., 2007; Denver, 2009b). Genes associated with the HPA axis have even been identified in invertebrate taxa, demonstrating the ancient origins of the HPA axis (Stefano, et al., 2002). Distinct similarities in the structure and function of the HPA axis among vertebrate taxa make it possible to use lower vertebrate models
to study the role of the stress response in animal development, and in physiological and behavioural adaptation to changes in the environment.

**Characteristics of the Stressor and Stress Profiles**

When an animal experiences an environmental challenge, it is the increase in circulating CORT that helps mediate the onset of physiological (e.g., increase in metabolism, change in cardiovascular function) and related morphological (e.g., increase in tail depth, decrease in body size) coping responses, which in conjunction with behavioural (e.g., flee, freeze, fight) coping responses, help to re-establish homeostasis and increase survival/fitness (Sapolsky, 2000; Hu et al., 2008). The reactivity of the stress response is dependent, in part, upon the characteristics of a stressor (e.g., type, severity, duration), which influence the profile of the stress response (e.g., acute, chronic) (Denver, 2009a). Moreover, stressors experienced at different stages of development can impact the fitness and life history (e.g., body condition, rate of development, reproductive success) of the animal later in life (Kulkarni and Buchholz, 2014).

The activity and profile of the stress response is influenced by a range of stressor types, such as competing for resources when placed in high density areas (Schreck, 1981; Glennemeier and Denver, 2002), food limitation (Crespi and Warne, 2013), and perceived predation risk (Middlemis Maher et al., 2013; Fischer et al., 2014). The magnitude of the response elicited by animals to these stressors is dependent on the magnitude of the perceived threat. For example, the response of prey to predation risk is greater when the predator is larger (Chivers et al., 2001). However, when the predator cannot be seen and detection of nearby risk is dependent upon other sensory cues (e.g., auditory, olfaction), the magnitude of the stress response in prey is not moderated by visually perceived severity and is dependent on innate or species specific perception of risk (Chivers et al., 2001). For example, rodents respond to odor cues from cat
feces, (Sullivan and Gratton, 1998), whereas tadpoles show a reactive stress response to
dragonfly nymph olfactory cues (Hossie et al., 2012; Middlemis Maher et al., 2013). For
tadpoles, the use of olfactory cues allows prey to detect inconspicuous predators through
kairomone cues released by the predator and/or via conspecific alarm cues from consumed prey
(e.g., cues from chewed or digested tadpoles) (Rajchard, 2006; Schoepner and Relyea, 2009).
Interestingly, kairomone and conspecific alarm cues induce variable anti-predator defense
mechanisms in tadpoles, where the detection of both kairomone and conspecific alarm cues is
required to induce both behavioural and morphological defenses in tadpoles (Schoepner and
Relyea, 2009; Hettyey et al., 2015). This example suggests that prey may be able to distinguish
between the severity of cues in order to mount an appropriate coping response, and thereby limit
the allocation and expenditure of energy (Steiner, 2007; Schoepner and Relyea, 2009; Hettyey
et al., 2015).

The duration of exposure to a stressor can also alter the perception of risk and therefore,
the elicited response to stressors may be short-term, ranging from seconds to minutes (i.e., acute
responses) (Wingfield et al., 2011; Dickens and Romero, 2013), or long-term, ranging from days
to months (i.e., chronic responses) (McEwen, 1998; Wingfield and Romero, 2001). A short-term,
or acute, stressor typically briefly activates physiological and behavioural coping mechanisms
that respond to the short-term duration of the stressor (Sapolsky, 2000; Hu et al., 2008). For
example, *Lithobates sylvaticus* tadpoles exposed to predator cues for 90 minutes have elevated
CORT levels during the first two minutes of exposure, however, CORT levels decreased shortly
thereafter (Bennett et al., 2016). In contrast, in response to a persistent or prolonged stressor,
CORT levels remain elevated and morphological changes are more typically observed
(Middlemis Maher et al., 2013). Importantly, chronically elevated CORT levels may be costly as
they can leave the animal susceptible to the harmful physiological effects (e.g., growth inhibition, decrease in immune function, decrease in reproductive success) (Romero, 2004). However, negative feedback mechanisms of the stress response are activated to reduce continuously elevated CORT levels (e.g., adaptation), which lower the risk of harmful effects of high CORT levels. For example, *Lithobates pipiens* tadpoles chronically exposed to predation risk show elevated CORT levels after two weeks as well as anti-predator morphological changes (e.g., greater tail depth) (Middlemis Maher et al., 2013), however, after three weeks of predator exposure in *Lithobates sylvaticus* tadpoles, the CORT levels decline to near base-line levels, reducing the harmful effects of elevated CORT on the tadpole (Bennett et al., 2016).

Acute and chronic CORT responses have been well established in mammals. For example, ground squirrels exposed to dog predation show an elevated CORT response in the first five minutes of exposure, followed by declining CORT levels two hours after exposure (Hubbs et al., 2000). In response to a chronic stressor, rodents exposed to handling stress for two weeks show elevated CORT levels. However, as the association of handling with a high degree of risk decreases after two weeks of handling without harm (i.e., they acclimate to the stressor), stressor-induced CORT levels decline significantly (Dobrakovova and Kvetnansy, 1993). A reduction in CORT levels after acute or long-term exposure may be influenced by the reduction of the perception of risk associated with the stressor. A decrease in the perceived severity of the stressor over time may allow the animal to reassess the degree of risk, and reduce CORT altering related physiological and behavioural responses to re-establish homeostasis. However, if the degree of perceived risk remains severe, elevated CORT levels will persist, and the negative consequences associated with elevated CORT levels may occur; the severity of these
consequences has been shown to be dependent on the life stage of the animal (e.g., neonatal, larval, adult) (Caldji et al., 2001; Kalinichev et al., 2002; Kulkarni and Buchholz, 2014).

Stressors experienced early in development have been shown to have powerful and long-lasting influences on the physiology and behaviour of an animal by altering brain development, affecting the reactivity of the stress response of the animal later in life (Caldji et al., 2001; Kalinichev et al., 2002). For example, neonatal handling stress in rodent pups handled every day for two weeks altered the reactivity of the stress response of the adult rodents to increase anxiety-related behaviours, such that nearly 50% lower CORT response to moderately severe stressors was observed when compared to rodents that were not handled as pups (Caldji et al., 2001; Kalinichev et al., 2002). Adult amphibians are also affected by stressors experienced early in life (i.e., prior to metamorphosis), the effects of which can alter the transition from the larval stage and even carryover into the adult stage (Kulkarni and Buchholz, 2014). For example, western spadefoot toad tadpoles continuously exposed to severe stressors (e.g., pond drying) early in ontogeny (e.g., after three weeks of development) accelerate metamorphic timing to shorten the larval period, but have poor growth and development (i.e., poor body condition) at the timing of metamorphosis (Gomez-Mestre et al., 2013; Kulkarni and Buchholz, 2014). However, if the severity of a stressor decreases later in ontogeny (e.g., six weeks of development), acceleration in growth and development (i.e., compensatory catch up growth) may be possible to re-establish homeostasis to reach optimal body size for metamorphosis (Dennis et al., 2011).

Although the HPA/I stress axis is the initial neuroendocrine pathway activated in response to environmental change, the stress response interacts with the sympathetic nervous system (a mediator of the fight-or-flight response), which contributes to the regulation of other hormonal pathways involved in the regulation of coping mechanisms allowing for behavioural
and physiological/morphological plasticity. Further, changes in the perception of the severity of a stressor can alter the function of the HPA/I axis, and dampen the CORT response to allow an animal to achieve normal life-history trajectory (e.g., growth and development, reproductive success, body condition) (Crespi and Warne, 2013).

The Influence of CORT on Physiological, Morphological, and Behavioural Systems

The increase in CORT during the stress response adaptively modulates internal energy stores and growth regulation by acting on endocrine pathways, including the thyroid hormone (TH) and growth hormone (GH) axes, where both hormonal axes influence metamorphic timing, and development in amphibians (Rousseau et al., 1999; Rousseau et al., 2007). Although CORT plays an integral part in energy allocation and growth modulation, maintaining homeostasis requires hormonal systems to work in concert to establish appropriate coping responses to environmental stressors.

 Environmental challenges are strong selective pressures that impact the onset and development of phenotypic plasticity (e.g., behavioural or physiological/morphological plasticity) (Hossie et al., 2012; Middlemis Maher et al., 2013). The activation of the stress response is essential for the development of adaptive responses to environmental stress (Denver, 1999). For example, behavioural responses in prey exposed to continuous predation risk show increased CORT and reduced foraging and activity in general to lessen the risk of detection (Hossie et al., 2012). As the exposure to predation risk continues (e.g., two to three weeks), the development of anti-predator morphology (e.g., greater tail depth) also is observed (Hossie et al., 2012; Middlemis Maher et al., 2013), and is correlated with increased CORT levels (Middlemis Maher et al., 2013). In addition, using exogenous CORT administration in conjunction with an inhibitor of CORT synthesis (metyrapone; MTP) demonstrated that MTP could suppress the
CORT-induced tail depth of tadpoles (Hossie et al., 2012; Middlemis Maher et al., 2013). This, illustrates a mediating role played by CORT on phenotypic responses.

The onset of anti-predator morphology (e.g., tail depth) alters energy allocation, resulting in developmental consequences (e.g., reduced growth, poor body condition) (Walzer et al., 2015), underlying the thrifty phenotype hypothesis (Barker and Clark, 1997). However, as a learned response to the lack of severity/threat of the stressor develops over time (i.e., habituation), and the animal becomes accustomed (i.e., acclimatized) to the presence of stressor, CORT levels decrease as the stressor is no longer perceived as a threat. The habituation- and acclimation-induced decreases in CORT levels causes re-allocation of energy resources in accordance with the life history of the animal (e.g., body size, developmental rate) (Romero, 2004; Capellan and Nicieza, 2007). Habituating and acclimating stress responses have been observed in both mammalian and non-mammalian species (Romero, 2004; Dahl et al., 2012). For example, habituation has been observed in tadpoles that were exposed to predation risk for three weeks, showed no change in CORT levels when reintroduced to the predator cue for 90 minutes (Bennett et al., 2016). However, a decrease in stressor-induced CORT levels observed in rats exposed to handling stress for two weeks has been described as an acclimating stress response (Dobrakovova and Kvetnansy, 1993). Although acclimating to a stressor reduces the detrimental physiological consequences associated with prolonged elevated CORT levels, acclimating to one stressor can heighten the CORT response to other future stressors, a concept known as facilitation (Romero, 2004).

The neuroendocrine stress axis is affected by many factors, including the characteristics of both the stressor (e.g., duration, type, severity) and the animal (e.g., genetics, past experiences), which interact to influence the magnitude and duration of the coping responses that
are mounted. Selective environmental pressures trigger the HPA/IC axis, which impacts the fitness of the animal and shapes life history traits (e.g., body condition, developmental timing, and reproductive success). Exploring the structure and function of the HPA/IC axis in lower vertebrates will unravel components of the stress response, and the relation to animal survival and fitness.

**Goal of Thesis**

Amphibian tadpoles provide a useful model to study the development of the stress response thanks to similarities in the functionality and structure of the HPI/A axis between tadpoles and higher vertebrate systems (including mammals and birds) (Denver, 2009a). The effects of prolonged predator exposure on tadpole ontogeny were examined at three and six weeks of exposure to test the hypothesis that the predator-induced stress response would diminish after six weeks of predator exposure. It was postulated that older, larger-bodied tadpoles would not perceive the continuous exposure to dragonfly nymph predation with the same degree of severity compared to younger, less developed tadpoles. Preliminary data (Longhi et al., unpublished) indicates an increase in CORT levels and greater tail depth in tadpoles after three weeks of predator exposure; however, it is unknown what type of stress response or morphological response would be observed beyond this time point. Past studies were unable to demonstrate a lack of tail depth variation between predator exposed and predator naïve tadpoles after six weeks of predator exposure, suggesting that predator exposure may not be perceived as a risk later in tadpole ontogeny (Hossie et al., 2010). Thus, tadpoles were exposed to an additional three weeks of predation risk (i.e. predator exposure for six weeks) to determine the relationship between tadpole CORT levels and morphology over this prolonged stressor. Following this, tadpole tail depth and body size were assessed to determine whether body size is
altered to compensate for the onset of defensive morphology (Chapter 2). A conclusive formation of the ideas from this work will focus on the relevance of this thesis to other areas of stress-related research by focusing on the activity of the stress response in relation to environmental stressors experienced throughout development, and the commonalities of the stress response observed among vertebrate systems. Suggestions for future research will include investigating different characteristics of a stressor (e.g., increased severity, different type of predator) and profiles of the stress response (e.g., facilitation) in lower vertebrate systems, which will aid in building a more comprehensive understanding of the similarities between vertebrate taxa (Chapter 3).
Chapter 2

Acclimating to Predation Risk: Decreasing Stress Hormones and Predator-Induced Morphology in *Lithobates pipiens* Tadpoles
ABSTRACT

Perceived predation risk can lead to changes in both morphology and behaviour of animals, and such changes ultimately may drive differential fitness and life history strategy. The neuroendocrine stress axis is known for mediating phenotypic plasticity in anuran tadpoles, with augmented corticosterone (CORT) coinciding with increased relative tail depth. The physiological processes underlying this response have yet to be elucidated, especially for the later stages of tadpole development when animals may be less reactive to prolonged exposure to perceived predation risk (PPR). We investigated the effect of PPR on stress hormone production and morphological development in *Lithobates pipiens* tadpoles, at both 3 and 6 weeks of exposure to predation risk cues. Whole-body CORT levels declined from 3 to 6 weeks of continuous exposure to PPR, corresponding to concurrent change in tadpole body mass and tail depth. Our findings suggest that the mediating effect of CORT on tadpole tail depth diminishes after prolonged exposure to perceived predation risk, implying that animals become desensitized to risk over time. Furthermore, tadpoles may be exhibiting changes in growth modulation, such as catch-up or compensatory growth later in ontogeny, to offset early developmental costs due to anti-predator changes in morphology.
1. Introduction

All organisms are exposed to environmental stressors, and the hypothalamic-pituitary-adrenal/interrenal (HPA/HPI) axis is the primary physiological system allowing stress responses to be calibrated according to the local environment and attendant risk levels (Glennemeier and Denver, 2002a; Denver, 2009). It is understood that animals respond physiologically to chronic perceived predation risk (PPR) by increasing whole body corticosterone levels (Denver, 2009), and in the case of anuran tadpoles, this response coincides with changes in morphology (Maher et al., 2013). Given that the addition of exogenous CORT to water increases tadpole tail depth in a similar manner as does exposure to chemical cues associated with predation risk (Glennemeier and Denver, 2002b; Maher et al., 2013), stressor-induced morphological changes have been attributed to increased circulating CORT levels. Additionally, blocking CORT synthesis at the adrenal level restricts tail depth growth when tadpoles are in the presence of predation risk cues (Hossie et al., 2010; Maher et al., 2013), further reinforcing the close linkage between perceived risk, CORT, and morphology.

Corresponding with the development of predator-induced traits, tadpoles experience lower body mass following exposure to PPR early in development (Relyea and Werner, 2000; Relyea, 2001). This reduced body mass likely reflects reallocation of energy resources from growth to production of anti-predator traits (Schoeppner and Relyea, 2008), although a reduced energy intake also may contribute to body mass reduction (Steiner, 2007). Tadpoles are highly responsive (behaviorally and morphologically) to PPR early in development (Relyea, 2003; Ireland et al., 2007), but show reduced sensitivity to such cues later in development (Hossie et al., 2012). Indeed, differences in tail morphology among tadpoles decline later in ontogeny, regardless of the level of predation risk (Hossie et al., 2012; Bennett and Murray, 2015),
although the underlying physiological mechanism behind this convergence has yet to be explored.

As perceived predation risk exposure and perception of the stressor extends through time, CORT levels may become down-regulated, a concept known as acclimation (Romero, 2004). Acclimation is suggested to arise when the stressor is no longer perceived by the animal with the same initial magnitude of threat (Romero, 2004), as is seen in adult rats in longitudinal response to chronic stressors such as handling (Dobrakova and Kvetnansky, 1993) and restraint (Stamp and Herbert, 1999; Simpkiss and Devine, 2003). Adapting to a stressor involves maintaining homeostasis via allostasis, which allows the animal to respond appropriately (e.g., hormonally, morphologically) to environmental stressors (McEwen, 1998; McEwen and Seeman, 1999). Furthermore, allostatic load can be reduced by stressor acclimation, thereby lessening the negative physiological consequences associated with long-term stress (CORT) exposure (McEwen, 1998; McEwen and Seeman, 1999). For example, acclimating to PPR can reverse CORT-induced effects on metabolic function (e.g., reduced growth), thereby alleviating energy costs associated with mounting predator-induced morphological changes. Thus, the effect of CORT on tadpole growth and body size may be reversed as the degree of PPR weakens over time, as is seen in other vertebrates (Schoech et al., 1997; Kitaysky et al., 1999). Yet, whether the down regulation of CORT can lead to reversal of expression of predator-induced traits (e.g., changes in tail depth) is unknown, as predation risk-induced changes in CORT levels have yet to be explored following prolonged exposure to PPR.

Current research has yet to examine the role of acclimation in the plastic responses of tadpoles to PPR. We examined CORT level, body size, and morphology of anuran larvae over six weeks, with and without exposure to predation risk cues, to determine whether tadpoles
acclimate to predator-induced stress through hormonal down regulation. We predicted that, as tadpoles acclimate to predation risk cues, whole body CORT will decline, corresponding to increased growth rates resulting in reduced differences in body size between predator-reared and predator-naïve tadpoles, later in ontogeny. Furthermore, as tadpoles typically increase relative tail depth late in ontogeny, regardless of exposure to PPR (Hossie et al., 2012; Bennett and Murray, 2015), we predicted that tail morphology would converge among older tadpoles notwithstanding PPR exposure and CORT levels.

2. Methods

2.1 Egg Collection

Northern leopard frog (Lithobates pipiens) egg masses (N=3) were collected near Trent University, Peterborough, ON (44°36’N -78°26’W) in the spring of 2014. Late-instar dragonfly nymphs (family: Aeshnidae) were collected from nearby ponds, and were used to induce PPR. Egg masses were housed outdoors in plastic bins in reverse-osmosis (RO) water reconstituted with replenish (5 mL in 20L of RO to raise General Hardness by 2 meq/L), and left to hatch and develop until Gosner stage (GS) 24 (Gosner, 1960). Upon reaching GS24, tadpoles were considered to be free-swimming larvae and were brought indoors where they were randomly placed into their respective treatments. Environmental chambers were maintained at 18°C on a 14:10 light:dark cycle.

2.2 Predator Exposure, Three or Six Weeks

During experimental trials (predator exposure for either three or six weeks) tadpoles were housed in eight glass aquaria (N=8 tadpoles per aquarium; 33.0 cm L x 15.20 cm W x 20.30 cm H) filled with 7L of reconstituted RO water. The predator treatment (N=4) consisted of one dragonfly nymph per tank, housed in a floating plastic cage (17.15 cm L x 7.62 cm W x 7.62 cm
allowing water flow through. Nymphs were fed three tadpoles each, every other day, for the duration of the experiment. Empty breeding cages were placed in control tanks (N = 4). Aquaria were cleaned on alternate days with a full water change and tadpoles were fed ground algae discs (Omega One Veggie Rounds™) *ad libitum*. Low mortality was observed at only 2%, and tadpoles were not replaced. Tadpoles were euthanized at the designated time (three or six weeks of exposure) by immersion into a 0.1% benzocaine solution (Boorse and Denver, 2003; Crespi and Warne, 2013), and were then weighed, staged (Gosner, 1960), and photographed against a scale bar using a Nikon D70 digital camera equipped with a Tamron 90 mm macro lens, prior to being flash frozen in liquid nitrogen and stored at -80°C. Tail depth was measured from the greatest fin depth observed in photographs using ImageJ software (Rasband, 2012).

2.3 Corticosterone Radioimmunoassay

Tadpole samples were processed for corticosterone radioimmunoassay (RIA) according to Bennett et al. (2016). Briefly, tadpoles were homogenized in 500 μL of water, sonicated on ice for 30 seconds, and stored overnight at -80°C. The next day, 500 μL of methanol was added to tadpole homogenate, vortexed for 30 seconds, and stored at -80°C until extractions were performed. Dichloromethane extraction (adapted from Newman et al., 2008) was used to collect corticosterone from tadpole homogenate (see Bennett et al., 2016 for details). A double antibody radioimmunoassay kit (MP Biomedicals, Orangeburg, NY, USA) was used to measure corticosterone levels in duplicate from extracted tadpole samples. Mean (± standard error) inter-assay variation was 11.76% (± 1.19%; N=78), and the mean intra-assay variation was 12.85% (± 2.31%; N=35).
### 2.4 Statistical Analysis

Relative tail depth was determined using the independent size metric size correction (ISMSC; Berner, 2011). Briefly, tail depth was regressed against log-transformed mass using ordinary least squares regression with the residuals from that regression considered to be a size-independent measurement of tail depth (hereafter referred to as relative tail depth). We used three linear mixed-effects models to evaluate the interaction between predator exposure (fixed factor: predator, no predator) and week of exposure (fixed factor: three weeks, six weeks) on whole body corticosterone (ng/g bwt), relative tail depth, and body mass (g), with individual tadpole as the random effect, whereas an ordinal multinomial regression was used on developmental stage. Significant main effects and interactions were analyzed by Tukey's HSD post hoc analyses.

### 3. Results

Overall, PPR-exposed tadpoles had higher whole body CORT and relative tail depth ($F_{1,35} = 67.58$, $p < 0.01$, Figure 1A, and $F_{1,52} = 13.05$, $p < 0.01$, Figure 1B, respectively), and lower body mass ($F_{1,52} = 11.92$, $p < 0.01$; Figure 1C), compared to predator-naïve tadpoles. Irrespective of PPR exposure, whole body CORT declined between three and six weeks of development ($F_{1,35} = 39.16$, $p < 0.01$, Figure 1A), whereas relative tail depth and body mass increased over time ($F_{1,52} = 23.72$, $p < 0.01$, Figure 1B, and $F_{1,52} = 10.12$, $p < 0.01$, Figure 1C, respectively). Although predator-exposed tadpoles were less developed overall than their predator-naïve counterparts (Wald Statistic = 11.46, $p < 0.01$), there was no significant development in either treatment condition from three to six weeks (Wald Statistic = 1.30, $p = 0.26$; Figure 1D). Significant interactions were observed between treatment and duration of treatment (time) on whole body CORT ($F_{1,35} = 29.94$, $p < 0.01$) and relative tail depth ($F_{1,35} =$
6.70, \( p = 0.01 \)). As expected, after three weeks of exposure to PPR whole body corticosterone (CORT) level in tadpoles was higher (approx. 320% higher; Tukey’s HSD: \( p < 0.01 \); Figure 1A), and relative tail depth also was higher (approx. 370% higher; Tukey’s HSD: \( p < 0.01 \); Figure 1B) than that observed among predator-naïve tadpoles. However after six weeks, relative tail depth of PPR-exposed tadpoles was similar to that of six-week old predator-naïve tadpoles (Tukey’s HSD: \( p = 0.52 \); Figure 1B). Although by six weeks of exposure, CORT levels in PPR-exposed tadpoles dropped by 205%, CORT levels still remained higher than those in predator-naïve tadpoles (Tukey’s HSD: \( p = 0.03 \); Figure 1A). We found no significant treatment X time interaction on tadpole body mass (\( F_{1,52} = 0.12, p = 0.73 \)), or developmental stage (Wald Statistic = 1.77, \( p = 0.18 \)). Qualitatively, body mass differences between PPR-exposed and predator-naïve tadpoles decreased after six weeks of exposure (Figure 1C), following a similar shift towards convergence between treatments, as was observed in whole body CORT (Figure 1A) and relative tail depth (Figure 1B). Notably, developmental stage tended to diverge through time, with development of predator-exposed tadpoles being suppressed compared to that of predator-naïve tadpoles (Figure 1D).

4. Discussion

Tadpoles with elevated CORT levels after three weeks of exposure to perceived predation risk had lower CORT levels after six weeks of exposure. This reduction in CORT after prolonged exposure to the experimental treatment (PPR) may represent acclimation to the stressor in *L. pipiens* tadpoles. Acclimation of the stress axis occurs when a stressor no longer is perceived as the same level of risk as was previously sensed by the animal (Romero, 2004). We know of a single prior study that has examined predator-induced CORT levels in anuran larvae over multiple weeks: Dahl et al. (2012) found a similar acclimation response in *Laurasiarana*
*temporaria* tadpoles after only 15 days of risk exposure. However, predator-induced CORT levels in a *Lithobates* species has been shown to remain elevated after 15 days (Maher et al., 2013), leaving uncertainty in the duration of the acclimation response in other anuran species. Indeed, differences in acclimation timing between species likely reflect variation in developmental rate (i.e., number of weeks), since prey response to predation risk (e.g., avoidance or change in growth and survival) is directly related to body size and developmental stage (i.e., Gosner stage) (Preisser and Orrock, 2012). Thus, if older, larger-bodied tadpoles perceive dragonfly nymph predation risk as less severe (Semlitsch, 1990; Eklov and Werner, 2000), there may be a developmental trade off allowing tadpoles to intrinsically grow at a faster rate, despite the delay in development stage. In turn, this should accelerate tadpole acclimation to predation risk, thereby further reducing risks associated with prolonged CORT exposure (Barton et al., 1987; Dahl et al., 2012). Although the activation of the CORT response to prolonged predation risk is not species-specific, it is likely that the duration of the stress response is dependent on intrinsic, specific factors, such as growth or development rate.

Within six weeks of exposure to PPR, acclimation or down regulation of CORT in PPR-exposed *L. pipiens* tadpoles coincided with an increase in tadpole body mass. In contrast, we failed to observe commensurate progression in development. The recovery of body mass in predator-exposed tadpoles over time suggests that CORT may be involved in the modulation and allocation of energy through allostasis (i.e., the thrifty phenotype hypothesis) (Barker and Clark, 1997; Crespi and Warne, 2013). The impact that CORT has on physiological functions in an animal may not be permanent, but can be reversible if the animal is successful in alleviating negative effects associated with chronic stress (i.e., acclimation to predation risk). As such, prey may exhibit catch-up or compensatory growth to balance costs associated with anti-predator
morphological changes (Dmitriew and Rowe, 2005; Walzer et al., 2015). Further, growth and development in predator-exposed prey may be accelerated under favourable conditions to reach optimal size prior to maturation (Hector and Nakagawa, 2012). This may be true for other vertebrates as well, given that a negative relationship between body mass and CORT has been demonstrated in both mammalian (Cabezas et al., 2007) and avian (Schoech et al., 1997; Kitaysky et al., 1999) taxa, regardless of the stressor. However, it is important to note that prey responses to prolonged predator exposure may not be consistent across taxa, and in fact may be species-specific (Capellan and Nicieza, 2007).

Tail morphology was not affected by acclimating CORT levels at six weeks of predator exposure, suggesting that other factors influence tail morphology later in development. Change in relative tail depth is a graded response to predation risk, such that tail morphology is reflective of the severity of predation risk (Laurila et al., 2004; Schoeppner and Relyea, 2008). However, our study suggests that increased relative tail depth eventually reaches a plateau regardless of PPR exposure, given the lack of difference in tail depth between three- and six-week predator-exposed tadpoles. Accordingly, it appears that predator-exposed tadpoles develop anti-predator morphology by accelerating the processes required to increase tail depth, as predator-naïve tadpoles will develop relative tail depths similar to predator-exposed tadpoles after five to six weeks of development (Hossie et al., 2012; Bennett and Murray 2015). The lack of phenotypic variation in tail depth between predator-exposed and predator-naïve tadpoles after six weeks of development likely indicates that increasing tail depth is part of tadpole development, and that although there is a relationship between high levels of CORT and tail depth in the earlier stages of development, other factors may become predominant in later developmental stages. The expected increase in tail depth late in ontogeny may reflect morphological compensation for drag
caused by developing limb buds (energy efficiency hypothesis) or an increase in vulnerability to predation risk as tadpoles near metamorphosis, regardless of extrinsic cues (innate predator defense hypothesis; Hossie and Murray 2012). Regardless of the specific selection pressure acting on developing a deeper tail later in ontogeny, we show that changes in relative tail depth late in tadpole development (i.e., six weeks) occur irrespective of changes in whole body CORT.

The neuroendocrine stress axis is a fundamental component in promoting the onset of adaptive morphological traits in larval anurans (Denver, 2009; Maher et al., 2013). In this study we demonstrate that *L. pipiens* tadpoles acclimate to prolonged predator exposure within six weeks, showing a negative relationship between predator-induced stress and tadpole body mass but no relationship between acclimating CORT levels and tail depth morphology. Although tail depth is mediated by CORT early in development, the lack of a similar effect later in development supports that acclimation is the outcome of prolonged exposure. Nonetheless, acclimating to one stressor does not prevent further activation of the stress axis, as is seen in animals exhibiting facilitation of the stress response (Romero, 2004). It follows that future research should assess the influence of the neuroendocrine stress axis on tadpole fitness and morphology in response to exposure to different types of stressors, as well as variation in the time and duration of stressor exposure, and combination of stressors. A comparative approach may be especially helpful in elucidating different stress responses and growth modulation patterns across anuran species. In light of the variety in type and magnitude of observed anti-predator responses in anurans (e.g., Hossie et al, 2016), such work will help shed light on the historical and contemporary selective pressures influencing tadpole morphology.
Figure 1. Mean (± SE) whole body corticosterone levels (ng/g bwt) (A), relative tail depth (B), body mass (g) (C), and developmental stage (Gosner) (D) of *Lithobates pipiens* tadpoles exposed to perceived predation risk for three and six weeks. The * and horizontal bracket indicates significant treatment X time interaction (Tukey HSD, p<0.05).
Chapter 3: General Discussion

The involvement of the hypothalamic-pituitary-adrenal/interrenal (HPA/I) stress axis in mediating predator-induced phenotypic responses has been shown during the first two weeks of tadpole ontogeny, when dragonfly nymph predation is perceived as a high risk scenario (Eklov and Werner, 2000; Dahl et al., 2012; Middlemis Maher et al., 2013). Yet, investigation of predator-induced CORT on morphological adaptation in the later stages of a developing tadpole hasn’t been carried out. This thesis demonstrates that tadpoles exposed to six weeks of prolonged predation risk, in the form of both kairomone and conspecific cues, show a significant decrease in CORT levels compared to 3 weeks, which may be representative of an acclimating stress response as a result of a decrease in perceived severity of the stressor (Romero, 2004). A reduction in CORT levels may cause redistribution of energy expenditures, thus allowing an animal to accelerate its growth and development (e.g., compensatory or catch up growth) to reach optimal body size prior to metamorphosis (Walzer et al., 2015). Phenotypic plasticity, which is mediated in part by CORT levels, incurs an energetic cost that may lead to developmental consequences as a result of reallocating energy (Crespi and Warne, 2013). A decrease in perceived stressor severity may reduce CORT levels, allowing the animal to compensate for phenotypic costs (i.e., thrifty phenotypic hypothesis) (Barker and Clarke, 1997; Crespi and Warne, 2013).

This thesis also demonstrates that predation accelerates the onset of morphological traits (i.e., tail depth) that can be used as defensive morphology to increase survival (Eklov and Werner, 2000; Hossie et al., 2012), where predator-exposed tadpoles show greater tail depth when exposed to predation in the early stages of development, yet the same morphological feature is observed in non-predator exposed tadpoles later in ontogeny. Despite the involvement
of CORT in mediating phenotypic plasticity in response to predation risk, changes in tadpole morphology occur later in development irrespective of changes in CORT.

**Acclimating to Predation and Compensatory Growth Modulation**

Exposing tadpoles to dragonfly nymph predation showed an initial significant increase in CORT after three weeks of exposure, followed by a steep decrease in CORT levels when exposed to perceived predation risk for six weeks (Chapter 2). Acclimating to predation risk after six weeks of exposure suggests that the stressor (i.e. the predator) is no longer perceived as a threat as older, larger bodied tadpoles become less responsive to dragonfly nymph predation (Semlitsch, 1990; Eklov and Werner, 2000). Decreasing CORT levels also may be associated with a learned response (i.e., habituation) due to the absence of attack on prey, which has been demonstrated across vertebrate taxa, such as in rats that were repeatedly exposed to cats (Figueirido et al., 2003), penguins exposed to frequent human visitations (Walker et al., 2006; Ellenberg et al., 2007), and in tadpoles chronically exposed to dragonfly nymph predation after two weeks of exposure (Dahl et al., 2012; Bennett et al., 2016). However, growth modulation in the presence of a decreasing CORT response (i.e., acclimation) has yet to be investigated. The results from this thesis suggest that developing prey become accustomed to the presence of predation due to the lack of threat associated with the predator over time, and compensate for the initial delay in growth by increasing growth rate to reach optimal body size and development prior to metamorphosis (i.e., the animal undergoes a compensatory growth period). Given that decreasing CORT levels (Figure 1A) coincided with an increase in body mass (Figure 1C), it is possible that CORT levels are related to growth inhibition early in tadpole ontogeny. However, as CORT levels decrease, the inhibition on tadpole growth is removed, which may influence compensatory growth responses in amphibians.
Compensatory and catch up growth strategies are well known among different vertebrate taxa, including mammals, birds, and amphibians (Wilson and Osbourn, 1960; Capellan and Nicieza, 2007; Dmitriew 2011), and are influenced by stressors such as food restriction, low temperature, and exposure to pesticides or toxic chemicals (Ashworth and Millward, 1986; Dmitriew, 2011; Hector and Nakagawa, 2012). However, evidence for compensatory or catch up growth in animals exposed to predation risk is surprisingly limited, even though it is suggested that predation negatively impacts growth rates in prey (Van Buskirk and Yurewicz, 1998; Benard, 2004; Capellan and Nicieza, 2007). Interestingly, the few studies that have examined growth modulation in the presence of predation risk have done so using tadpoles (Altwegg and Reyer, 2003; Capellan and Nicieza, 2007). Yet, in contrast to the results suggested by this thesis, *Rana temporaria* tadpoles exposed to dragonfly nymphs fed daily for either short (1-20 days) or long-term (until metamorphosis; approximately 42 days) duration did not compensate for reduced body size induced by predation risk, which may be due to the severity of risk perceived by the tadpole (Altwegg and Reyer, 2003; Capellan and Nicieza, 2007). For example, the severity of predation can be influenced by the amount of prey consumed by the predator, and how often the predator is fed, which affects the magnitude of morphological and behavioural responses (Laurila et al., 2004; Schoeppner and Relyea, 2008). Aquatic prey are able to assess conspecific and predator cue concentrations to evaluate the degree of risk to mount an appropriate coping responses (Schoeppner and Relyea, 2008). Thus, predators that are fed daily (Capellan and Nicieza, 2007) may be perceived as a greater risk due to greater conspecific concentrations compared to predators fed every other day as was done for the study in this thesis (Chapter 2). The variation in prey consumption by a predator may be sufficient in changing the
perception of predation risk from moderate to severe, thus affecting the growth modulation of the animal.

Experimental findings from this thesis highlight the complex nature of the stress response and the role CORT may play in the growth modulation of phenotypic responses in lower vertebrates. However, further experimentation is required to develop a better understanding of the factors that influence different types of CORT responses (e.g., acclimation, habituation, facilitation), and how hormonal changes alter energy allocation to modulate growth and development in larval prey to enhance survival and fitness.

**Future Directions**

The main body of this work demonstrates that prey exposed to prolonged predation risk may exhibit an acclimating CORT response as the perception of predation decreases in severity, and the resulting reduction of CORT levels is related to a compensatory increase in growth rate. Further, the decline in CORT levels to one stressor may prime the animal to respond more robustly to a similar or different stressor (e.g., sensitization, facilitation) (Dallman and Bhatnagar, 2001; McEwen and Wingfield, 2003; Figueiredo et al., 2003; Romero, 2004). That is, different brain/limbic areas may be involved in the stress response at different times during exposure to a stressor (including predation risk), and therefore may differentially influence the activity of the HPA/I axis. Future studies should examine different brain areas (e.g. amygdala, pallium) to build on the understanding of the pathways involved in acclimation, habituation and facilitation.

Acclimating or habituating to a stressor not only reduces CORT levels, these processes also alters other hormonal factors that may in turn influence the reactivity of the stress response. Thus, assessing additional hormonal factors influencing the HPA axis can aid in characterizing
the type of stress response exhibited by the animal. For example, when an animal acclimates to a stressor, ACTH levels decrease, yet norepinephrine levels remain elevated, suggesting that the enhanced CORT response to a new stressor may be due to the potentiated norepinephrine response (Dobrakovova et al., 1993). This type of hormonal response has been observed in rats exposed to handling stress for two weeks, where acclimating to the stressor prepares the animal to respond to new stressors with a heightened CORT response, thus exhibiting a facilitating stress response (Dobrakovova et al., 1993). However, rats pre-exposed to handling chambers without allowing for the onset of acclimation as evidenced as decreasing CORT levels, also exhibited a heightened CORT response to a new stressor, but it is suggested that this type of response represents a sensitization stress response (Figueiredo et al., 2003). Indeed, assessing stressor-induced CORT responses, including acclimation and habituation, provides important information regarding the reactivity of the stress response; however, it is evident that there are additional physiological pathways (e.g., growth, metabolic, immunological) that influence the stress CORT axis in prey. Therefore, developing a better understanding of the hormonal changes induced by the varying nature of predation risk (e.g., type, severity, duration), will provide further advancement in the types of CORT responses observed in prey.

Growth modulation observed in tadpoles suggests that prey may accelerate growth and development to compensate for phenotypic consequences as a result of defensive morphological traits. Although the involvement of the stress axis is apparent in the onset of defensive morphology (Middlemis Maher et al., 2013; Chapter 2), it would be beneficial to understand the role of other neuroendocrine pathways that regulate phenotypic responses to predation risk. Both the growth hormone (GH) and thyroid hormone (TH) axes are important regulators of the growth and development of an animal, and are important hormonal pathways to investigate in
understanding the growth modulation during phenotypic changes. The modulation of the GH axis is influenced by changes in CORT levels, such that elevated CORT levels may inhibit the production and release of GH (Hu et al., 2008), and alter growth factors responsible for inhibiting and promoting developmental responses (e.g., cell apoptosis, tissue and organ growth) (Cornils et al., 2011). Therefore, a predator-induced stress response may alter the activity of the GH axis to modulate the onset of predator-induced morphology (e.g., accelerate tail depth growth) and invoke the redistribution of energy, following a decrease in stressor severity that compensates for the developmental delays. Nevertheless, the involvement of the GH axis in compensatory growth responses in prey has yet to be investigated, but remains a likely possibility since the axis plays a key role in compensatory growth in mammals and fish under conditions of food restriction (Blum et al., 1985; Ali et al., 2003).

The TH axis also plays an integral role in the growth modulation of an animal, where developmental timing and the onset of metamorphosis are influenced by increasing TH levels (Denver, 1993; Laudet, 2011). In amphibian systems, the HPA neurohormone, CRH, acts on TSH to stimulate the release of TH in the body of the animal (Denver, 1993; Laudet, 2011). However, TH alone is not sufficient in carrying metamorphosis to completion and additional hormonal involvement from corticosteroids, such as CORT and aldosterone (ALDO), are required (Kulkarni et al., 2014). As the mineralocorticoid in frogs, ALDO works synergistically with CORT to promote the completion of metamorphosis (Kulkarni et al., 2014). The hormonal role of corticosteroids in developmental and metamorphic timing in tadpoles has been experimentally demonstrated using exogenous compounds (e.g., CORT, CRH, ALDO) (Denver, 1993; Denver, 1997; Boorse and Denver, 2002). However, it is essential to understand hormonal involvement in an environmental context (i.e., predation risk), given that the use of exogenous
compounds may lead to misinterpretation of how the physiology of the HPA axis is affected by a stressor (Denver, 1997).

It is unlikely that CORT is the only hormone involved in mediating and regulating phenotypic change in response to environmental stressors. However, investigating the role of CORT provides a foundation in developing an understanding of the function and physiology of the HPA axis in lower vertebrates, paving the way to explore other components of the stress response and additional neuroendocrine hormonal systems. Further investigation of the characteristics of the CORT response, and factors influencing growth modulation in prey will enhance and improve our understanding of the how the HPA axis works in concert with other physiological systems to aid in the survival of the animal.

**Concluding Remarks**

Investigating the role of the stress response in lower vertebrates helps build an enhanced understanding of how CORT aids in the survival and fitness of an animal in challenging environments. Lower vertebrate models can be studied to understand how certain stressors (e.g., pesticides, toxic substances, and viral/bacterial infections) impact the activity of the stress CORT response and other physiological systems to develop a better understanding in higher vertebrate systems. In addition to demonstrating compensatory growth modulation in the presence of predation risk, this thesis demonstrates that the stress response in anurans may have the same complexity and sensitivity to respond to changes in cue severity as is demonstrated in mammalian models.
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