ECOLOGICAL AND EVOLUTIONARY CONTEXT OF THE STRESS RESPONSE IN LARVAL ANURANS

by

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TABLE OF CONTENTS

LIST OF FIGURES

ABSTRACT

Describing and understanding the interactions between environment and phenotype, and the resulting consequences for populations and communities, is a central goal of ecological and evolutionary research and theory. In vertebrates, the endocrine stress response provides a mechanistic connection between environmental variation and organism phenotype, with resulting effects on fitness. Using larval anurans as a model system, I combine field surveys, mesocosm experiments, and exogenous hormone manipulation to explore the complex interactions among the predator stress response, phenotype, and fitness, with respect to other contemporary stressors and species evolutionary history. First, I developed a comprehensive understanding of the predator stress response over time, mapping hormone production to changes in behavior and morphology, and demonstrating the effects on predation survival. I found that tadpoles modulate their predator stress response to maximize survival under predation risk, and additionally showed that the stress hormones produced during a predator response initiates morphological changes indistinguishable from predator-induced morphology. Next, I used a comparative approach to place the predator stress response into an evolutionary context, testing the hypothesis that species that predictably encounter high larval predator densities reduce the costs of chronic endocrine activation. Across four anuran species arrayed in conspecific pairs, I found support for this hypothesis, showing that species typically under high predation risk reduce the magnitude of their stress

response to predator presence relative to conspecifics. I next considered the combined effects of inbreeding and predator presence, and showed that inbred tadpoles also elevate stress hormone production, although there is no additive effect in combination with predator presence. The resulting behavioral and morphological changes in inbred tadpoles may place them at a disadvantage under some circumstances. Finally, I showed that reduced food resource availability also increases stress hormone production independently of predator presence. Together, these results connect perceived predation risk, endocrine response, and phenotypic plasticity and place them in an ecological and evolutionary context. Furthermore, this research highlights the importance of using an integrative approach to investigate the impacts of developmental history on organism-environment interactions.

CHAPTER 1

INTRODUCTION

The role of endocrine stress activity as a phenotypic response to the environment has been increasingly recognized and integrated into ecological thought (Creel 2001, Rollins-Smith 2001, Wingfield and Kitaysky 2002, Romero 2004, Sih et al. 2004, Tracy et al. 2006, Wikelski and Cooke 2006, Hawlena and Schmitz 2010). Previously, the term "environmental stressor" has been broadly used to describe any challenging event or selective force that could impact fitness, but only recently have the tools of stress physiology been applied to ecology on a wide scale (Romero 2004, Tracy et al. 2006). The result has been a more rigorous definition of stress, one that measures the physiological responses to the environmental challenge as well as the ecological and evolutionary consequences. The integration of a deeper understanding of stress physiology within an ecological context advances our predictive understanding of the mechanistic connections between environment and phenotype for organisms.

The influence of physiological ecology has morphed our understanding of stress from a descriptor of something inherently negative for the organism to an endocrine response that increases organismal survival during the stressful event by inducing an "emergency life history stage" (Wingfield et al. 1998, Wingfield and Kitaysky 2002). In the face of an acute physical, psychological, or environmental challenge, vertebrates react by increasing circulating levels of glucocorticoid stress hormones, leading to suppression

of reproduction and immune function and mobilization of energy stores (Wingfield et al. 1997, Sapolsky et al. 2000). Over time, however, these same effects that can improve initial survival eventually may become more costly than beneficial (McNamara and Buchanan 2005, Rich and Romero 2005, Cyr and Romero 2007). Thus, conservation biologists have adopted measurements of stress hormone production as an indicator of population health (Creel et al. 2002, Homan et al. 2003, Tempel and Gutierrez 2003, Cockrem 2005, Wikelski and Cooke 2006, Teixeira et al. 2007, Cyr and Romero 2008). Indeed, increased circulating stress hormones and reactivity of the stress axis have been shown to have negative impacts on individual fitness and population dynamics in a number of taxa (Boonstra and Singleton 1993, Krebs et al. 1995, Hubbs and Boonstra 1997, Boonstra et al. 1998, Rollins-Smith 2001, Romero and Wikelski 2001, Clinchy et al. 2004, Romero and Wikelski 2010). Therefore, we need to not only investigate the causes and phenotypic effects of the glucocorticoid stress response, but also consider how time and individual condition modulate the ecological and evolutionary consequences.

In my dissertation research, I have placed the glucocorticoid stress response in an ecological and evolutionary context, investigating the stressors that induce this response in larval anurans and the consequences for individuals, populations, and species. Larval anurans present an excellent system to examine the interactions between environment and phenotypic plasticity, since ecological and individual fitness consequences of these interactions have been well-studied, in particular the direct and indirect effects of predation and competition on tadpole phenotype and life history (e.g. Berven 1990, Werner and McPeek 1994, Skelly 1995, Werner and Anholt 1996, Van Buskirk and Yurewicz 1998, Anholt et al. 2000, Relyea 2000, Van Buskirk 2000, 2002, Werner and

Peacor 2003, Relyea 2004, Relyea and Auld 2005, Peacor et al. 2007). In addition, the causes and consequences of the glucocorticoid stress response have been investigated in several larval amphibian systems (Rollins-Smith and Blair 1993, Hayes and Wu 1995, Rollins-Smith et al. 1997, Denver 1998, Belden and Kiesecker 2005, Belden et al. 2005, Crespi and Denver 2005, Belden et al. 2007, Hu et al. 2008, Denver 2009), providing the background and tools necessary to apply it in my research. Throughout the following studies, I have considered the tadpole stress response from an ecological perspective, emphasizing its role as one aspect of overall phenotype while exploring interactions with plasticity in behavior, morphology, growth, and resulting fitness.

Predators have long been labeled as stressors (Scheuerlein et al. 2001, Clinchy et al. 2004, Adamec et al. 2006, Barcellos et al. 2007, Roseboom et al. 2007) , but temporal modulation of the glucocorticoid stress response, predation vulnerability, and phenotypic responses in prey has not previously been explored in detail. In Chapter 2, I investigate the role of predator presence as a stressor in larval wood frogs (*Rana sylvatica*), mapping the shape of the hormonal stress response over time and using experimental manipulations to link the stress response to changes in tadpole morphology and behavior. My results demonstrate the role of stress hormones in mediating anti-predator defense phenotypes, showing that morphologies typical of predator-induction can be initiated through exogenous corticosterone application alone. In addition, I demonstrate through a time-series analysis of the predator stress response, combined with predation survivorship trials, that temporal pattern of stress hormone production to predator cue exposure may be adapted to maximize survival in the face of both acute and chronic predation risk.

Variation in predation risk across habitat gradients is an important determinant of anuran species habitat choice and survival success (Skelly et al. 1999). This is in large part a consequence of evolved differences in the expression of phenotypic plasticity exhibited by each species (Werner and Anholt 1993, Skelly 1995, Smith and Van Buskirk 1995, Anholt and Werner 1998, Anholt et al. 2000, Relyea and Werner 2000, Schiesari et al. 2006). Owing to these documented differences among anuran species and the demonstration of the involvement of the stress axis in inducing predator defense phenotypes, I hypothesize in Chapter 3 that species living at either end of the predation risk gradient may also show predictable differences in stress reactivity to predator presence. In particular, I suggest that due to the potentially damaging effects of mounting a strong chronic stress response, species that predictably encounter high predator densities in their larval environment would demonstrate a blunted predator stress response. My results from a comparison of four larval anuran species raised in a common garden support this hypothesis, and suggest a further mechanism for species success along the pond-permanence gradient.

Predator presence is by no means the only stressor that individuals are likely to encounter, however, and investigations of combined stressors are becoming increasingly important to predicting demographic patterns in wild populations (Relyea and Mills 2001, Sih et al. 2004, Hayes et al. 2006, Relyea 2006). Various stressors may co-occur with predators, including how the environment interacts with an individual's own genetic background. As habitats are fragmented and gene flow interrupted, increased levels of inbreeding are likely to become more commonplace among populations (Epps et al. 2005, Coulon et al. 2006, Cushman 2006, Riley et al. 2006, Vandergast et al. 2007), leading to

the potential for inbreeding-environment interactions to strengthen inbreeding depression under stressful conditions (Armbruster and Reed 2005, Fox and Reed 2011). In Chapter 4, I examine the potential for inbreeding to impact stress physiology, both alone and in combination with predator presence, in larval wood frogs. In addition, I look for signatures of synergistic interactions among inbreeding and predator presence on aspects of tadpole morphology, behavior, and growth. My results demonstrate that either inbreeding or predators alone may be sufficient to elicit a response and that there may be a ceiling effect on the stress response that would blunt adaptive reactions to novel challenges.

Organisms must also balance their response to predator presence with their investment in foraging gain. There is a well-developed literature on the theoretical and empirical demonstrations of this trade-off, for which larval anurans have become a model system (Werner and Anholt 1993, Anholt and Werner 1998, Anholt et al. 2000, Schiesari et al. 2006). In addition, both predator presence and food deprivation can induce a glucocorticoid stress response in various organisms (Kitaysky et al. 1999, Scheuerlein et al. 2001, Glennemeier and Denver 2002, Clinchy et al. 2004, Crespi and Denver 2005). In Chapter 5, I investigate the potential for low food resources to induce a stress response and interact with the predator stress response, and explore the consequences for tadpole growth. My results support previous demonstrations of a glucocorticoid response to reduced forage availability (Glennemeier and Denver 2002, Crespi and Denver 2005) but only at later sampling points, suggesting that energetic reserves need to significantly decline before initiation of a stress response. Tadpole growth under these conditions

matched a predicted pattern of reduced foraging gain in predator presence only under non-limiting resources (Peacor and Werner 2004).

In the concluding chapter, I integrate the results of the four previous chapters and explain the significance of my results for individual fitness and interactions between larval anurans and the pond community. I also explore the potential for carry-over effects among stages of the complex life cycle, and how developmental environment may impact juvenile and adult phenotype through the action of stress hormones. Finally, I discuss current research goals and suggest directions for future research.

References

Adamec, R., D. Head, J. Blundell, P. Burton, and O. Berton. 2006. Lasting anxiogenic effects of feline predator stress in mice: Sex differences in vulnerability to stress and predicting severity of anxiogenic response from the stress experience. Physiology & Behavior **88**:12-29.

Anholt, B. R., E. Werner, and D. K. Skelly. 2000. Effect of food and predators on the activity of four larval ranid frogs. Ecology **81**:3509-3521.

Anholt, B. R. and E. E. Werner. 1998. Predictable changes in predation mortality as a consequence of changes in food availability and predation risk. Evolutionary Ecology **12**:729-738.

Armbruster, P. and D. H. Reed. 2005. Inbreeding depression in benign and stressful environments. Heredity **95**:235-242.

Barcellos, L. J. G., F. Ritter, L. C. Kreutz, R. M. Quevedo, L. B. da Silva, A. C. Bedin, J. Finco, and L. Cericato. 2007. Whole-body cortisol increases after direct and visual contact with a predator in zebrafish, Danio rerio. Aquaculture **272**:774-778.

Belden, L. K. and J. M. Kiesecker. 2005. Glucocorticosteroid hormone treatment of larval treefrogs increases infection by *Alaria sp* trematode cercariae. Journal Of Parasitology **91**:686-688.

Belden, L. K., I. T. Moore, J. C. Wingfield, and A. R. Blaustein. 2005. Corticosterone and growth in Pacific treefrog (*Hyla regilla*) tadpoles. Copeia:424-430.

Belden, L. K., M. J. Rubbo, J. C. Wingfield, and J. M. Kiesecker. 2007. Searching for the physiological mechanism of density dependence: Does corticosterone regulate

tadpole responses to density? Physiological And Biochemical Zoology **80**:444-451.

Berven, K. A. 1990. Factors affecting population fluctuations in larval and adult stages of the wood frog (*Rana sylvatica*). Ecology **71**:1599-1608.

Boonstra, R., D. Hik, G. R. Singleton, and A. Tinnikov. 1998. The impacts of predator-induced stress on the snowshoe hare cycle. Ecological Monographs **68**:371-394.

Boonstra, R. and G. R. Singleton. 1993. Population declines in the snowshoe hare and the role of stress. General And Comparative Endocrinology **91**:126-143.

Clinchy, M., L. Zanette, R. Boonstra, J. C. Wingfield, and J. N. M. Smith. 2004. Balancing food and predator pressure induces chronic stress in songbirds. Proceedings Of The Royal Society Of London Series B-Biological Sciences **271**:2473-2479.

Cockrem, J. F. 2005. Conservation and behavioral neuroendocrinology. Hormones And Behavior **48**:492-501.

Coulon, A., G. Guillot, J. F. Cosson, J. M. A. Angibault, S. Aulagnier, B. Cargnelutti, M. Galan, and A. J. M. Hewison. 2006. Genetic structure is influenced by landscape features: empirical evidence from a roe deer population. Molecular Ecology **15**:1669-1679.

Creel, S. 2001. Social dominance and stress hormones. Trends In Ecology & Evolution **16**:491-497.

Creel, S., J. E. Fox, A. Hardy, J. Sands, B. Garrott, and R. O. Peterson. 2002. Snowmobile activity and glucocorticoid stress responses in wolves and elk. Conservation Biology **16**:809-814.

Crespi, E. J. and R. J. Denver. 2005. Roles of stress hormones in food intake regulation in anuran amphibians throughout the life cycle. Comparative Biochemistry And Physiology A-Molecular & Integrative Physiology **141**:381-390.

Cushman, S. A. 2006. Effects of habitat loss and fragmentation on amphibians: A review and prospectus. Biological Conservation **128**:231-240.

Cyr, N. E. and L. M. Romero. 2007. Chronic stress in free-living European starlings reduces corticosterone concentrations and reproductive success. General And Comparative Endocrinology **151**:82-89.

Cyr, N. E. and L. M. Romero. 2008. Fecal glucocorticoid metabolites of experimentally stressed captive and free-living starlings: Implications for conservation research. General And Comparative Endocrinology **158**:20-28.

Denver, R. J. 1998. Hormonal correlates of environmentally induced metamorphosis in the Western spadefoot toad, Scaphiopus hammondii. General And Comparative Endocrinology **110**:326-336.

Denver, R. J. 2009. Stress hormones mediate environment-genotype interactions during amphibian development. General And Comparative Endocrinology **164**:20-31.

Epps, C. W., P. J. Palsboll, J. D. Wehausen, G. K. Roderick, R. R. Ramey, and D. R. McCullough. 2005. Highways block gene flow and cause a rapid decline in genetic diversity of desert bighorn sheep. Ecology Letters **8**:1029-1038.

Fox, C. W. and D. H. Reed. 2011. Inbreeding depression increases with environmental stress: An experimental study and meta-analysis. Evolution **65**:246-258.

Glennemeier, K. A. and R. J. Denver. 2002. Role for corticoids in mediating the response of *Rana pipiens* tadpoles to intraspecific competition. Journal Of Experimental Zoology **292**:32-40.

Hawlena, D. and O. J. Schmitz. 2010. Physiological Stress as a Fundamental Mechanism Linking Predation to Ecosystem Functioning. American Naturalist **176**:537-556.

Hayes, T. B., P. Case, S. Chui, D. Chung, C. Haeffele, K. Haston, M. Lee, V. P. Mai, Y. Marjuoa, J. Parker, and M. Tsui. 2006. Pesticide mixtures, endocrine disruption, and amphibian declines: Are we underestimating the impact? Environmental Health Perspectives **114**:40-50.

Hayes, T. B. and T. H. Wu. 1995. Role of corticosterone in anuran metamorphosis and potential role in stress-induced metamorphosis. Netherlands Journal Of Zoology **45**:107-109.

Homan, R. N., J. V. Regosin, D. M. Rodrigues, J. M. Reed, B. S. Windmiller, and L. M. Romero. 2003. Impacts of varying habitat quality on the physiological stress of spotted salamanders (*Ambystoma maculatum*). Animal Conservation **6**:11-18.

Hu, F., E. J. Crespi, and R. J. Denver. 2008. Programming neuroendocrine stress axis activity by exposure to glucocorticoids during postembryonic development of the frog, *Xenopus laevis*. Endocrinology **149**:5470-5481.

Hubbs, A. H. and R. Boonstra. 1997. Population limitation in Arctic ground squirrels: Effects of food and predation. Journal Of Animal Ecology **66**:527-541.

Kitaysky, A. S., J. C. Wingfield, and J. F. Piatt. 1999. Dynamics of food availability, body condition and physiological stress response in breeding Black-legged Kittiwakes. Functional Ecology **13**:577-584.

Krebs, C. J., S. Boutin, R. Boonstra, A. R. E. Sinclair, J. N. M. Smith, M. R. T. Dale, K. Martin, and R. Turkington. 1995. Impact of food and predation on the snowshoe hare cycle. Science **269**:1112-1115.

McNamara, J. M. and K. L. Buchanan. 2005. Stress, resource allocation, and mortality. Behavioral Ecology **16**:1008-1017.

Peacor, S. D., L. Schiesari, and E. E. Werner. 2007. Mechanisms of nonlethal predator effect on cohort size variation: Ecological and evolutionary implications. Ecology **88**:1536-1547.

Peacor, S. D. and E. E. Werner. 2004. Context dependence of nonlethal effects of a predator on prey growth. Israel Journal of Zoology **50**:139-167.

Relyea, R. A. 2000. Trait-mediated indirect effects in larval anurans: reversing competition with the threat of predation. Ecology **81**:2278-2289.

Relyea, R. A. 2004. Fine-tuned phenotypes: Tadpole plasticity under 16 combinations of predators and competitors. Ecology **85**:172-179.

Relyea, R. A. 2006. The effects of pesticides, pH, and predatory stress on amphibians under mesocosm conditions. Ecotoxicology **15**:503-511.

Relyea, R. A. and J. R. Auld. 2005. Predator- and competitor-induced plasticity: how changes in foraging morphology affect phenotypic trade-offs. Ecology **86**:1723-1729.

Relyea, R. A. and N. Mills. 2001. Predator-induced stress makes the pesticide carbaryl more deadly to gray treefrog tadpoles (*Hyla versicolor*). Proceedings Of The National Academy Of Sciences Of The United States Of America **98**:2491-2496.

Relyea, R. A. and E. E. Werner. 2000. Morphological plasticity in four larval anurans distributed along an environmental gradient. Copeia:178-190.

Rich, E. L. and L. M. Romero. 2005. Exposure to chronic stress downregulates corticosterone responses to acute stressors. American Journal of Physiology - Regulatory Integrative and Comparative Physiology **288**:R1628-R1636.

Riley, S. P. D., J. P. Pollinger, R. M. Sauvajot, E. C. York, C. Bromley, T. K. Fuller, and R. K. Wayne. 2006. A southern California freeway is a physical and social barrier to gene flow in carnivores. Molecular Ecology **15**:1733-1741.

Rollins-Smith, L. A. 2001. Neuroendocrine-immune system interactions in amphibians - Implications for understanding global amphibian declines. Immunologic Research **23**:273-280.

Rollins-Smith, L. A., K. S. Barker, and A. T. Davis. 1997. Involvement of glucocorticoids in the reorganization of the amphibian immune system at metamorphosis. Developmental Immunology **5**:145-152.

Rollins-Smith, L. A. and P. J. Blair. 1993. The effects of corticosteroid hormones and thyroid hormones on lymphocyte viability and proliferation during development and metamorphosis of *Xenopus laevis*. Differentiation **54**:155-160.

Romero, L. M. 2004. Physiological stress in ecology: lessons from biomedical research. Trends In Ecology & Evolution **19**:249-255.

Romero, L. M. and M. Wikelski. 2001. Corticosterone levels predict survival probabilities of Galapagos marine iguanas during El Nino events. Proceedings Of The National Academy Of Sciences Of The United States Of America **98**:7366-7370.

Romero, L. M. and M. Wikelski. 2010. Stress physiology as a predictor of survival in Galapagos marine iguanas. Proceedings Of The Royal Society B-Biological Sciences **277**:3157-3162.

Roseboom, P. H., S. A. Nanda, V. P. Bakshi, A. Trentani, S. M. Newman, and N. H. Kalin. 2007. Predator threat induces behavioral inhibition, pituitary-adrenal activation and changes in amygdala CRF-binding protein gene expression. Psychoneuroendocrinology **32**:44-55.

Sapolsky, R. M., L. M. Romero, and A. U. Munck. 2000. How do glucocorticoids influence stress responses? Integrating permissive, suppressive, stimulatory, and preparative actions. Endocrine Reviews **21**:55-89.

Scheuerlein, A., T. J. Van't Hof, and E. Gwinner. 2001. Predators as stressors? Physiological and reproductive consequences of predation risk in tropical stonechats (*Saxicola torquata axillaris*). Proceedings Of The Royal Society Of London Series B-Biological Sciences **268**:1575-1582.

Schiesari, L., S. D. Peacor, and E. E. Werner. 2006. The growth-mortality tradeoff: evidence from anuran larvae and consequences for species distributions. Oecologia **149**:194-202.

Sih, A., A. M. Bell, and J. L. Kerby. 2004. Two stressors are far deadlier than one. Trends In Ecology & Evolution **19**:274-276.

Skelly, D. K. 1995. A behavioral trade-off and its consequences for the distribution of Pseudacris treefrog larvae. Ecology **76**:150-164.

Skelly, D. K., E. E. Werner, and S. A. Cortwright. 1999. Long-term distributional dynamics of a Michigan amphibian assemblage. Ecology **80**:2326-2337.

Smith, D. C. and J. Van Buskirk. 1995. Phenotypic design, plasticity, and ecological performance in two tadpole species. American Naturalist **145**:211-233.

Teixeira, C. P., C. S. De Azevedo, M. Mendl, C. F. Cipreste, and R. J. Young. 2007. Revisiting translocation and reintroduction programmes: the importance of considering stress. Animal Behaviour **73**:1-13.

Tempel, D. J. and R. J. Gutierrez. 2003. Fecal corticosterone levels in California spotted owls exposed to low-intensity chainsaw sound. Wildlife Society Bulletin **31**:698-702.

Tracy, C. R., K. E. Nussear, T. C. Esque, K. Dean-Bradley, C. R. Tracy, L. A. DeFalco, K. T. Castle, L. C. Zimmerman, R. E. Espinoza, and A. M. Barber. 2006. The importance of physiological ecology in conservation biology. Integrative and Comparative Biology **46**:1191-1205.

Van Buskirk, J. 2000. The costs of an inducible defense in anuran larvae. Ecology **81**:2813-2821.

Van Buskirk, J. 2002. A comparative test of the adaptive plasticity hypothesis: relationships between habitat and phenotype in anuran larvae. American Naturalist **160**:87-102.

Van Buskirk, J. and K. L. Yurewicz. 1998. Effects of predators on prey growth rate: relative contributions of thinning and reduced activity. Oikos **82**:20-28.

Vandergast, A. G., A. J. Bohonak, D. B. Weissman, and R. N. Fisher. 2007. Understanding the genetic effects of recent habitat fragmentation in the context of evolutionary history: phylogeography and landscape genetics of a southern

California endemic Jerusalem cricket (Orthoptera : Stenopelmatidae : Stenopelmatus). Molecular Ecology **16**:977-992.

Werner, E. E. and B. R. Anholt. 1993. Ecological consequences of the trade-off between growth and mortality rates mediated by foraging activity American Naturalist **142**:242-272.

Werner, E. E. and B. R. Anholt. 1996. Predator-induced behavioral indirect effects: Consequences to competitive interactions in anuran larvae. Ecology **77**:157-169.

Werner, E. E. and M. A. McPeek. 1994. Direct and indirect effects of predators on two anuran species along an environmental gradient. Ecology **75**:1368-1382.

Werner, E. E. and S. D. Peacor. 2003. A review of trait-mediated indirect interactions in ecological communities. Ecology **84**:1083-1100.

Wikelski, M. and S. J. Cooke. 2006. Conservation physiology. Trends In Ecology & Evolution **21**:38-46.

Wingfield, J. C., C. W. Breuner, and J. D. Jacobs. 1997. Corticosterone and behavioral responses to unpredictable events.*in* R. J. Etches and S. Harvey, editors. Avian Endocrinology. J. Endocrinology Ltd., Bristol.

Wingfield, J. C. and A. S. Kitaysky. 2002. Endocrine responses to unpredictable environmental events: Stress or anti-stress hormones? Integrative and Comparative Biology **42**:600-609.

Wingfield, J. C., D. L. Maney, C. W. Breuner, J. D. Jacobs, S. Lynn, M. Ramenofsky, and R. D. Richardson. 1998. Ecological bases of hormone-behavior interactions: The "emergency life history stage". American Zoologist **38**:191-206.

CHAPTER 2

COMPLEX INTERACTIONS AMONG PREDATOR PRESENCE, STRESS RESPONSE, AND PHENOTYPE IN LARVAL WOOD FROGS

Introduction

The link between environmental stressors and the expression of an organism's phenotype is key to understanding the consequences of these stressors on individual fitness and interactions within populations and communities. In vertebrates, many environmental stressors lead to activation of the neuroendocrine stress axis, and the hormones produced are known or hypothesized to mediate behavioral, physiological and developmental responses to environmental change (Denver 2009). From an ecological perspective, it would be useful to measure an organism's physiological response to stressors directly in order to generate predictions regarding non-lethal effects of environmental conditions; i.e., to trace the phenotypic effects "upstream" to the proximate mechanisms that generate the phenotype. Such proximate physiological responses may then serve as a proxy measure for subsequent phenotypic expression leading to higher order organismal and population effects. In addition, if these responses are experienced early in life they often can lead to long-term, stable changes in phenotypic expression in subsequent life history stages (Meaney et al. 2007, Denver 2009).

Vertebrates normally react to environmental, physiological, or psychological stressors by increasing circulating levels of glucocorticoid (GC) stress hormones. The

presence of increased GCs is presumed to direct energy expenditure away from less critical activities and boost survival responses in the face of an acute stressor (Sapolsky et al. 2000, Wingfield and Romero 2001). However, when a stressor is chronic many of these effects become damaging, and therefore organisms must balance responding adaptively to survive a stressor in the short-term and contending with the long-term consequences of a chronic response. Due to the negative effects of stress, stress physiology is an emerging proximate measure of ecologically relevant consequences, including behavioral and sub-lethal effects, of environmental conditions (e.g. Wasser et al. 1997, Creel et al. 2002). To understand the phenotype induced by a stressful environment, however, requires identifying the salient stressor and how the physiological response is manifest in fitness correlates.

Predation risk is a common source of stress for prey (Blanchard et al. 1998, Scheuerlein et al. 2001, Canoine et al. 2002, Adamec et al. 2006, Barcellos et al. 2007, Roseboom et al. 2007), and larval amphibians have been used as a model system for examining the direct and indirect effects of predator presence on individuals and their interactions with other species (e.g. Skelly 1994, Van Buskirk and Yurewicz 1998, Relyea 2000, Van Buskirk and Schmidt 2000, Relyea 2004). Tadpoles are able to detect the presence of invertebrate predators using chemical cues produced by the capture of conspecific prey (McCollum and Leimberger 1997, Relyea 2000, Gallie et al. 2001), and as a result change their behavior and morphology (Van Buskirk and McCollum 2000, Relyea 2001b, Relyea and Auld 2005) in ways that increase fitness under high predation risk (Van Buskirk et al. 1997). However, the complex interactions among predator presence, tadpole stress physiology, and effects on fitness correlates are not well

understood.

In this study, we explored the relationship between stressors in the larval pond environment, primarily predator presence, and stress hormone production in wood frog tadpoles (*Rana sylvatica*), as well as the role of stress hormones in producing predator-induced phenotypic responses. First, we surveyed natural ponds to test for correlations between tadpole GC activity and three potentially-interacting taxa: snails (an amphibian parasite vector), predators, and competitors, and confirmed the predator relationship with an experimental study. We then used sequential sampling of corticosterone from groups of tadpoles exposed to caged feeding predators in order to track the time course of the neuroendocrine stress response to predator presence. Finally, we applied stress hormone or GC-synthesis blocker in an experimental context with chemical cues of lethal predators to determine whether there is a direct causal link between stress hormones on tadpole behavior, predator-induced morphology, and predation trial survivorship. These studies combine field data and experimental manipulation to provide important insight into the connections between larval pond environment, physiological response, and phenotypic and fitness consequences.

Methods

Study 1: Field survey

Wood frog (*Rana sylvatica*) tadpoles at Gosner stages 27-30 (Gosner 1960) were collected from 10 natural ponds on the University of Michigan's E.S. George Reserve (ESGR) in Livingston Co., MI. Data from a long-term survey enabled us to choose ponds that have relatively consistent wood frog populations and represent a range of pond sizes

and environmental characteristics (4 are closed-canopy ponds and 6 are open-canopy; Werner et al. 2007). Due to their small size (~100-200 mg), 2-3 tadpoles were pooled as a single sample to provide sufficient tissue for steroid hormone analysis. Ten samples were taken from each pond for baseline corticosterone measurement, and each sample was flash-frozen in a test tube immersed in an ethanol-dry ice bath within 3 min of capture.

Within the same week, ponds were sampled for amphibian larvae, their invertebrate and vertebrate predators, and snails as part of an annual survey program (for methods see Werner et al. 2007). Tadpoles were identified to species, measured, and length-weight regressions employed to estimate biomass, which was then used as a measure of potential competitive pressure. Biomass of potential tadpole predators in these ponds (adult newts, adult and larval dytiscids, larval hydrophylids, adult belostomatids, and larval aeshnids) was used as a measure of predator pressure. Finally, snail biomass was also assessed because snails may be a potential source of trematode parasites or competition for tadpoles.

Study 2: Experimental mesocosm experiment

Experimental mesocosms were set up as described in (Relyea 2001a) using tanks filled with 1000 L of well water, 200 g of leaves (primarily *Quercus*) for substrate, a 4 L aliquot of pond water for zooplankton and periphyton colonization, and 15 g of rabbit chow as an initial food source. Six wood frog egg masses were collected from Cattail Marsh on the ESGR, pooled, and allowed to hatch in outdoor tanks. Once hatchlings were free-swimming, 40 were haphazardly chosen and placed in each of the experimental mesocosms. Each mesocosm was then randomly assigned to one of two treatments, non-lethal predator presence or no-predator, each replicated 10 times. Predator treatment

tanks received three cages each containing one late-instar dragonfly larva (*Anax* sp.); no-predator treatments received empty cages. Predators were fed approximately 0.3 g of live wood frog tadpoles three times per week to produce chemical cues of predator presence (McCollum and Leimberger 1997).

On day 25 of the experiment, 4 tadpoles (between Gosner stages 28-29) were collected from each tank, pooled into 2 samples, and immediately flash-frozen for later estimation of baseline whole-body corticosterone content. As the remaining tadpoles reached metamorphosis, individuals with tail stubs at or less than 50% of their snout-vent length were collected from mesocosm tanks and placed into plastic containers maintained in the lab to complete metamorphosis. Once metamorphosis was complete (tail stub completely resorbed, 2-3 days following removal from tank), 2 juveniles originating from each tank were pooled and immediately flash-frozen for baseline corticosterone analysis. A further 2 juveniles from each tank were subjected to a confinement stressor (enclosure in a 16.5 cm x 15 cm plastic zip-top bag) for 90 min, after which they were pooled by tank and flash-frozen.

Study 3: Shape of the stress response over time

Wood frog tadpoles for this experiment came from 15 partial egg masses collected from a private pond in Southeast Michigan and allowed to hatch in outdoor pools. Experimental outdoor wading pools (200 L) were filled with well water and covered with shade cloth to prevent colonization by other organisms. Ten pools received a caged later-instar *Anax* (non-lethal predator treatment) while the remaining 10 pools received an empty cage (control treatment). At approximately Gosner stage 27, groups of 5 tadpoles were each placed into 1.5-L plastic containers, topped by a screen mesh to

allow water to flow though, and containing 3-4 g of rabbit chow to provide sufficient food for the 8-day experimental period. Containers were then haphazardly assigned to the two treatments: non-lethal predator and control. Each outdoor pool received 5 containers; just before addition of the containers to the pools, the caged Anax larvae in the non-lethal predator treatment were fed 3 live tadpoles to produce chemical cue and were fed every day thereafter to maintain a near-constant level of cue in the water throughout the experiment (cages in control tanks were similarly manipulated to simulate any disturbance). Samples were removed and tadpoles pooled and flash-frozen at 4 hr, 24 hr, 2 d, 4 d, and 8 d following initiation of the experiment. Tadpoles intended for different sample timepoints were housed in separate containers throughout this experiment to minimize disturbance: sequential removals of containers from a pool was determined to pose less disturbance than haphazardly hand-netting free tadpoles. However the samples at the 2 d timepoint were lost and results are therefore not reported here.

Study 4: Morphology and behavior experiment

Wood frog tadpoles for this experiment were collected as eggs from a privately-owned pond in Livingston County, Michigan: 10 partial egg masses were removed from the pond shortly after laying and allowed to hatch in outdoor wading pools. Once tadpoles had developed to approximately Gosner stage 26, mixed-family groups of 12 were haphazardly chosen and transferred to 10-L plastic tanks in the lab filled with 8-L of aged well water and maintained on a 14:10 light:dark cycle. Treatments consisted of application of predator cue (Pred; see below), predator cue + GC-synthesis blocker metyrapone (Pred+MTP), corticosterone (CORT), or control; each was replicated 16

times and boxes were randomly assigned to treatment. All treatments were administered via the tank water. To produce predator cue, 12-18 late-instar Anax were each fed 2-3 live wood frog tadpoles and given 2-3 hours to consume the tadpoles. A 50-ml aliquot of the resulting "cue water" was then added to each of the treatment boxes. CORT was dissolved in ethanol and added to the appropriate treatment boxes to produce a final concentration of 130 nM (Glennemeier and Denver 2002b). MTP was also dissolved in ethanol and added to the appropriate treatments for a final concentration of 110 uM (Glennemeier and Denver 2002b). Control tanks received a 50 ml aliquot of vehicle control with 0.0005% ethanol (to match final concentrations in hormone treatments). Water changes were performed every other day, after which the above treatments were added to the water and the tadpoles were fed *ad lib* rabbit chow. Treatment continued for 14 days.

On the third day of treatment, behavioral observations were taken 1 hour after the administration of the treatments described above. The number of active tadpoles in each box was recorded every 10 minutes for 1 hour. At the termination of the experiment, four tadpoles from each box were haphazardly chosen for morphological analysis and preserved in formalin. A second haphazard sample of six tadpoles was flash-frozen for hormone analysis. Very low mortality was observed: only two tadpoles died during the course of the experiment, one from the Pred+MTP treatment and one from the Pred treatment. These tadpoles were not replaced.

Formalin-preserved tadpoles were digitally photographed for morphological measurements. Body length (BL), body width (BW), tail length (TL), and tail width (TW) were measured using Image J 1.36b (National Institutes of Health, Bethesda, MD)

using the landmarks provided by Relyea (2000). Morphological measurements were plotted against body mass and the residuals were used in statistical analyses to remove effects of tadpole size. To further explore one particular morphological pattern, tail depth was regressed on body length and the residuals analyzed.

Study 5: Survivorship study

Survivorship with a lethal predator was assessed under two conditions: short- and long-term exposure to CORT or MTP. Wood frog tadpoles for the short-term exposure experiment came from the same 10-family pool described in the morphology-behavior study above. At approximately Gosner stage 28, 3 groups of 80 tadpoles were haphazardly assigned to tanks housed in the lab and pre-treated for 3 hours with either CORT, MTP, or an ethanol vehicle control (CORT and MTP were at doses used in the behavior-morphology study above, and ethanol to match the 0.0005% solution present in the treatment tanks).

Earlier the same day, 60 10-L tanks were filled with aged well water, stocked with one late-instar *Anax*, and randomly assigned to one of the above tadpole treatments. A strip of vinyl screen approximately 4 cm wide by 40-50 cm long was added to each tank to provide structure. Once the 3-hour pre-treatment period was complete, four tadpoles were added to each tank according to the assigned treatment (producing 20 tanks with CORT-treated tadpoles, 20 with MTP-treated tadpoles, and 20 with control tadpoles). It is important to note that no CORT or MTP was present in the water of the tank containing the *Anax* predator. The number of remaining tadpoles in each tank was then counted every 30 min for 5 hours.

Tadpoles for the long-term exposure experiment came from 15 egg masses collected from Southeast Marsh on the ESGR and allowed to hatch in outdoor wading pools. At approximately Gosner stage 26, tadpoles were haphazardly assigned 10-L tanks in the lab that had been filled with 8-L of aged well water. CORT, MTP, and ethanol control treatments (at the same doses used in the short-term survivorship study) were each randomly assigned to 16 replicate tanks and treatment was continued for 12 days. As in the behavior-morphology study, water changes and treatment application occurred every other day and tadpoles were fed *ad lib* rabbit chow. Following the 12-day treatment period, tadpoles were transferred to tanks containing aged well water, screen, and a late-instar Anax predator. The remaining tadpoles were counted every 30 min for 5 hours.

Corticosterone analysis

Whole-tadpoles were extracted for corticosterone analysis as described in Denver (1998) and analyzed by radioimmunoassay (RIA) as described by Licht et al. (1983). The anti-corticosterone serum was purchased from MP Biomedicals (Irvine, CA, USA). Samples from a single study were assayed in either a single RIA or multiple RIAs conducted on the same day. Potency estimates from the RIA were corrected for recoveries, which ranged from 20 to 40%. Inter- and intra-assay coefficients of variation were calculated using a quality control standard, and averaged 13% and 8-12%, respectively, for each study.

Statistics

In Study 1, a multiple regression was performed using corticosterone concentration as the dependent variable and predator biomass, tadpole competitor

biomass, and snail biomass as predictors, and pond was used as the experimental unit. In the remaining experiments, experimental or rearing tank were used as the experimental unit in all analyses. In Study 2, the mesocosm experiment, independent-samples t-tests were employed to determine whether there was an effect of predator treatment on larval baseline stress hormone production and BW at metamorphosis. To determine whether larval treatment and stress measure (baseline or response to a novel stressor) produced differences in corticosterone concentration among juveniles, a one-way ANOVA was computed using corticosterone as the dependent variable and larval treatment and stress measure as fixed factors.

In Study 3, ANOVA was used to assess differences between the two treatment groups across the sample time periods. In Study 4, the behavior and morphology study, a repeated-measures ANOVA was used to analyze differences among treatment groups. Morphological measurements from tadpoles housed in the same box were averaged and the resulting replicate mean was used as the experimental unit. In three tadpoles, damage to the tail during preservation made accurate measurement impossible and those data were discarded. ANOVA was used to assess differences among treatments with regard to tail depth, tail length, body depth, and body length. Unless otherwise noted, all above analyses were conducted using SPSS 14.0 (SPSS Inc., Chicago, IL, USA). In Study 5, survivorship analyses were conducted in JMP (SAS Institute Inc, Cary, NC, USA) to determine whether experimental treatments increased or decreased the latency to first tadpole caught by the predator relative to the control treatment.

Results

Study 1: Field survey

In the field experiment, tadpole stress hormone levels increased in the presence of predators and snails. Whole-body corticosterone content was positively associated with predator ($t = 2.777$, $p = 0.032$) and snail ($t = 3.195$, $p = 0.019$) biomass in ponds, but not with tadpole competitor biomass ($t = -0.194$, $p = 0.379$; whole model: $F_{3,9} = 6.035$, $R^2 = 0.751$, $p = 0.030$; Fig 2.1).

Study 2: Mesocosm experiment

In mesocosms, the presence of caged predators also increased stress hormone content in tadpoles and recently metamorphosed juveniles, confirming the causal relationship suggested by Study 1. Tadpoles reared in tanks containing caged *Anax* predators exhibited significantly greater whole-body corticosterone content than controls ($t_9 = 2.387$, $p = 0.041$; Fig 2.2). There was a significant effect of predator treatment on corticosterone content of juveniles ($F_{1,38} = 1.462$, $p = 0.049$), a nonsignificant effect of the stress measure ($F_{1,38} = 2.813$, $p = 0.102$), and a significant treatment x stress measure interaction ($F_{1,38} = 4.882$, $p = 0.034$). With regards to the interaction effect, juveniles that emerged from tanks with caged predators exhibited increased whole-body corticosterone content compared to control juveniles (see Fig 2.2). The significant treatment x stressor interaction demonstrates that juveniles emerging from no-predator mesocosms responded to confinement by increasing corticosterone production above baseline, but juveniles emerging from mesocosms with caged predators exhibited no stress response (see Fig 2.2). Mass at metamorphosis was not significantly affected by larval treatment, although there was a slight trend towards smaller juveniles emerging from predator tanks ($t_{68} = -$

1.209, $p = 0.231$).

Study 3: Time course of the predator-induced stress response

Sample time ($F = 8.382$, $p < 0.001$) and treatment ($F = 6.795$, $p = 0.011$) both had significant effects on corticosterone content in tadpoles. In addition, the time x treatment interaction was also significant ($F = 9.415$, $p < 0.001$), demonstrating that response to predation cue changed over time (Fig. 2.3).

Study 4: Morphology and behavior experiment

Exogenous corticosterone application increased tadpole activity at 3 days, and increased tail depth and decreased body length similar to predator cue-exposed tadpoles at 14 days. Application of MTP (CORT-synthesis blocker) with predator cues, on the other hand, reversed the predator-induced effects.

Tadpole behavior was significantly different among treatments ($F_{3,60} = 40.823$, $p < 0.0001$; Fig 2.4), and CORT-treated tadpoles were significantly more active than all other treatments (CORT vs. Control: $p = 0.031$, CORT vs. Pred and Pred+MTP $p < 0.0001$), and predator and predator + MTP treatments significantly less active than control and CORT treatments ($p < 0.0001$ in both cases). Predator cue and predator cue + MTP treatments did not significantly differ from one another ($p = 0.824$).

Tadpole tail depth ($F_{3,60} = 7.310$, $p = 0.003$) and body length ($F_{3,60} = 9.406$, $p < 0.0001$; Fig 2.5) responded to treatments, such that CORT-treated and predator-cue tadpoles both developed relatively deeper tails (Pred: $p = 0.0003$; CORT: $p = 0.019$) and shorter bodies (Pred: $p = 0.0001$; CORT nonsignificant trend: $p = 0.101$) relative to controls. CORT and Pred tadpoles did not significantly differ from one another in either measure (tail depth: $p = 0.998$; body length: $p = 0.171$). Pred+MTP tadpoles had

shallower tails (p = 0.033) and longer bodies (p = 0.0004) than predator-cue tadpoles, although there was no significant difference with CORT tadpoles (tail depth: p = 0.792; body length: p = 0.248). Tail length ($F_{3,60}$ = 1.430, p = 0.243) and body width ($F_{3,60}$ = 0.469, p = 0.705) showed no significant differences among groups. To further explore the relationship between tail depth (TD) and body length (BL), residuals of a regression of TD on BL were compared among groups (Fig 2.6), showing that Pred and CORT tadpoles do not differ (p > 0.9) and Control and Pred + MTP tadpoles do not differ (p = 0.202), but the two treatment pairs significantly differ from one another (p < 0.0001).

Study 5: Survivorship study

Survival probability of CORT-treated tadpoles showed opposite patterns when hormone exposure was short (3 hr) versus long (8 d). In the short-term hormone exposure (3 hr) survivorship study, the CORT-treated tadpoles were more likely to be eaten than the MTP-treated and control tadpoles (Wilcoxon X^2 = 8.299, p = 0.016), but MTP-treated and control tadpoles did not significantly differ (Wilcoxon X^2 = 1.224, p = 0.268). In the long-term hormone exposure (8 d) survivorship study, however, the CORT-treated tadpoles trended towards being less likely to be eaten than both MTP-treated and control tadpoles (Wilcoxon X^2 = 4.751, p = 0.093), while the MTP-treated and control tadpoles did not differ (Wilcoxon X^2 = 0.981, p = 0.322).

Discussion

Here we show for the first time, to our knowledge, the time course and phenotypic consequences of the predator stress response in larval anurans, using a combination of field surveys, mesocosm experiments, and laboratory manipulations. We first showed

that natural ponds with high predator and snail biomass produce tadpoles with high baseline stress hormone levels, and the mesocosm exposure demonstrated this relationship is causal in the case of predators. The same mesocosm study showed that predator-exposed tadpoles metamorphose into juveniles that retain a high baseline corticosterone level and do not mount a stress response to a novel stressor. Next, we extended our investigation beyond a single measure of stress hormone content and performed a time series analysis of the predator stress response. We found that when tadpoles are exposed to predator cue, endogenous CORT content is initially reduced, but between 24 hours and 4 days of exposure CORT production is increased and maintained at level above no-predator controls, matching the results seen under long-term exposure in ponds and mesocosms.

We then focused on determining the phenotypic consequences of increased corticosterone production, using exogenous hormone application. Our results show that tadpoles that exposed to exogenous CORT increase activity relative to other treatments, but over a two-week treatment time develop morphology similar to predator-cue exposed tadpoles. In addition, treatment with MTP blocks this predator-induced morphology. Finally, we investigated the potential for the glucocorticoid axis to impact fitness through predation survival, and showed that while short-term CORT treatment decreases survivorship with lethal predators, long-term CORT treatment has a trend towards increasing survivorship. As a whole, these results demonstrate the complexity of the interaction between predator presence, tadpole physiological and morphological response, and fitness effects.

The non-lethal effects of predators on tadpoles have been well-studied (e.g.

McCollum and Leimberger 1997, Relyea 2001b), and predators have been shown to reduce tadpole activity and growth and alter morphology (Van Buskirk and Yurewicz 1998, Relyea 2001b, Peacor 2002). These trait responses in turn can cause important trait-mediated indirect interactions (TMIIs, Abrams et al. 1996) in ecological communities (Werner and Peacor 2003). Wood frogs are an excellent system for demonstrating the mechanistic underpinnings of phenotypic plasticity to predators, since populations of wood frogs inhabit a range of pond types and associated predation risks (Werner et al. 2007), and therefore tadpoles exhibit a high degree of both behavioral and morphological plasticity that is locally adapted to their larval environment (Relyea 2002, 2004). The results presented here demonstrate that some of the phenotypic effects induced by predators may be mediated through the stress axis, and furthermore that the tadpole stress response may follow a temporal pattern that maximizes survival by increasing hormone production in the long-term to induce anti-predator morphology.

In this study, tadpoles initially reduce endogenous stress hormone levels in response to threat of predation (Study 3), which is consistent with results from a similar tadpole-predator system (Fraker et al. 2009). This initial reduction in CORT production and resulting behavioral quiescence is likely an adaptive response that increases survivorship: treatment with exogenous CORT causes increased activity (Study 4), which increases predator encounter rate and may lead to the decreased survivorship demonstrated after brief CORT exposure in Study 5. However, the field and mesocosm data (Studies 1 and 2) show that chronic predator presence is directly linked to long-term increases in CORT production, which Study 3 shows happens within the first week of continuous exposure to predator cues. Furthermore, the resulting high CORT levels play

an important role in the development of morphological changes (Study 4) that increase survival probability under lethal predation (Study 5, Van Buskirk et al. 1997). Thus, the results presented here show that the predator-induced response of the neuroendocrine stress axis in tadpoles appears to be timed to trigger both adaptive behavioral and morphological changes. In addition, as CORT is involved in feeding behavior, the rise in CORT production following several days of predator exposure, and resulting behavioral quiescence, may stimulate appetite while inducing morphology that reduces predation efficiency. These results highlight the importance of considering the time scale over which a response is measured, as well as the potentially competing demands that shape that response.

Our results develop a more complete understanding of the tadpole response to predator stress than has been, to our knowledge, demonstrated previously. Our results are consistent with previous studies, and help serve to coalesce what has been found in other systems. As mentioned earlier, Fraker et al. (2009) showed that CORT content of *Rana clamitans* decreases during the first 4 hours following predator cue exposure, during which time behavioral quiescence also occurs, and we have confirmed this finding in wood frogs here. In addition, it has been shown previously that CORT may be part of the mechanism responsible for inducing morphology associated with predators: Hossie (2010) showed that *Rana pipiens* tadpoles whose CORT synthesis was inhibited with MTP exhibited shallow tails compared to predator-cue tadpoles, and Glennemeier and Denver (2002) also saw changes in tail shape following CORT application, both of which we also show here. However, this study goes beyond previous work by demonstrating the full temporal pattern of the stress response, namely that tadpoles exposed to predator

cue continuously, as would likely occur in ponds colonized by invertebrate predators, eventually produce chronically high levels of CORT, and that this response is measurable in field populations. In addition, we investigated the fitness effects of this stress response using survivorship analysis, showing that an initial reduction in CORT may be beneficial by allowing reduced activity, whereas long-term increased production induces adaptive morphology. However, a chronic stress response may have lasting effects on phenotype and fitness, the investigation of which may require study over the entire life cycle.

We have focused our investigation on the interaction between tadpoles and their predators, but the field survey also generated some intriguing results regarding other potential stressors: the survey also showed an association between CORT content and snail biomass. Although this association remains correlative without further experimentation, there are several potential mechanisms through which snails may directly affect tadpole performance. First, snails also consume periphyton and therefore could be resource competitors with tadpoles; however, this explanation seems unlikely, given that tadpole competitors were not a significant stressor and yet were found at biomasses up to an order of magnitude higher than snails (see Fig 1). A second possible explanation is the potential presence of trematode parasites in ponds where snails are abundant: snails are the first intermediate host of trematodes, and mature cercariae shed from the infected snails subsequently infect tadpoles (Najarian 1953). One of the most abundant snail species sampled in this study was *Stagnicola elodes*, which is known to carry a tadpole-infecting echinostome, *Echinostoma revolutum* (Schell 1970, Marino and Holland *pers. comm.*) which leads to lower growth and high rates of mortality in infected tadpoles (Holland et al. 2007). Tadpoles of other species have been shown to detect

infected snails and cercariae (Rohr et al. 2009) so it is possible that the presence of parasites or infected snails might induce a stress response. Such a reaction would have potentially interesting implications: prolonged elevation of GCs generally has negative effects on immune function (Sapolsky et al. 2000), and therefore it may be that predator-stressed tadpoles also become more prone to micro- and macro-parasites (Belden and Kiesecker 2005). This could have important implications for tadpoles in natural ponds, where predators and parasites co-exist. Furthermore, the adaptive value of producing an acute stress response to parasite presence may differ from a response to the more chronic condition of parasite infection.

In contrast to predators and snails, tadpole competitor biomass was not a significant predictor of tadpole stress hormone levels in the field. Belden et al. (2007) conducted a similar field survey of six wood frog populations found at different densities, and reported that tadpole corticosterone levels varied among ponds but was not associated with tadpole density. Density-dependent growth and developmental rates have been studied extensively in amphibian larvae (e.g. Wilbur 1977, Berven 1990, Van Buskirk and Smith 1991, Loman 2004), but both this study (above) and Belden et al. (2007) suggest that corticosterone is not necessarily associated with the reduced growth of tadpoles developing at high densities in the field. Given a number of examples of stress hormone involvement in consumption and growth (e.g. Hayes et al. 1993, Glennemeier and Denver 2002a, Hossie et al. 2010), however, this result does not necessarily imply that competition in all amphibian larval communities does not involve mediation by stress hormones.

As we map out the causes and effects of environmental stress in the larval stage, it

is also important to consider the potential for hormone-mediated long-term impacts on the phenotype expressed in the terrestrial juvenile stage. Most studies of such "carry-over effects" in amphibians have focused on the influence of pond conditions on age and size at metamorphosis (e.g. Wilbur and Collins 1973), but other aspects of phenotype may also similarly carry over to the adult stage and affect fitness (e.g. Relyea 2001a, Benard and Fordyce 2003, Scott et al. 2007). These "carry-over effects" are especially interesting in species that exhibit a complex life cycle, where life stages are ecologically distinct and thus early developmental experiences are unlikely to be predictive of future environmental conditions (Wilbur 1980). Thus, the effects of early environment may potentially impact interactions in very different communities through carry-over effects from larval (aquatic) environments on subsequent (terrestrial) life history stages. In the present study, individuals that experienced cues of predator presence during the larval period continued to show elevated basal corticosterone levels after metamorphosis. Moreover, when presented with a novel confinement stressor, predator-treatment juveniles were unable to further elevate their corticosterone levels to produce a stress response, suggesting that they were already at maximal hormone production. Since these measurements were taken just after completion of metamorphosis, we cannot rule out the possibility that we were measuring residual hormones from the larval period. However, a hormonal carry-over effect is a plausible explanation given the results of previous research; Hu et al. (2008) demonstrated a reduced stress response in *Xenopus* juveniles as long as 3 months after larval stress. A similar pattern has been documented in other species as well: laboratory experiments in birds and rodents have shown that stressed juveniles develop into adults that exhibit altered stress activity, either through increasing

reactivity to stressful stimuli or failure to mount a normal stress response (Anisman et al. 1998, Meaney 1999, Hayward and Wingfield 2004, Parfitt et al. 2004).

Therefore, potential for larval stress to affect juvenile and adult stress response in our system deserves further consideration. Since the physiological stress response is considered adaptive for escaping or surviving difficult and/or unpredictable situations (Wingfield and Kitaysky 2002), the inability to produce a stress response is likely costly. In addition, chronic elevation of stress hormones becomes harmful to the organism due to such effects as suppression of immune function, reduced growth rate, and neuronal cell death (Sapolsky et al. 2000, Wingfield and Romero 2001). Hu et al. (2008) also found that early life exposure to elevated GCs caused decreased GC receptor expression in regions of the juvenile frog brain involved in behavioral stress responses, suggesting that anxiogenic and predator avoidance behaviors could be altered in these animals.

The results presented here also have important implications for studies that measure basal GC levels in the field as a measure of the "health" of a population or response to an environmental perturbation (e.g. Wasser et al. 1997, Creel et al. 2002). If early experiences have a "programming" effect on the stress axis (e.g. Hu et al. 2008), then the stress activity exhibited by individuals in a population are a function of previous experience and may or may not reflect current conditions. Indeed, previously-stressed individuals in this study did not mount a response to a novel stressor, meaning that a physiological reaction to an environmental perturbation later in life may not be detectable in organisms that experienced a high-stress ontogeny.

In addition, and perhaps more importantly, our results demonstrate that environmental "stress" does not always manifest itself with high stress hormone

production, but rather that the action of the stress hormone on phenotype, and the time period over which those changes occur, may differ based on the context and fitness consequences of a given response. Therefore, stress hormone measurements from wild populations should be interpreted carefully, and consideration should be given to the possibility that previous experiences and/or context-dependent physiological responses may be shaping the current measurement. In addition, populations that differ in their exposure to stressors, even early in development, may exhibit correlated differences in growth rates, dispersal probabilities, immunity, or reproductive strategies due to the widespread effects of stress on phenotype (Sapolsky et al. 2000, Wingfield and Romero 2001). Taken as a whole, this emphasizes the utility and importance of understanding physiological mechanisms to assess how environment can impact ecological interactions and long-term fitness.

References

Abrams, P. A., B. A. Menge, G. G. Mittalbach, D. Spiller, and P. Yodzis. 1996. The role of indirect effects in food webs. Pages 371-395 *in* G. A. Polis and K. O. Winemiller, editors. Food webs: Integration of pattern and dynamics. Chapman & Hill, New York.

Adamec, R., D. Head, J. Blundell, P. Burton, and O. Berton. 2006. Lasting anxiogenic effects of feline predator stress in mice: Sex differences in vulnerability to stress and predicting severity of anxiogenic response from the stress experience. Physiology & Behavior **88**:12-29.

Anisman, H., M. D. Zaharia, M. J. Meaney, and Z. Merali. 1998. Do early-life events permanently alter behavioral and hormonal responses to stressors? International Journal Of Developmental Neuroscience **16**:149-164.

Barcellos, L. J. G., F. Ritter, L. C. Kreutz, R. M. Quevedo, L. B. da Silva, A. C. Bedin, J. Finco, and L. Cericato. 2007. Whole-body cortisol increases after direct and visual contact with a predator in zebrafish, Danio rerio. Aquaculture **272**:774-778.

Belden, L. K. and J. M. Kiesecker. 2005. Glucocorticosteroid hormone treatment of larval treefrogs increases infection by *Alaria sp* trematode cercariae. Journal Of Parasitology **91**:686-688.

Benard, M. F. and J. A. Fordyce. 2003. Are induced defenses costly? Consequences of predator-induced defenses in western toads, *Bufo boreas*. Ecology **84**:68-78.

Berven, K. A. 1990. Factors affecting population fluctuations in larval and adult stages of the wood frog (*Rana sylvatica*). Ecology **71**:1599-1608.

Blanchard, R. J., J. N. Nikulina, R. R. Sakai, C. McKittrick, B. McEwen, and D. C. Blanchard. 1998. Behavioral and endocrine change following chronic predatory stress. Physiology & Behavior **63**:561-569.

Canoine, V., T. J. Hayden, K. Rowe, and W. Goymann. 2002. The stress response of European stonechats depends on the type of stressor. Behaviour **139**:1303-1311.

Creel, S., J. E. Fox, A. Hardy, J. Sands, B. Garrott, and R. O. Peterson. 2002. Snowmobile activity and glucocorticoid stress responses in wolves and elk. Conservation Biology **16**:809-814.

Denver, R. J. 2009. Stress hormones mediate environment-genotype interactions during amphibian development. General And Comparative Endocrinology **164**:20-31.

Fraker, M. E., F. Hu, V. Cuddapah, S. A. McCollum, R. A. Relyea, J. Hempel, and R. J. Denver. 2009. Characterization of an alarm pheromone secreted by amphibian tadpoles that induces behavioral inhibition and suppression of the neuroendocrine stress axis. Hormones And Behavior **55**:520-529.

Gallie, J. A., R. L. Mumme, and S. A. Wissinger. 2001. Experience has no effect on the development of chemosensory recognition of predators by tadpoles of the American toad, Bufo americanus. Herpetologica **57**:376-383.

Glennemeier, K. A. and R. J. Denver. 2002a. Role for corticoids in mediating the response of *Rana pipiens* tadpoles to intraspecific competition. Journal Of Experimental Zoology **292**:32-40.

Glennemeier, K. A. and R. J. Denver. 2002b. Small changes in whole-body corticosterone content affect larval *Rana pipiens* fitness components. General And Comparative Endocrinology **127**:16-25.

Gosner, K. L. 1960. A simplified table for staging anuran embryos and larvae with notes on identification. Herpetologica **16**:183-190.

Hayes, T., R. Chan, and P. Licht. 1993. Interactions of temperature and steroids on larval growth, development, and metamorphosis in a toad (*Bufo boreas*). Journal Of Experimental Zoology **266**:206-215.

Hayward, L. S. and J. C. Wingfield. 2004. Maternal corticosterone is transferred to avian yolk and may alter offspring growth and adult phenotype. General And Comparative Endocrinology **135**:365-371.

Holland, M. P., D. K. Skelly, M. Kashgarian, S. R. Bolden, L. M. Harrison, and M. Cappello. 2007. Echinostome infection in green frogs (*Rana clamitans*) is stage and age dependent. Journal Of Zoology **271**:455-462.

Hossie, T. J., B. Ferland-Raymond, G. Burness, and D. L. Murray. 2010. Morphological and behavioural responses of frog tadpoles to perceived predation risk: A possible role for corticosterone mediation? Ecoscience **17**:100-108.

Loman, J. 2004. Density regulation in tadpoles of *Rana temporaria*: A full pond field experiment. Ecology **85**:1611-1618.

McCollum, S. A. and J. D. Leimberger. 1997. Predator-induced morphological changes in an amphibian: predation by dragonflies affects tadpole shape and color. Oecologia **109**:615-621.

Meaney, M. J. 1999. Early environmental experience and lifelong changes in behavior and in gene expression. Biological Psychiatry **45**:44S-44S.

Meaney, M. J., M. Szyf, and J. R. Seckl. 2007. Epigenetic mechanisms of perinatal programming of hypothalamic-pituitary-adrenal function and health. Trends in Molecular Medicine **13**:269-277.

Najarian, H. H. 1953. The life history of *Echinoparyphium flexum* (Linton 1892) (Dietz 1910) (Trematoda, Echinostomidae). Science **117**:564-565.

Parfitt, D. B., J. K. Levin, K. P. Saltstein, A. S. Klayman, L. M. Greer, and D. L. Helmreich. 2004. Differential early rearing environments can accentuate or attenuate the responses to stress in male C57BL/6 mice. Brain Research **1016**:111-118.

Peacor, S. D. 2002. Positive effect of predators on prey growth rate through induced modifications of prey behaviour. Ecology Letters **5**:77-85.

Relyea, R. A. 2000. Trait-mediated indirect effects in larval anurans: reversing competition with the threat of predation. Ecology **81**:2278-2289.

Relyea, R. A. 2001a. The lasting effects of adaptive plasticity: predator-induced tadpoles become long-legged frogs. Ecology **82**:1947-1955.

Relyea, R. A. 2001b. Morphological and behavioral plasticity of larval anurans in response to different predators. Ecology **82**:523-540.

Relyea, R. A. 2002. Local population differences in phenotypic plasticity: predator-induced changes in wood frog tadpoles. Ecological Monographs **72**:77-93.

Relyea, R. A. 2004. Fine-tuned phenotypes: Tadpole plasticity under 16 combinations of predators and competitors. Ecology **85**:172-179.

Relyea, R. A. and J. R. Auld. 2005. Predator- and competitor-induced plasticity: how changes in foraging morphology affect phenotypic trade-offs. Ecology **86**:1723-1729.

Rohr, J. R., A. Swan, T. R. Raffel, and P. J. Hudson. 2009. Parasites, info-disruption, and the ecology of fear. Oecologia **159**:447-454.

Roseboom, P. H., S. A. Nanda, V. P. Bakshi, A. Trentani, S. M. Newman, and N. H. Kalin. 2007. Predator threat induces behavioral inhibition, pituitary-adrenal activation and changes in amygdala CRF-binding protein gene expression. Psychoneuroendocrinology **32**:44-55.

Sapolsky, R. M., L. M. Romero, and A. U. Munck. 2000. How do glucocorticoids influence stress responses? Integrating permissive, suppressive, stimulatory, and preparative actions. Endocrine Reviews **21**:55-89.

Schell, S. C. 1970. How to know the trematodes. Wm. C. Brown Company, Dubuque, IA.

Scheuerlein, A., T. J. Van't Hof, and E. Gwinner. 2001. Predators as stressors? Physiological and reproductive consequences of predation risk in tropical stonechats (*Saxicola torquata axillaris*). Proceedings Of The Royal Society Of London Series B-Biological Sciences **268**:1575-1582.

Scott, D. E., E. D. Casey, M. F. Donovan, and T. K. Lynch. 2007. Amphibian lipid levels at metamorphosis correlate to post-metamorphic terrestrial survival. Oecologia **153**:521-532.

Skelly, D. K. 1994. Activity level and the susceptibility of anuran larvae to predation. Animal Behaviour **47**:465-468.

Van Buskirk, J. and S. A. McCollum. 2000. Functional mechanisms of an inducible defence in tadpoles: morphology and behaviour influence mortality risk from predation. Journal of Evolutionary Biology **13**:336-347.

Van Buskirk, J., S. A. McCollum, and E. E. Werner. 1997. Natural selection for environmentally induced phenotypes in tadpoles. Evolution **51**:1983-1992.

Van Buskirk, J. and B. R. Schmidt. 2000. Predator-induced phenotypic plasticity in larval newts: trade-offs, selection, and variation in nature. Ecology **81**:3009-3028.

Van Buskirk, J. and D. C. Smith. 1991. Density-dependent population regulation in a salamander. Ecology **72**:1747-1756.

Van Buskirk, J. and K. L. Yurewicz. 1998. Effects of predators on prey growth rate: relative contributions of thinning and reduced activity. Oikos **82**:20-28.

Wasser, S. K., K. Bevis, G. King, and E. Hanson. 1997. Noninvasive physiological measures of disturbance in the Northern Spotted Owl. Conservation Biology **11**:1019-1022.

Werner, E. E. and S. D. Peacor. 2003. A review of trait-mediated indirect interactions in ecological communities. Ecology **84**:1083-1100.

Werner, E. E., D. K. Skelly, R. A. Relyea, and K. L. Yurewicz. 2007. Amphibian species richness across environmental gradients. Oikos **116**:1697-1712.

Wilbur, H. M. 1977. Density-dependent aspects of growth and metamorphosis in *Bufo americanus*. Ecology **58**:196-200.

Wilbur, H. M. 1980. Complex life-cycles. Annual Review Of Ecology And Systematics **11**:67-93.

Wilbur, H. M. and J. P. Collins. 1973. Ecological aspects of amphibian metamorphosis. Science **182**:1305-1314.

Wingfield, J. C. and A. S. Kitaysky. 2002. Endocrine responses to unpredictable environmental events: Stress or anti-stress hormones? Integrative and Comparative Biology **42**:600-609.

Wingfield, J. C. and L. M. Romero. 2001. Andrenocortical responses to stress and their modulation in free-living vertebrates. Pages 211-234 *in* B. S. McEwen and H. M. Goodman, editors. Handbook of Physiology; Section 7: The Endocrine System; Volume IV: Coping with the Environment: Neural and Endocrine Mechanisms. Oxford University Press.

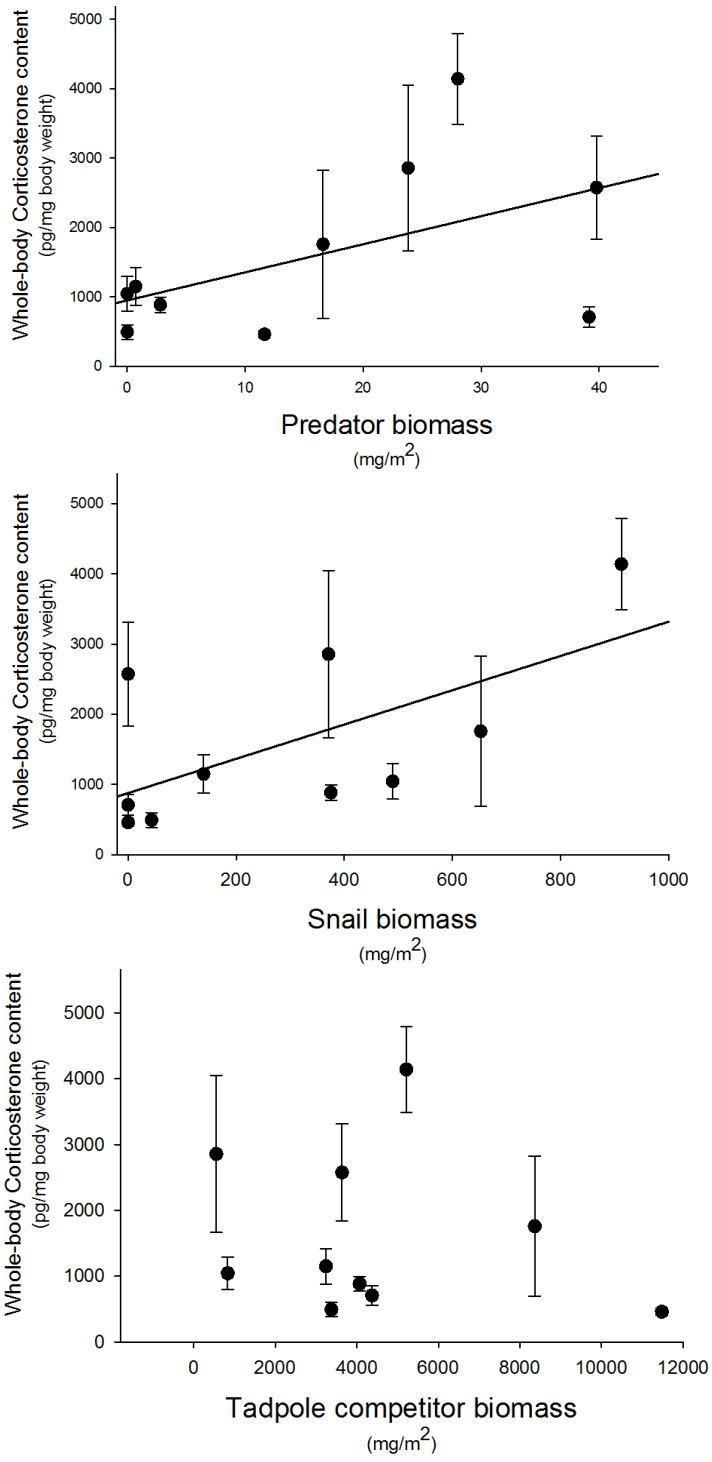


Figure 2.1. Relationships between tadpole corticosterone content and predator, snail, and competitor biomass in the field survey. Points represent pond means with +/- 2 S.E. Whole-body corticosterone content was positively associated with predator (t = 2.777, *p*

= 0.032) and snail (t = 3.195, p = 0.019) biomass in ponds, but not with tadpole competitor biomass (t = -0.194, p = 0.379; whole model: $F_{3,9}$ = 6.035, R^2 = 0.751, p = 0.030).

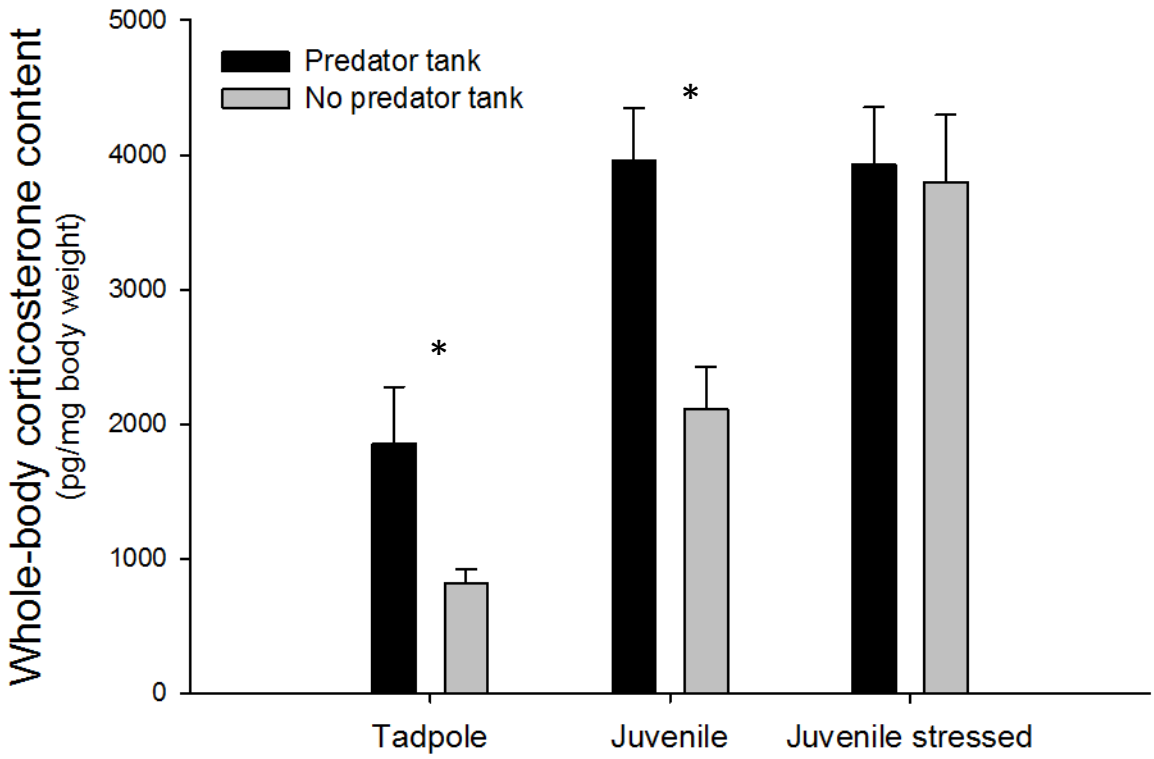


Figure 2.2. Whole-body corticosterone content from tadpoles and juveniles in the mesocosm experiment. Tadpole CORT content significantly differed among predator and no-predator treatments ($t_9 = 2.387$, $p = 0.041$). Juveniles showed a similar pattern in baseline measures but when exposed to confinement stress, treatments did not differ (significant treatment x stress measure interaction; $F_{1,38} = 4.882$, $p = 0.034$). Gray bars represent no predator treatments while black show predator treatments; error bars represent 2 S.E.

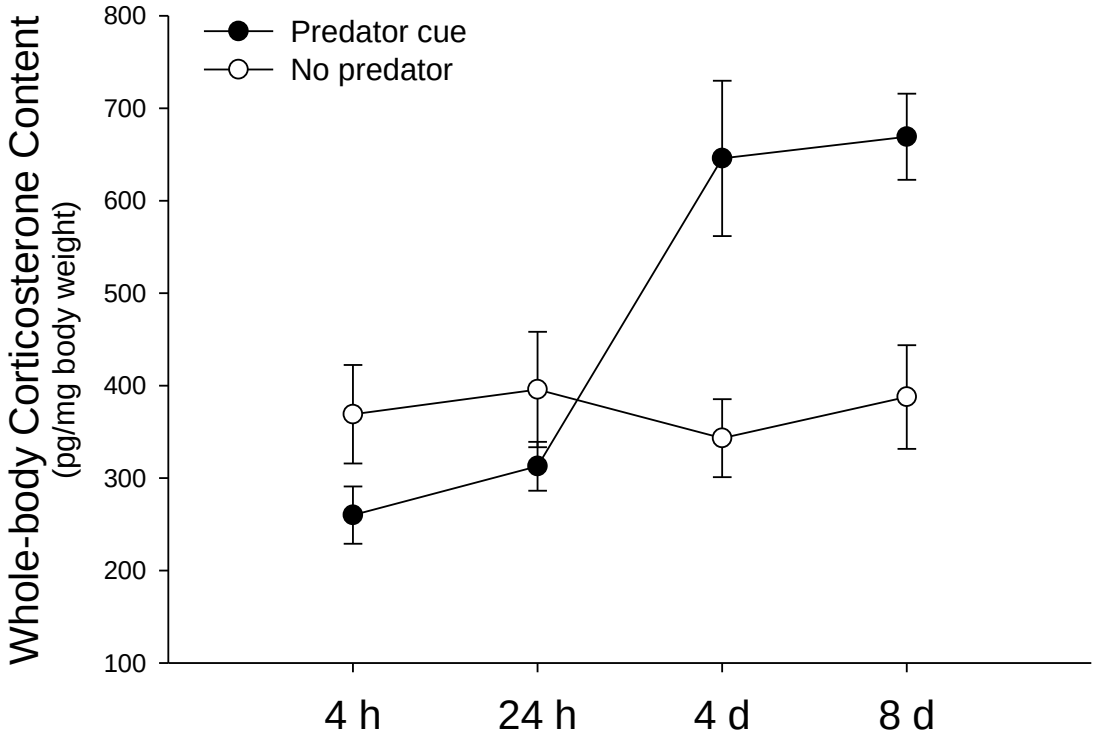


Figure 2.3. Time series of the tadpole predator stress response. Sample time ($F = 8.382$, $p < 0.001$) and treatment ($F = 6.795$, $p = 0.011$) both had significant effects on corticosterone content in tadpoles. In addition, the time x treatment interaction was also significant ($F = 9.415$, $p < 0.001$), demonstrating that response to predation cue changed over time. Open symbols represent no predator treatments, filled represent predator; points are sample time means with 2 S.E.

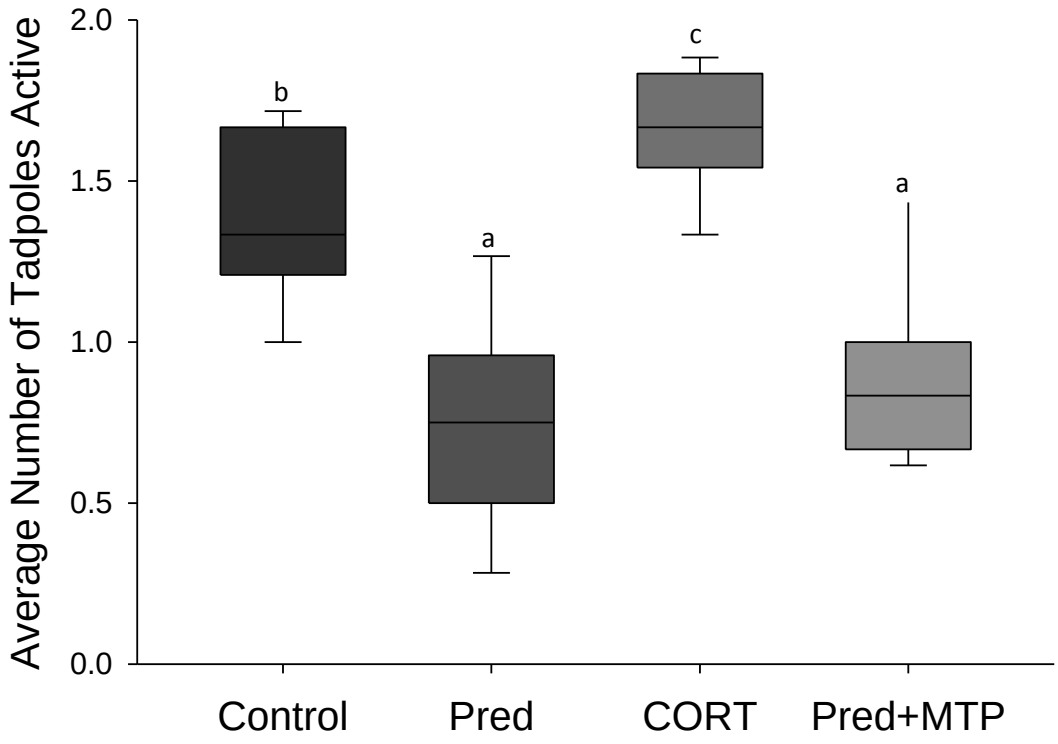


Figure 2.4. Tadpole activity in the hormone manipulation experiment (Study 4). Prior to observation, tadpoles had been treated for 3 days with exogenous corticosterone (CORT), predator cue (Pred), predator cue plus a glucocorticoid-synthesis blocker metyrapone (Pred + MTP), or given a vehicle control (Control). CORT tadpoles were more active than Control ($p = 0.031$), Pred ($p < 0.0001$), and Pred+MTP ($p < 0.0001$). Pred and Pred + MTP treatments did not significantly differ from one another ($p = 0.824$), and were significantly less active than both control and CORT treatments ($p < 0.0001$).

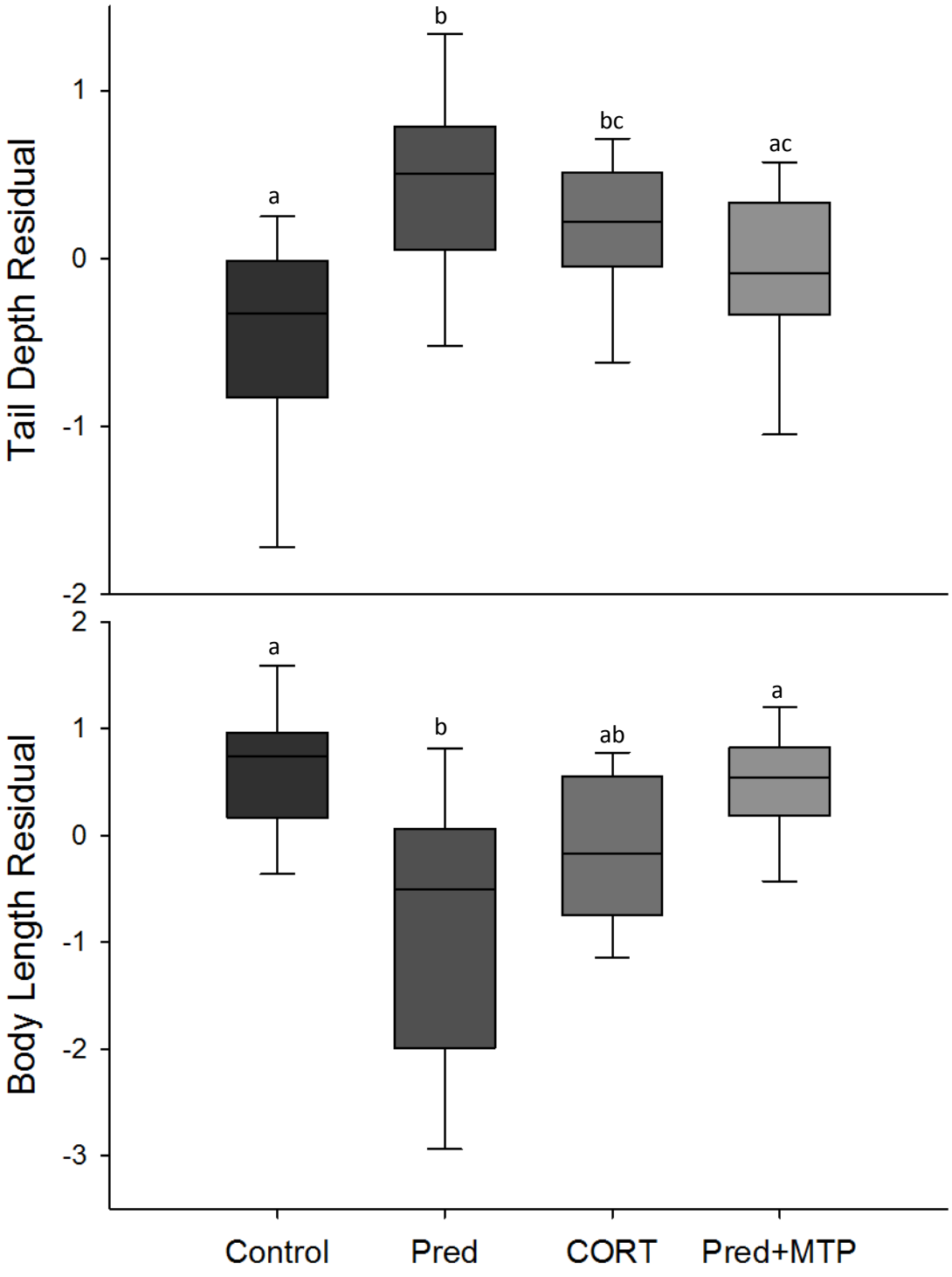


Figure 2.5. Tadpole morphology in the hormone manipulation experiment (Study 4). After 14 days of treatment, tadpoles were preserved and morphological measurements

were regressed onto body mass. Tadpole tail depth ($F_{3,60} = 7.310$, $p = 0.003$) and body length ($F_{3,60} = 9.406$, $p < 0.0001$) responded to treatments, such that CORT-treated and predator-cue tadpoles both developed relatively deeper tails (Pred: $p = 0.0003$; CORT: $p = 0.019$) and shorter bodies (Pred: $p = 0.0001$; CORT nonsignificant trend: $p = 0.101$) relative to Controls. CORT and Pred tadpoles did not significantly differ from one another in either measure (tail depth: $p = 0.998$; body length: $p = 0.171$). Pred+MTP tadpoles had shallower tails ($p = 0.033$) and longer bodies ($p = 0.0004$) than Pred tadpoles, although there was no significant difference with CORT tadpoles (tail depth: $p = 0.792$; body length: $p = 0.248$).

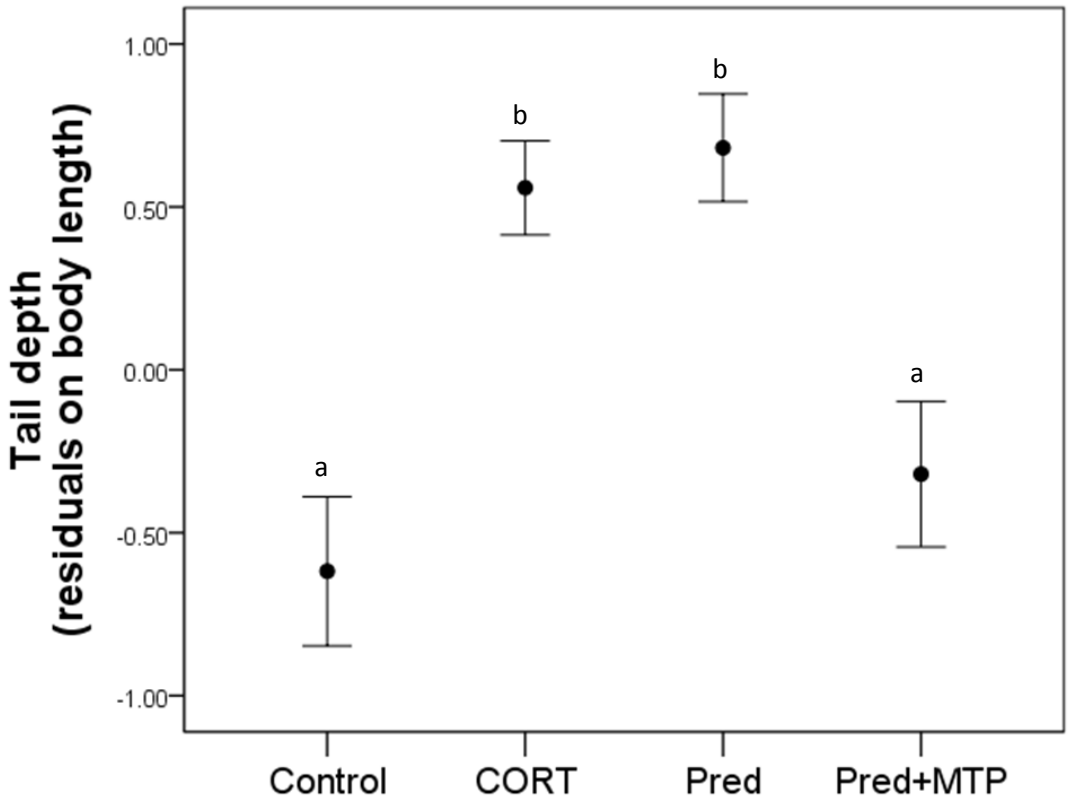


Figure 2.6. Tail depth as a function of body length. To further illustrate the relationship between morphological traits, tail depth was regressed on body length and the residuals plotted here. Pred and CORT tadpoles do not differ ($p > 0.9$) and Control and Pred + MTP tadpoles do not differ ($p = 0.202$), but the two treatment pairs significantly differ from one another ($p < 0.0001$). Points represent treatment means with +/- 2 S.E.

CHAPTER 3

PREDATOR STRESS RESPONSE AND MAGNITUDE OF RISK IN LARVAL ANURANS

Introduction

Environmental variation in ecological pressures, such as predation, can be an important driver of evolution of phenotypic response among populations and species. Predators impose selective pressure on traits that influence prey survivorship, but such defensive traits often come at a cost (Van Buskirk et al. 1997, Van Buskirk and Relyea 1998, Relyea 2000). Therefore, when environments differ in the risk of predation, organisms should be selected for different magnitudes of defense phenotype expression, in order to maximize the benefit/cost relationship of maintaining plasticity in that trait (West-Eberhard 1989).

One important phenotypic reaction to predation threat is the initiation of a stress response (Boonstra et al. 1998, Scheuerlein et al. 2001, Clinchy et al. 2004, Barcellos et al. 2007, Roseboom et al. 2007), which usually increases circulating levels of glucocorticoid (GC) stress hormones in a chronic response (Chapter 2). Increased production of GCs is presumed to direct energy expenditure away from unnecessary activities and boost survival responses in the face of an acute stressor such as predation risk (Sapolsky et al. 2000, Wingfield and Romero 2001). Although the GC stress response may be adaptive in emergency situations (Wingfield et al. 1998), prolonged

expression of GCs comes at a physiological cost, including reductions in growth, immunocompetence, and reproductive activity (Sapolsky et al. 2000, Wingfield and Romero 2001).

From a mechanistic viewpoint, an "optimal" stress response, i.e. allowing the organism to respond to the immediate challenge while avoiding the negative effects of sustained GC elevation, would appear to be a combination of low GC baseline, fast elevation, and rapid induction of negative feedback (Breuner et al. 2008). Realistically, however, stressors such as predation risk are often prolonged or repeated events that do not allow for a rapid recovery of low baseline, and thus the mechanistic optimum is not always achievable. The magnitude and duration of an organism's GC response to a stressor thus may be an important determinant of fitness, and yet the fitness consequences of a particular response to a stressor appear to be highly context-dependent (Bonier et al. 2009). Therefore, it is likely that the magnitude and frequency of a stressor in turn imposes selection on the organism's response to that stressful event; for example, organisms that experience a chronic and/or predictable stressor may alter their response to that event in such a way that avoids the costs of prolonged GC elevation.

The larval stage of pond-breeding amphibians has been a model system for investigating predator-prey interactions, in part because larvae exhibit a number of adaptive behavioral and morphological responses to predation risk (Van Buskirk et al. 1997, Van Buskirk and Relyea 1998). This system also lends itself to comparative study, since pond-breeding species face a gradient of habitat types that vary in level and type of predation risk: from ephemeral ponds whose invertebrate predator populations must recolonize each spring, to permanent ponds with stable populations of invertebrate and

fish predators (Wellborn et al. 1996). Species that distribute themselves differently along this gradient thus face a different set of selective pressures on such aspects of life history as reproductive phenology, developmental timing, and investment in predator defense strategies. For example, among species that utilize different pond types, there is evidence of both differing degrees of morphological plasticity to predator presence (Van Buskirk 2002) as well as local adaptation among populations (Van Buskirk and Relyea 1998, Relyea 2002). Thus, it seems plausible that species living along a predation risk gradient may exhibit differing degrees of response to that predictable stressor, and subsequently adaptively reduce the cost of the chronic GC response. The aim of this experiment was to use a comparative method to determine whether the magnitude of stress response can be adaptively managed by species living under chronically or predictably predator-rich environments.

In this study, I chose four anuran species that are arrayed along the hydrological period gradient, employing survey data to determine each species' "typical" habitat. As a result, these species also differ in the level of predation risk they normally encounter, which I also verified using survey data. The focal species represented two pairs of congeners, with one member of each pair tending to develop in relatively high-predator ponds and the other experiencing relatively low levels of predators. The decision to use congener pairs was made in order to control for any phylogenetic effects on physiology, and the choice of one high-predator and one low-predator species within each pair allowed me to focus on those differences in species-typical habitat. I raised tadpoles in a common garden and exposed each species to both nonlethal predator presence and no predator treatments, and measured the relative glucocorticoid (specifically corticosterone,

CORT) response to predation risk. I then compared the magnitude of response within congener pairs to assess any potentially adaptive pattern between physiology and species-typical habitat type, specifically to test whether species under chronic stress are able to manage their stress response in such a way to reduce the costs of prolonged GC exposure.

Methods

Experimental species and determination of species habitat type

To identify appropriate species for this comparative experiment, data were used from a long-term annual survey of amphibian larvae and their vertebrate and invertebrate predators in ponds on the University of Michigan's Edwin S. George Reserve (ESGR; for detailed methods see Werner et al. 2007). Biomass of potential tadpole predators in breeding ponds (adult newts, adult and larval dytiscids, larval hydrophylids, adult belostomatids, and larval aeshnids) was used as a measure of predator pressure. Twelve years (1996-2008) of tadpole occupancy and predator biomass data were available for each of four selected species: *Pseudacris triseriata*, *P. crucifer*, *Rana sylvatica*, and *R. pipiens*. Predator biomass for each pond-year in which tadpoles of the same congener pair did not co-occur (*P. triseriata* n = 30, *P. crucifer* n = 111, *R. sylvatica* n = 252, *R. pipiens* n = 19) was compared using independent-samples t-tests.

Egg collection

Individuals of the four anuran species were collected as eggs from breeding ponds (or breeding pairs removed from ponds) in southeast Michigan during early spring. On 8 April 2008, 6 *P. triseriata* egg masses were collected from Dreadful Swamp on the ESGR. On the same day, several pairs of adult *P. triseriata* were also collected from

Dreadful Swamp and allowed to lay eggs in breeding boxes in the lab. All resulting hatchling *P. triseriata* were mixed and pooled into 2 outdoor holding tanks. Adult *P. crucifer* were collected from Star Pond and West Marsh #6 on the ESGR between 7-12 April 2008, paired in breeding buckets, and left along the pond margin overnight to lay eggs. Seven of these pairings resulted in fertilized eggs. Hatchling *P. crucifer* were mixed and pooled into 2 outdoor holding tanks. On 9 April 2008, 10 partial *R. sylvatica* egg masses were collected from a privately-owned pond near Gregory, MI. Hatchling *R. sylvatica* were mixed and placed in 6 outdoor holding tanks. On 10 April 2008, 5 *R. pipiens* egg masses were collected from a privately-owned pond near Pinckney, MI. Hatchling *R. pipiens* were mixed and placed into 4 outdoor holding tanks.

Experimental treatments

On 18 April, 2008, 40 200 L wading-pool mesocosms were set up with leaf litter, an aliquot of pond water for colonization of zooplankton and phytoplankton, and 5 g of rabbit chow. Mesocosms were covered with a shade-cloth lid to prevent colonization of aquatic insects and other amphibians. Six days later, tadpoles of *R. sylvatica*, *R. pipiens*, *P. crucifer*, and *P. triseriata* were introduced into their assigned mesocosms. For the pair of ranids, 50 tadpoles were placed into each of 10 mesocosms per species. For the pair of Pseudacris species (which are smaller-bodied), 70 tadpoles were placed into each of 10 mesocosms per species. Tadpoles received 5 g supplementary rabbit chow every four days.

On 25 April, 2008, predator and no-predator treatments were initiated. In predator-treatment mesocosms (5 of the 10 mesocosms for each species), a single late-instar larval odonate (genus *Anax*) was caged and placed in each mesocosm. Predators

were fed approximately 200 mg of conspecific tadpoles every three days to produce cues of lethal predator presence (McCollum and Leimberger 1997). No-predator mesocosms received an empty cage, which was disturbed every three days to control for the effects of predator feedings.

On 11 May 2008, tadpoles of *R. sylvatica*, *R. pipiens*, and *P. triseriata* were collected for hormone analysis. Tadpoles of *P. crucifer* were collected on 27 May due to their slower developmental rate. All tadpoles were between Gosner 28-29 at time of sampling, to control for effects of ontogeny on hormone content. Samples for corticosterone content analysis were collected from each mesocosm by immediately flash-freezing tadpoles after capture. To procure enough tissue for hormone extraction, a single sample contained multiple individuals collected from the mesocosm: 6-8 tadpoles of the larger ranids or 10-12 of the smaller hylids. Samples were maintained on dry ice until return to the laboratory, where they were stored at -20 C until hormone extraction and radioimmunoassay.

Corticosterone analysis

Whole-body tadpole samples were extracted as described in Denver (1998). In short, tissue was homogenized in ethyl acetate, centrifuged, and the supernatant dried. Extracts were then fractionated using thin-layer chromatography (TLC) on silica plates; ethyl ether was used to extract the CORT from the silica after fractionation. After drying under nitrogen, the extract was resuspended in PBS-gelatin (0.02 M, pH 7.3) for corticosterone radioimmunoassay (RIA). The RIA protocol followed that of Licht et al. (1983). Anti-corticosterone was purchased from MP Biomedicals (Irvine, CA, USA). Samples from a single species pair were assayed in multiple RIAs conducted on the same

day. Inter- and intra-assay coefficients of variation were calculated using a quality control standard, and ranged between 10-15%.

Statistical Analysis

To test the hypothesis that a species' predation risk exposure (based on typical predator biomass in ponds) modifies its physiological response to predator presence, a generalized linear model (GLM) of the data was analyzed in R 2.8.1 (R Core Development Team 2008). Whole-body corticosterone concentration was used as the response variable, and predator treatment, species predation risk exposure, congener pair, and species were included as fixed effects. Mesocosm was used as the experimental unit. To specifically test the question posed here, an interaction effect between predator treatment and species-typical habitat was also included in the model.

Results

Determination of species predation risk exposure

The survey data showed significant differences in the predation risk typically encountered by each species within a congener pair. Within the *Rana* species pair, *R. pipiens* occupies ponds with significantly higher predator biomass than does *R. sylvatica* (corrected for unequal variances: $t_{18} = 2.953$, $p = 0.0085$; Fig 3.1). Within the *Pseudacris* species pair, *P. crucifer* regularly occupies ponds with a significantly higher predator biomass than *P. triseriata* (corrected for unequal variances: $t_{115} = 4.133$, $p = 0.0001$; Fig 3.1).

Experiment

Predator treatment ($F_{4,68}$= 30.1821, $p < 0.0001$), congener pair ($F_{4,68}$ = 18.8456, $p < 0.0001$), and species ($F_{4,68}$ = 25.8693, $p < 0.0001$) had significant main effects on corticosterone content in tadpoles. Species-typical habitat had a marginally significant effect ($F_{4,68}$ = 3.3588, $p = 0.0713$). However, the question posed by this study is concerned with whether species that typically encounter high predation risk also differentially respond to predator presence (e.g. with a reduced corticosterone stress response) compared to a congener. This question is addressed by the species-typical habitat x treatment interaction, which was significant ($F_{4,68}$ = 6.0254, $p = 0.0167$; Fig 3.2).

Discussion

To my knowledge, this is the first comparative study that shows a predictable pattern between stress response phenotype and a species' environment. Results support the hypothesis tested here, that species that inhabit predictably high-predator environments have adapted to reduce their stress response to predators. Specifically, a significant interaction between species-typical habitat and predator treatment indicates that the predator level that species normally encounter influences their response to the presence of predators in a common garden (Fig 3.1). Predator treatment, congener pair, and species identity also had significant main effects on corticosterone concentration in experimental tadpoles. The effect of predator treatment indicates that the presence of predators reliably causes increased stress hormone production across species, compared to tadpoles in control (no predator) mesocosms. In Chapter 2, this effect was demonstrated only in wood frogs.

Significant effects of congeneric pair and species identity are unsurprising, but demonstrate the validity of the experimental design. Species were arranged in pairs of congeners in order to control for any phylogenetic effects on physiology and to focus on the differences in species predation risk normally encountered in the field. The significant effect of congener pair on stress hormone production suggests that phylogenetic effects do indeed exist, and therefore this design was necessary. In addition, the significant effect of species identity suggests that physiology differs among species and raw corticosterone concentrations are not necessarily directly comparable across taxa. For this reason, multi-species comparisons in stress ecology should include measurements of not only the stress response but also baseline hormone level for calibration purposes. Furthermore, it is important to know the experiential background of experimental animals: stress physiology is primarily a plastic system in which baseline hormone levels show some degree of heritability, but are also a product of the organism's previous experiences (Fride et al. 1986, Cooper et al. 2003, Knuth and Etgen 2007, Spencer and Verhulst 2007). In the present study, the common garden approach served to reduce the impact of previous experience on stress physiology, and rather focus on differences in plasticity and magnitude of response among species from different environments.

The significant interaction between species-typical habitat and predator treatment, the main result from this study, suggests that maintaining a strong physiological stress response to a predictable stressor is costly, and that species mitigate this cost by adaptively reducing the magnitude of the hormonal response to commonly-encountered stressors compared to species that encounter the stressor to a lesser extent. Although a

brief, strong stress response to an environmental challenge is considered beneficial for an organism, (Sapolsky 1992, Wingfield et al. 1998), chronically high stress hormone levels are known to become damaging due to the costly effects of glucocorticoids on necessary functions such as energy storage, growth, immune response, and breeding activity (Sapolsky et al. 2000, Wingfield and Romero 2001). It is clear from work both in the lab and field that long-term stress hormone elevation can damage health, survival, and reproductive success (e.g. Lee and McDonald 1985, Schoech et al. 1991, Boonstra and Singleton 1993, Creel 2001, Belden and Kiesecker 2005, Spencer et al. 2005, Cyr and Romero 2007) and thus exposure and subsequent response to chronic stress is likely costly to an animal. Therefore, we expect that animals would adaptively balance the benefits of an acute adrenocortical response with the costs of exhibiting a chronic stress response, and thus down-regulate the stress response to common or predictable stressors in their environment. Results from the present experiment support this prediction, and suggest that species living across a gradient of predation risk adaptively manage their response to predator presence in order to minimize the costs of chronic activation of the stress axis.

These results are supported by data in other systems that demonstrate differences in stress hormone production among populations within a species that experience different levels of a stressor. For example, a population of Belding's ground squirrels (*Spermophilus beldingi*) living under higher predation risk (reduced visibility) exhibited greater vigilance and antipredator behavior but lower fecal cortisol levels (Mateo 2007). Populations of fish from high-predator environments have also been shown to exhibit a reduced stress response, measured by opercular beat rate, which the authors suggest may

be an adaptation to reduce energy expenditure (Brown et al. 2005). In addition, populations of European blackbirds (*Turdus merula*) that have adapted to an urban lifestyle also exhibit reduced stress response to handling, despite being raised in a common garden (Partecke et al. 2006). These data suggest that stress physiology is under ongoing natural selection, and populations may adapt in order to differentially respond to stressors based on their predictability in the local environment. The results presented in the current study, however, show how a "calibrated" measure of the stress response to predator presence can be used to demonstrate an adaptive pattern among different species. In addition, this study joins a wealth of knowledge regarding the larval anuran system, adding a further layer of mechanistic understanding to the patterns in behavior, morphology, growth, and habitat use already observed in these species (Skelly 1995, Smith and Van Buskirk 1995, Relyea 2000, Werner et al. 2009).

The existence of a species-level trade-off between high and low stress response to predator presence in the larval environment has a number of implications for the ability to respond to novel stressors, the evolution of phenotypic plasticity, and species habitat occupancy. First, the results of this study raise the question of whether down-regulation of the stress response to predators is confined to predator-stress only, or may also be reflected in the response to other stressors. Individuals encounter a variety of stressors, ranging from predators (Blanchard et al. 1998, Scheuerlein et al. 2001, Brown et al. 2005, Barcellos et al. 2007) to social conflicts (Sapolsky 1992, Creel 2001) to anthropogenic activity (Wasser et al. 1997, Creel et al. 2002), and it is important that they respond appropriately to each stressor in order to maximize fitness (Wingfield et al. 1998, Wingfield and Kitaysky 2002, Cohen et al. 2006). However, the magnitude of an

organism's hormonal stress response may differ for each stressor encountered (Canoine et al. 2002), so it is possible that species are able to adaptively modulate their stress response to predators independently of the response to other environmental perturbations and therefore allow a customized response to each stressor or set of related stressors. If this is not the case, however, and the action of the stress response is correlated across stressors, then adaptation to a common stressor may come at the cost of an appropriate response to a novel stressor.

The species of tadpoles used in this study have also been demonstrated to have morphological and behavioral plasticity that responds to predator presence in the pond environment (Skelly 1995, Van Buskirk et al. 1997, Relyea 2000). Since corticosterone is involved in mediating aspects of behavioral and morphological change (Chapter 2), it is possible that the range of stress response observed in this study correlates with the degree of predator-induced phenotypic plasticity demonstrated by these species. In the case of the *Pseudacris* species pair, such a difference in plasticity has already been demonstrated: *P. triseriata* exhibit greater behavioral and morphological plasticity than their congener *P. crucifer* (Skelly 1995, Smith and Van Buskirk 1995). This pattern is thought to emerge because *P. triseriata* tadpoles encounter predators less often and therefore the cost of increased activity has not narrowed the range of plasticity in those traits (Skelly 1995), and therefore the variation in larval habitat determines the expression of phenotypic plasticity (Smith and Van Buskirk 1995). A similar, perhaps even related, mechanism may explain the difference in hormonal stress response in these two species. In addition, as both Skelly (1995) and Smith and Van Buskirk (1995) show, the difference in range of plasticity exhibited by the two congeners strongly determines each

species' success along the pond-permanence, and resulting predator-density, gradient, and plays a large part in determining habitat occupancy on the landscape (Werner et al. 2009).

Within the *Rana* species pair, comparisons of morphological plasticity are not as clear-cut as the *Pseudacris* example. Both *R. pipiens* and *R. sylvatica* induce changes in tail and body shape when confronted with predation risk, but it is unclear whether *R. sylvatica* is consistently more plastic across all traits measured (Relyea and Werner 2000). However, similar to the *Pseudacris* species, the two *Rana* species have adapted the direction and range of their phenotypic plasticity to fit the relative strength of predation in their respective larval environments. Like *P. triseriata*, *R. sylvatica* exhibits phenotypic traits such as greater activity that increase its competitive success when no predators are present, whereas *R. pipiens*, like *P. crucifer*, exhibits phenotypic traits such as increased growth rate that fare relatively better under predator presence (Relyea 2000).

Systems where we can compare phenotypes that have evolved under contrasting environmental pressures are essential to achieving the goal of matching phenotype to fitness outcome. Just as we can use differences in growth rate or behavior among populations as evidence for evolution on an ecologically-relevant scale (Cooch et al. 2002, Carlson et al. 2004, Peacor et al. 2007), we can also examine variation in mechanistic physiological responses, such as corticosterone production, that may impact a range of life-history traits. Environmentally-induced stress is an important agent of selection on phenotypes, and tracing the potential adaptability of the hormonal response to common stressors augments our understanding of, and may even explain, the observed range of other stress-induced phenotypic traits.

References

Barcellos, L. J. G., F. Ritter, L. C. Kreutz, R. M. Quevedo, L. B. da Silva, A. C. Bedin, J. Finco, and L. Cericato. 2007. Whole-body cortisol increases after direct and visual contact with a predator in zebrafish, Danio rerio. Aquaculture **272**:774-778.

Belden, L. K. and J. M. Kiesecker. 2005. Glucocorticosteroid hormone treatment of larval treefrogs increases infection by *Alaria sp* trematode cercariae. Journal Of Parasitology **91**:686-688.

Blanchard, R. J., J. N. Nikulina, R. R. Sakai, C. McKittrick, B. McEwen, and D. C. Blanchard. 1998. Behavioral and endocrine change following chronic predatory stress. Physiology & Behavior **63**:561-569.

Bonier, F., P. R. Martin, I. T. Moore, and J. C. Wingfield. 2009. Do baseline glucocorticoids predict fitness? Trends In Ecology & Evolution **24**:634-642.

Boonstra, R. and G. R. Singleton. 1993. Population declines in the snowshoe hare and the role of stress. General And Comparative Endocrinology **91**:126-143.

Brown, C., C. Gardner, and V. A. Braithwaite. 2005. Differential stress responses in fish from areas of high- and low-predation pressure. Journal Of Comparative Physiology B-Biochemical Systemic And Environmental Physiology **175**:305-312.

Canoine, V., T. J. Hayden, K. Rowe, and W. Goymann. 2002. The stress response of European stonechats depends on the type of stressor. Behaviour **139**:1303-1311.

Carlson, S. M., A. P. Hendry, and B. H. Letcher. 2004. Natural selection acting on body size, growth rate and compensatory growth: an empirical test in a wild trout population. Evolutionary Ecology Research **6**:955-973.

Cohen, H., J. Zohar, Y. Gidron, M. A. Matar, D. Belkind, U. Loewenthal, N. Kozlovsky, and Z. Kaplan. 2006. Blunted HPA axis response to stress influences susceptibility to posttraumatic stress response in rats. Biological Psychiatry **59**:1208-1218.

Cooch, E. G., E. Cam, and W. Link. 2002. Occam's shadow: levels of analysis in evolutionary ecology - where to next? Journal Of Applied Statistics **29**:19-48.

Cooper, C., E. M. Dennison, M. K. Javaid, H. E. Syddall, S. Shore, K. M. Godfrey, R. Oreffo, H. I. Roach, and N. K. Arden. 2003. The Barker hypothesis: Early life influences on bone and cardiovascular diseases. Journal Of Bone And Mineral Research **18**:1356-1356.

Creel, S. 2001. Social dominance and stress hormones. Trends In Ecology & Evolution **16**:491-497.

Creel, S., J. E. Fox, A. Hardy, J. Sands, B. Garrott, and R. O. Peterson. 2002. Snowmobile activity and glucocorticoid stress responses in wolves and elk. Conservation Biology **16**:809-814.

Cyr, N. E. and L. M. Romero. 2007. Chronic stress in free-living European starlings reduces corticosterone concentrations and reproductive success. General And Comparative Endocrinology **151**:82-89.

Fride, E., Y. Dan, J. Feldon, G. Halvey, and M. Weinstock. 1986. Effects of prenatal stress on vulnerability to stress in prepubertal and adult rats. Physiological Behavior **37**:681-687.

Knuth, E. D. and A. M. Etgen. 2007. Long-term behavioral consequences of brief, repeated neonatal isolation. Brain Research **1128**:139-147.

Lee, A. K. and I. R. McDonald. 1985. Stress and population regulation in small mammals. Oxford Reviews Of Reproductive Biology **7**:261-304.

Mateo, J. M. 2007. Ecological and hormonal correlates of antipredator behavior in adult Belding's ground squirrels (*Spermophilus beldingi*). Behavioral Ecology And Sociobiology **62**:37-49.

Partecke, J., I. Schwabl, and E. Gwinner. 2006. Stress and the city: Urbanization and its effects on the stress physiology in European Blackbirds. Ecology **87**:1945-1952.

Peacor, S. D., L. Schiesari, and E. E. Werner. 2007. Mechanisms of nonlethal predator effect on cohort size variation: Ecological and evolutionary implications. Ecology **88**:1536-1547.

Relyea, R. A. 2000. Trait-mediated indirect effects in larval anurans: reversing competition with the threat of predation. Ecology **81**:2278-2289.

Relyea, R. A. 2002. Local population differences in phenotypic plasticity: predator-induced changes in wood frog tadpoles. Ecological Monographs **72**:77-93.

Relyea, R. A. and E. E. Werner. 2000. Morphological plasticity in four larval anurans distributed along an environmental gradient. Copeia:178-190.

Sapolsky, R. M. 1992. Cortisol concentrations and the social significance of rank instability among wild baboons. Psychoneuroendocrinology **17**:701-709.

Sapolsky, R. M., L. M. Romero, and A. U. Munck. 2000. How do glucocorticoids influence stress responses? Integrating permissive, suppressive, stimulatory, and preparative actions. Endocrine Reviews **21**:55-89.

Scheuerlein, A., T. J. Van't Hof, and E. Gwinner. 2001. Predators as stressors? Physiological and reproductive consequences of predation risk in tropical

stonechats (*Saxicola torquata axillaris*). Proceedings Of The Royal Society Of London Series B-Biological Sciences **268**:1575-1582.

Schoech, S. J., R. L. Mumme, and M. C. Moore. 1991. Reproductive endocrinology and mechanisms of breeding inhibition in cooperatively breeding Florida Scrub Jays (*Aphelocoma coerulescens*). Condor **93**:354-364.

Skelly, D. K. 1995. A behavioral trade-off and its consequences for the distribution of Pseudacris treefrog larvae. Ecology **76**:150-164.

Smith, D. C. and J. Van Buskirk. 1995. Phenotypic design, plasticity, and ecological performance in two tadpole species. American Naturalist **145**:211-233.

Spencer, K. A. and S. Verhulst. 2007. Delayed behavioral effects of postnatal exposure to corticosterone in the zebra finch (Taeniopygia guttata). Hormones And Behavior **51**:273-280.

Spencer, K. A., J. H. Wimpenny, K. L. Buchanan, P. G. Lovell, A. R. Goldsmith, and C. K. Catchpole. 2005. Developmental stress affects the attractiveness of male song and female choice in the zebra finch (*Taeniopygia guttata*). Behavioral Ecology And Sociobiology **58**:423-428.

Van Buskirk, J. 2002. A comparative test of the adaptive plasticity hypothesis: relationships between habitat and phenotype in anuran larvae. American Naturalist **160**:87-102.

Van Buskirk, J., S. A. McCollum, and E. E. Werner. 1997. Natural selection for environmentally induced phenotypes in tadpoles. Evolution **51**:1983-1992.

Van Buskirk, J. and R. A. Relyea. 1998. Selection for phenotypic plasticity in *Rana sylvatica* tadpoles. Biological Journal Of The Linnean Society **65**:301-328.

Wasser, S. K., K. Bevis, G. King, and E. Hanson. 1997. Noninvasive physiological measures of disturbance in the Northern Spotted Owl. Conservation Biology **11**:1019-1022.

Wellborn, G. A., D. K. Skelly, and E. E. Werner. 1996. Mechanisms creating community structure across a freshwater habitat gradient. Annual Review Of Ecology And Systematics **27**:337-363.

Werner, E. E., R. A. Relyea, K. L. Yurewicz, D. K. Skelly, and C. J. Davis. 2009. Comparative landscape dynamics of two anuran species: climate-driven interaction of local and regional processes. Ecological Monographs **79**:503-521.

West-Eberhard, M. J. 1989. Phenotypic plasticity and the origins of diversity. Annual Review Of Ecology And Systematics **20**:249-278.

Wingfield, J. C. and A. S. Kitaysky. 2002. Endocrine responses to unpredictable environmental events: Stress or anti-stress hormones? Integrative and Comparative Biology **42**:600-609.

Wingfield, J. C., D. L. Maney, C. W. Breuner, J. D. Jacobs, S. Lynn, M. Ramenofsky, and R. D. Richardson. 1998. Ecological bases of hormone-behavior interactions: The "emergency life history stage". American Zoologist **38**:191-206.

Wingfield, J. C. and L. M. Romero. 2001. Andrenocortical responses to stress and their modulation in free-living vertebrates. Pages 211-234 *in* B. S. McEwen and H. M. Goodman, editors. Handbook of Physiology; Section 7: The Endocrine System; Volume IV: Coping with the Environment: Neural and Endocrine Mechanisms. Oxford University Press.

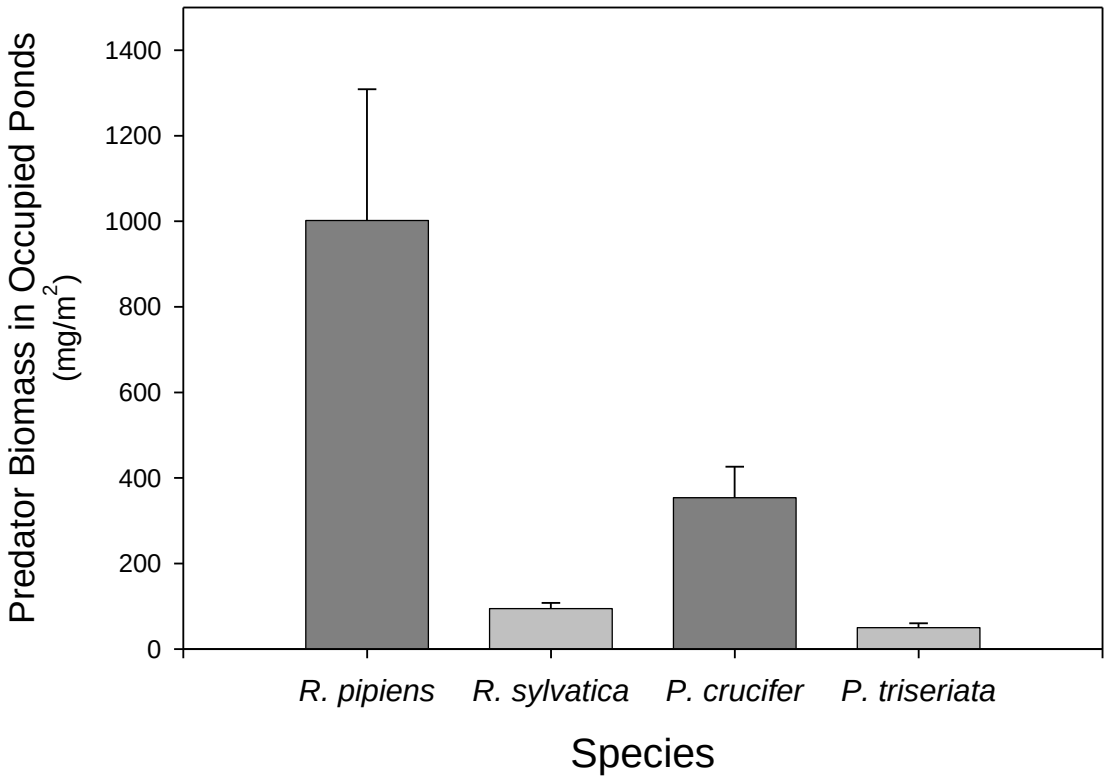


Figure 3.1. Predator biomass found in ponds containing the four study species: *Rana pipiens*, *R. sylvatica*, *Pseudacris crucifer*, and *P. triseriata.* Darker bars indicate the species within each congener pair that occupies significantly more predator-dense ponds (*Rana* pair: p = 0.0085, *Pseudacris* pair: p = 0.0001). Error bars represent +/- 2 S.E.

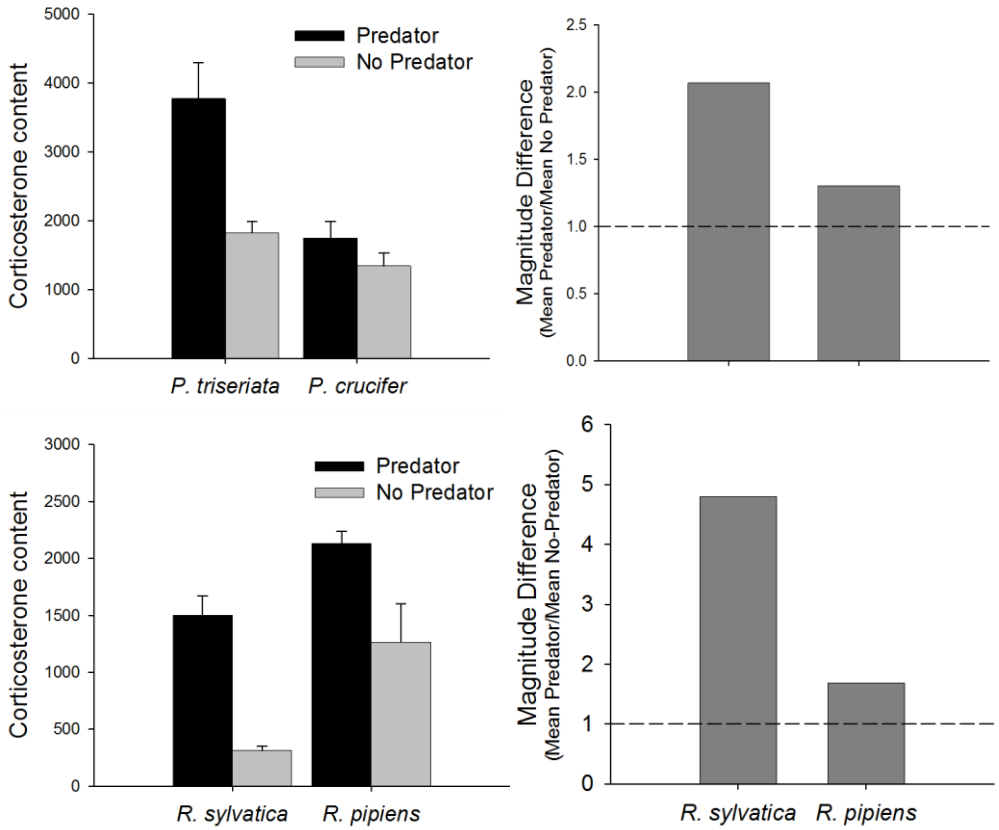


Figure 3.2. Comparison of stress hormone content between treatments and within species pairs. Within the *Pseudacris* species pair, *P. crucifer* normally inhabits high-predator ponds, and likewise fits the prediction of a reduced physiological response to the presence of predators. Within the *Rana* species pair, *R. pipiens* normally inhabits high-predator ponds, and also has a lower response magnitude to predators (significant species-typical habitat x treatment interaction: $F_{4,68} = 6.0254$, $p = 0.0167$). Dashed line represents no stress response to predators (no difference between predator treatment and no-predator treatment). Corticosterone content is expressed in pg/mg body weight.

CHAPTER 4

COMBINED EFFECTS OF INBREEDING AND PREDATOR PRESENCE ON STRESS PHYSIOLOGY, BEHAVIOR, AND MORPHOLOGY IN LARVAL AND POST-METAMORPHIC WOOD FROGS

Introduction

Inbreeding increases genomic similarity within populations, and commonly results in a loss of fitness presumed due to increased expression of deleterious recessive alleles (Charlesworth and Charlesworth 1987, Husband and Schemske 1996). The resulting inbreeding depression has been implicated as a cause for reduced viability of populations leading to the decline and extinction of species, particularly those species without prior history of extensive inbreeding (Frankham 2005). In addition, the magnitude and expression of inbreeding depression is highly dependent on the level of environmental stress in which is it measured, due to genotype x environment interactions (GxE, Armbruster and Reed 2005). Inbreeding x environment interactions, a subtype of GxE, may become apparent under environmental stress, and provide the conditions necessary for inbreeding to negatively affect population growth (Bijlsma et al. 1999, Coltman et al. 1999, Crnokrak and Roff 1999, Reed 2007).

Since inbreeding depression produces individuals that struggle to thrive in their environment, we might expect inbred individuals to respond as if stressed in order to cope with the perceived environmental challenges (Wingfield et al. 1998, Wingfield and

Kitaysky 2002). Such an observation is likely to become more commonplace as levels of inbreeding increase due to loss and fragmentation of habitats and associated declines in population sizes and genetic connectivity (Epps et al. 2005, Coulon et al. 2006, Cushman 2006, Riley et al. 2006, Vandergast et al. 2007). As inbred populations become more common, they will often intersect with other natural stressors, such as predation risk. Due to the increasing likelihood that individuals will encounter multiple stressors concurrently in their environment, the potential for synergistic effects is receiving increasing attention (for a review, see Sih et al. 2004). However, the connection between inbreeding and stress remains unclear: although numerous researchers have suggested that inbreeding depression should increase with stress, experimental results demonstrating it have been inconsistent (e.g. Armbruster and Reed 2005, Marr et al. 2006, Reed et al. 2007, Szulkin and Sheldon 2007, Kristensen et al. 2008, Waller et al. 2008, Fox and Reed 2011).

Amphibians in particular represent a system in which the effects of multiple stressors may reduce fitness in a nonadditive way and produce potential for population decline (e.g. Relyea and Mills 2001, Kiesecker 2002, Blaustein et al. 2003, Johnson et al. 2006, Davidson et al. 2007, Jones et al. 2011). In this study, we used the wood frog (*Rana sylvatica*) to investigate the relationship between inbreeding and the physiological stress response, using common-garden mesocosms to first remove the effects of environment, and in a second study, to manipulate the presence of predation risk along a gradient of relatively outbred to inbred populations. We have shown previously that predator presence is a salient stressor to wood frog tadpoles, causing a chronic hormonal stress response when predators are maintained in the environment (Chapters 2 and 3).

Here, we begin by looking for an association between inbreeding coefficient (F_{IS}) and tadpole corticosterone content in a common garden experiment (Study 1). We next consider the combined effects of inbreeding and predator presence in a factorial experiment measuring tadpole stress hormone response, behavior, morphology, and post-metamorphic phenotype (Study 2).

Methods

Study I: Inbreeding and stress hormone production

Samples for this study came from a larger common-garden experiment developed by Amanda Zellmer. In this experiment, tadpoles from 16 populations in S. E. Michigan whose inbreeding coefficients were known (Zellmer and Knowles 2009) were raised in an outdoor common-garden in order to assess differences among populations that are not attributable to environmental effects. To populate the experiment, 100 eggs were collected from each of 10 egg masses from each population (in the case of one pond (Cassidy 1) approximately 15 amplectant pairs were instead caught and returned to the lab to breed, from which 10 masses (laid within 24 hours of collection) were kept for this study). All eggs were placed in outdoor wading pools covered by shade cloth until hatching.

Common-garden mesocosms (1000 L tanks) were filled with aged well water, inoculated with zooplankton and approximately 6 L of filtered pond water to initiate phytoplankton growth, supplemented with approximately 300 g of leaves, and covered with 60% shade cloth to prevent colonization by aquatic insects and other anurans. Each tank was supplemented with approximately 30g of rabbit chow. Tanks were arranged in

4 replicate blocks, and populations randomly assigned within each block for a total of 64 tanks. Each tank received 100 tadpoles from its assigned population. When tadpoles had developed to Gosner stages 28-29 (18 days after the experiment had been initiated), a single sample of 6 tadpoles was removed from each tank and immediately flash-frozen for baseline corticosterone analysis.

Study II: Combined effects of inbreeding and predator presence on phenotype

This study (also in collaboration with A. Zellmer) used a subset of the populations from Study I: three relatively inbred and three relatively outbred populations. Ten partial *R. sylvatica* egg masses were collected within 24 hr of breeding from each of the six ponds, and hatchings were cultured in small wading pools and fed rabbit chow *ad lib* until the experiment began. Experimental tanks were 48 100-L wading pools filled with aged well water, ~100 g of oak leaves for substrate, a 1-L aliquot of phytoplankton and zooplankton, and 5 g of rabbit chow as an initial food source. Tanks were covered with shade cloth to prevent colonization of other aquatic organisms.

Once hatchlings were free-swimming, 50 tadpoles from a single population (pond-of-origin) were added to each of eight experimental tanks, half of which were assigned to a predator treatment and the other half assigned to a no-predator treatment. Predator treatment tanks contained a single caged (nonlethal) dragonfly (*Anax sp.*) larva, and no-predator tanks contained an empty cage. Predators were fed approximately 200 mg of live *R. sylvatica* tadpoles every 3 days to produce chemical cues of predation (cages in no-predator tanks were removed and replaced to produce the same level of disturbance). These two treatments were applied to the six populations to produce a 2 x 6

factorial design, replicated across 4 blocks. A supplement of 5 g of rabbit chow was added each week to maintain normal tadpole growth in experimental tanks.

Approximately 3.5 weeks into the experiment, tadpole behavioral measurements were taken at the same time over two consecutive days: every ten minutes for one hour, a trained observer noted the number of tadpoles active in the water column. Following the behavioral observation, a haphazard sample of 6 tadpoles from each tank was collected and immediately flash-frozen for baseline corticosterone analysis. Another 6 tadpoles were placed in approximately 30 ml of water and occasionally gently agitated for 90 minutes to produce a confinement stress response; these tadpoles were flash-frozen together following the 90-min stressor. Later that week, a haphazard sample of 10 tadpoles per tank was collected and preserved in 10% formalin for morphological analysis. The remaining tadpoles were allowed to develop to metamorphosis, at which time a haphazard sample of 6 metamorphs per tank were collected and preserved in 10% formalin.

Samples for corticosterone analysis from both studies were stored, extracted, and underwent radioimmunoassay (RIA) as described in Chapter 2. Tadpole morphology was also measured as described in Chapter 2. Four morphological measurements were taken from preserved metamorphs: head width (HW), snout-vent length (SVL), and both right and left leg length (LL). In addition, a fifth measurement, the difference in the two leg lengths, was computed to look for patterns of right-left asymmetry. Measurements were taken using Image J 1.36b (National Institutes of Health, Bethesda, MD), regressed on body weight, and the residuals were used in all subsequent analyses.

Statistical Analysis

In Study I, a regression analysis using tank as the experimental unit was employed to assess the relationship between F_{IS} and CORT content in tadpoles. In Study II, all analyses (with the exception of activity data) used an ANCOVA model with predator treatment as a fixed factor, F_{IS} as a covariate, and block as a random factor, and the interaction between predator treatment and inbreeding was included along with the main effects listed above. The morphological measurements produced multiple data points from a single tank, so to prevent pseudoreplication individual measurements within a tank were averaged and the tank mean was used as the experimental unit. Activity data over the observation period was also averaged to create a tank mean, and input into a least squares regression model with "day" as a random factor. Analyses were done in SPSS 16.0 (SPSS Inc., Chicago, IL, USA) and JMP 9.0 (SAS Institute Inc, Cary, NC, USA).

Results

In the common-garden (Study 1), corticosterone levels increased with population level of inbreeding ($R^2 = 0.348$, $p = 0.016$; Fig. 4.1). In Study 2, baseline corticosterone (Fig 4.2A) was significantly affected by predator presence ($F = 8.266$, $p = 0.006$) and inbreeding ($F = 7.243$, $p = 0.010$), and there was a nonsignificant trend towards an inbreeding x predator presence interaction ($F = 2.916$, $p = 0.095$). Block had no effect ($F = 1.736$, $p = 0.175$). However, when response to a novel confinement stressor was tested (Fig 4.2B), tadpole corticosterone content did not differ with respect to predator treatment ($F = 0.937$, $p = 0.339$), F_{IS} ($F = 0.635$, $p = 0.430$), or predator x F_{IS} ($F = 0.028$, $p = 0.869$), and block had no significant effect ($F = 1.708$, $p = 0.180$).

Activity of tadpoles in the water column (Fig 4.3) was decreased in predator treatments ($t = 5.08$, $p < 0.0001$) and there was a similar nonsignificant trend among F_{IS} values ($t = -1.84$, $p = 0.069$). The interaction between predators and inbreeding was significant ($t = -2.21$, $p = 0.029$). Tadpole mass (Fig 4.4) was significantly increased in inbred tadpoles ($F = 8.835$, $p = 0.005$) but was not affected by predator treatment ($F = 2.206$, $p = 0.145$), block ($F = 2.055$, $p = 0.122$), or the interaction ($F = 1.974$, $p = 0.168$).

Morphological traits showed different patterns depending on the trait measured (Fig 4.5). Tail depth (Fig 4.5A) was significantly larger in predator treatments ($F = 24.692$, $p < 0.0001$) and increased with inbreeding ($F = 4.608$, $p = 0.038$). The interaction effect was not significant ($F = 2.248$, $p = 0.141$) and there was no significant block effect ($F = 2.062$, $p = 0.12$). Tail length (Fig 4.5B) showed a nonsignificant trend to increase with inbreeding ($F = 2.497$, $p = 0.122$) but there was no predator effect ($F = 1.287$, $p = 0.263$) and no interaction effect ($F = 1.185$, $p = 0.283$). Body depth was not significant across predator treatments ($F = 0.224$, $p = 0.639$), populations ($F = 0.183$, $p = 0.671$), and there was no interaction effect ($F = 0.005$, $p = 0.944$). Predator-exposed tadpoles had significantly shorter bodies across populations ($F = 26.713$, $p < 0.0001$; Fig 4.5C), but there was no significant effect of population ($F = 0.221$, $p = 0.641$), block ($F = 0.776$, $p = 0.514$), and no interaction effect ($F = 0.429$, $p = 0.516$) on body length.

Metamorph mass did not differ across predator treatments ($F = 2.702$, $p = 0.108$), F_{IS} values ($F = 0.367$, $p = 0.548$), block ($F = 1.615$, $p = 0.201$), and there was no predator treatment x F_{IS} interaction ($F = 0.585$, $p = 0.449$). There were also no significant trends across populations or treatments with regards to morphological measurements. Metamorph SVL was not affected by predator treatment ($F = 2.132$, $p = 0.152$), level of

inbreeding ($F = 0.016$, $p = 0.899$), block ($F = 1.328$, $p = 0.279$), or the predator treatment x inbreeding interaction ($F = 0.559$, $p = 0.459$). Head width was similarly unaffected by predator treatment ($F = 1.161$, $p = 0.211$), level of inbreeding ($F = 0.926$, $p = 0.342$), block ($F = 0.416$, $p = 0.743$), or the interaction ($F = 2.462$, $p = 0.125$). Metamorph leg length was not significantly affected by predator treatment ($F = 0.489$, $p = 0.489$), F_{IS} ($F = 0.444$, $p = 0.509$), or the interaction ($F = 1.128$, $p = 0.294$), but there was a marginally nonsignificant block effect ($F = 2.693$, $p = 0.059$). There was also no detectable asymmetry in leg length associated with predator treatments ($F = 0.055$, $p = 0.816$), F_{IS} ($F = 0.375$, $p = 0.544$), block ($F = 2.134$, $p = 0.112$), or the predator treatment x inbreeding interaction ($F = 0.001$, $p = 0.972$).

Discussion

In Study 1, we have shown that inbred populations express higher stress hormone levels in a common garden environment. Although we cannot say that inbreeding directly causes this stress response, this finding is consistent with the hypothesis that inbred individuals perceive a common environment as more stressful than do their outbred conspecifics. This association between inbreeding and stress hormone production has not, to our knowledge, been demonstrated previously. However, the potential for inbreeding x environment interactions to produce relatively stressed animals has been suggested by other authors (Fox and Reed 2011), with the capacity for overwhelming the organism's ability to respond to environmental perturbations (Kristensen et al. 2006) and thus causing increased inbreeding depression under stressful environmental conditions.

An alternative mechanism for our results may be provided by maternal effects. It has been demonstrated in other taxa that egg-laying mothers can pass on stress hormones to their offspring through yolk contents (Hayward and Wingfield 2004). In such as case, we would expect to see this pattern if mothers also have higher stress hormone levels. However, since wood frogs tend to breed in their natal pond (Vasconcelos and Calhoun 2004) and genetic connectivity is reduced in inbred populations, mothers in inbred populations would also likely be inbred themselves. Thus, a maternal effects mechanism would also support the original hypothesis, that inbreeding x environment interactions cause inbred individuals to perceive an environment as more stressful than their outbred counterparts, if the pattern we observed in the tadpoles was indirectly mediated through the mother. Further research should consider the possibility of generational effects on stress reactivity in this system, and include measurement of post-metamorphic phenotype and fitness.

When predator presence and inbreeding were examined in combination (Study 2), a number of interesting phenotypic patterns emerged. First, baseline tadpole corticosterone content was high in predator-exposed tadpoles across all populations, but increased in no-predator tadpoles as F_{IS} increased (a marginally significant predator treatment x inbreeding interaction). Interestingly, there was no difference in CORT levels between predator and no-predator tadpoles in the most inbred population, i.e., the combination of inbreeding and predator presence did not additively increase CORT production. In addition, the novel confinement stress response showed no differences among populations or treatments, and did not raise corticosterone levels above those seen in predator treatments or highly inbred populations. Together, these results suggest that

there is a ceiling on stress hormone production, and either inbreeding or predation risk independently can cause a tadpole to reach that response ceiling. Once the ceiling is reached, there is no additional response to further stressors. Such a ceiling has important implications for the ability of individuals to respond adaptively to a novel stressor, since the acute stress response would be blunted or nonexistent in inbred populations or in individuals already under stress (Wingfield et al. 1998, Breuner et al. 2008).

Tadpole morphology also demonstrated some interesting patterns in this experiment, responding to both predator presence and inbreeding but lacking interactive effects with the combined stressors. Tail depth and body length responded to predator presence in directions that have been demonstrated previously in predator-cue treatments (e.g. Relyea 2004). However, in the case of tail depth and, to some extent, tail length, there was a trend towards an interaction between predator treatment and inbreeding (Fig 4.5), although this trend was not significant. In particular, tail depth increased in inbred tadpoles to the point of matching the morphology of predator-exposed tadpoles. The mechanism of that phenotypic change may be CORT-induced, as demonstrated in Chapter 2. Deeper tails are adaptive in response to predator presence (Van Buskirk et al. 1997), but it is unclear whether there would be any functionality to morphological change in inbred tadpoles. In fact, such a morphological change may come at a cost to inbred tadpoles in populations without large predator communities (Van Buskirk 2000, Relyea and Auld 2005), potentially manifesting even after metamorphosis (Benard and Fordyce 2003).

Similarly, measurement of tadpole activity in this experiment showed responses to both inbreeding and predator presence. Tadpoles exhibited a decrease in activity in

predator-exposed treatments, which is not unexpected: tadpoles of vulnerable size are often found to reduce activity upon detection of predator chemical cue, and this behavioral quiescence reduces encounter rate with a sit-and-wait predator (Van Buskirk and McCollum 2000). In addition, we observed a marginal increase in activity among the most inbred population, possibly reflecting increased foraging in this group (Bridges 2002, Fraker 2008) consistent with the result that tadpoles were also larger in the inbred treatment. It is possible that the larger size of inbred tadpoles may also be due to density effects, since the most-inbred population experienced reduced survivorship to metamorphosis (Zellmer 2010). However, a density-mediated growth explanation does not fit all of the data, since Zellmer (2010) also demonstrated lower survivorship in the most outbred populations, and outbred tadpoles did not grow larger in this experiment. Increased activity among inbred tadpoles may also be costly in environments with predators, since tadpoles would fall prey to predators at a higher rate (Van Buskirk and McCollum 2000).

No differences in metamorph morphological measurements were detected among groups. In particular, we looked for signs of fluctuating asymmetry in leg length, since asymmetry is often implicated to be a function of poor developmental conditions and environmental stress (Kristensen et al. 2003a, Bize et al. 2004, Wells et al. 2006, Soderman et al. 2007, Vogt et al. 2008). The lack of detectable asymmetry in metamorph leg length, however, is not entirely unsurprising, since asymmetry is expected to manifest more often in non-functional traits (such as ornaments) than in functional morphology (Moller and Hoglund 1991, Moller 1992). Further analysis of the potential for

fluctuating asymmetry in this system should focus on non-functional traits such as color patterning.

One of the main goals of this study (particularly Study 2) was to investigate the potential for synergistic interactions among environmental stressors. It is commonly argued that the fitness consequences of exposure to environmental stressors should vary with inbreeding level and, reciprocally, that the magnitude of inbreeding depression should be greater in stressful environments. Supporting this prediction, inbreeding depression has been demonstrated to be stronger under stressful conditions in a variety of taxa (Fox and Reed 2011). However, despite demonstrating that inbreeding itself is associated with higher stress hormone content, we did not find any strong synergistic effects of combining inbreeding and predator presence on fitness correlates. The only apparent exception is the nonsignificant trend towards an interaction effect on baseline corticosterone production: the glucocorticoid activity induced by inbreeding and by predator presence alone each had the same maximum effect on phenotype as the two in combination. As mentioned earlier, the potential for a maximal response ceiling is suggested by the lack of further corticosterone response to a novel confinement stress test. Although this hypothesis requires further testing, reduction of stress reactivity could endanger inbred or predator-exposed individuals if a strong adrenocortical response is necessary to survive a novel environmental perturbation (Wingfield et al. 1998, Wingfield and Kitaysky 2002). It has recently been shown that the ability to down-regulate the stress response, presumably in preparation for the new stressor, is a strong predictor of survival in marine iguanas (Romero and Wikelski 2010). In addition, chronic activation of the neuroendocrine stress axis has a number of negative

consequences, such as reproductive and immune suppression (Sapolsky et al. 2000, Wingfield and Romero 2001). Thus, the long-term maintenance of a maximal glucocorticoid response is likely detrimental to fitness.

However, it is also possible that the lack of strong interaction effects in Study 2 is due to our choice of populations and rearing environment. Recent work has supported the prediction that there should be greater potential for inbreeding-environmental stress interactions under novel environmental conditions, to which the organism is not adapted or acclimated (Kristensen et al. 2003b). In our study, tadpoles all came from open-canopy ponds, which tend to support a denser predator community (Werner et al. 2007) and the mesocosm environment more closely resembles an open-canopy pond environment in terms of temperature and food availability (*personal observation*). Since wood frogs have been shown to be locally adapted to natal pond environment along the open-closed canopy gradient (Relyea 2002), the experimental conditions were potentially not novel enough to elicit a strong inbreeding-environmental stress interaction in many of the traits measured. Future work should test this hypothesis to determine whether novel stressors may increase the likelihood of synergistic effects or whether a ceiling exists on maximal stress activity, possibly imposing constraints on further responses and therefore impacting survival.

In addition, the potential for individual history and genotype to set a maximal response threshold has important implications for using stress hormone measurements as an indicator of environmental quality or population "health" (Creel et al. 2002, Homan et al. 2003). As habitats are fragmented and gene flow among populations interrupted, increased levels of inbreeding are likely to become more commonplace (Epps et al. 2005,

Coulon et al. 2006, Cushman 2006, Riley et al. 2006, Vandergast et al. 2007). In addition, due to the causes of habitat loss and fragmentation, many such populations would be found near human activity whose effects on populations would be of interest to conservation biologists using endocrine tools in the field. Given the effects of inbreeding on stress physiology, however, individuals from these populations may not only be less likely to successfully respond to and survive an environmental challenge, but single-measurement studies of stress hormone levels will also be less able to capture the result of contemporary stressors. Therefore, careful consideration of individual history and population genetic connectivity will become increasingly necessary for the successful employment of endocrine techniques in conservation biology in the near future.

References

Armbruster, P. and D. H. Reed. 2005. Inbreeding depression in benign and stressful environments. Heredity **95**:235-242.

Benard, M. F. and J. A. Fordyce. 2003. Are induced defenses costly? Consequences of predator-induced defenses in western toads, *Bufo boreas*. Ecology **84**:68-78.

Bijlsma, R., J. Bundgaard, and W. F. Van Putten. 1999. Environmental dependence of inbreeding depression and purging in Drosophila melanogaster. Journal of Evolutionary Biology **12**:1125-1137.

Bize, P., A. Roulin, and H. Richner. 2004. Parasitism, developmental plasticity and bilateral asymmetry of wing feathers in alpine swift, *Apus melba*, nestlings. Oikos **106**:317-323.

Blaustein, A. R., J. M. Romansic, J. M. Kiesecker, and A. C. Hatch. 2003. Ultraviolet radiation, toxic chemicals and amphibian population declines. Diversity And Distributions **9**:123-140.

Breuner, C. W., S. H. Patterson, and T. P. Hahn. 2008. In search of relationships between the acute adrenocortical response and fitness. General And Comparative Endocrinology **157**:288-295.

Bridges, C. M. 2002. Tadpoles balance foraging and predator avoidance: effects of predation, pond drying, and hunger. Journal of Herpetology **36**:627-634.

Charlesworth, D. and B. Charlesworth. 1987. Inbreeding depression and its evolutionary consequences. Annual Review Of Ecology And Systematics **18**:237-268.

Coltman, D. W., J. G. Pilkington, J. A. Smith, and J. M. Pemberton. 1999. Parasite-mediated selection against inbred Soay sheep in a free-living, island population. Evolution **53**:1259-1267.

Coulon, A., G. Guillot, J. F. Cosson, J. M. A. Angibault, S. Aulagnier, B. Cargnelutti, M. Galan, and A. J. M. Hewison. 2006. Genetic structure is influenced by landscape features: empirical evidence from a roe deer population. Molecular Ecology **15**:1669-1679.

Creel, S., J. E. Fox, A. Hardy, J. Sands, B. Garrott, and R. O. Peterson. 2002. Snowmobile activity and glucocorticoid stress responses in wolves and elk. Conservation Biology **16**:809-814.

Crnokrak, P. and D. A. Roff. 1999. Inbreeding depression in the wild. Heredity **83**:260-270.

Cushman, S. A. 2006. Effects of habitat loss and fragmentation on amphibians: A review and prospectus. Biological Conservation **128**:231-240.

Davidson, C., M. F. Benard, H. B. Shaffer, J. M. Parker, C. O'Leary, J. M. Conlon, and L. A. Rollins-Smith. 2007. Effects of chytrid and carbaryl exposure on survival, growth and skin peptide defenses in foothill yellow-legged frogs. Environmental Science & Technology **41**:1771-1776.

Epps, C. W., P. J. Palsboll, J. D. Wehausen, G. K. Roderick, R. R. Ramey, and D. R. McCullough. 2005. Highways block gene flow and cause a rapid decline in genetic diversity of desert bighorn sheep. Ecology Letters **8**:1029-1038.

Fox, C. W. and D. H. Reed. 2011. Inbreeding depression increases with environmental stress: An experimental study and meta-analysis. Evolution **65**:246-258.

Fraker, M. E. 2008. The effect of hunger on the strength and duration of the antipredator behavioral response of green frog (Rana clamitans) tadpoles. Behavioral Ecology And Sociobiology **62**:1201-1205.

Frankham, R. 2005. Genetics and extinction. Biological Conservation **126**:131-140.

Hayward, L. S. and J. C. Wingfield. 2004. Maternal corticosterone is transferred to avian yolk and may alter offspring growth and adult phenotype. General And Comparative Endocrinology **135**:365-371.

Homan, R. N., J. V. Regosin, D. M. Rodrigues, J. M. Reed, B. S. Windmiller, and L. M. Romero. 2003. Impacts of varying habitat quality on the physiological stress of spotted salamanders (*Ambystoma maculatum*). Animal Conservation **6**:11-18.

Husband, B. C. and D. W. Schemske. 1996. Evolution of the magnitude and timing of inbreeding depression in plants. Evolution **50**:54-70.

Johnson, P. T. J., E. R. Preu, D. R. Sutherland, J. M. Romansic, B. Han, and A. R. Blaustein. 2006. Adding infection to injury: Synergistic effects of predation and parasitism on amphibian malformations. Ecology **87**:2227-2235.

Jones, D. K., J. I. Hammond, and R. A. Relyea. 2011. Competitive stress can make the herbicide Roundup more deadly to larval amphibians Environmental Toxicology And Chemistry **30**:446-454.

Kiesecker, J. M. 2002. Synergism between trematode infection and pesticide exposure: A link to amphibian limb deformities in nature? Proceedings Of The National Academy Of Sciences Of The United States Of America **99**:9900-9904.

Kristensen, T. N., J. S. F. Barker, K. S. Pedersen, and V. Loeschcke. 2008. Extreme temperatures increase the deleterious consequences of inbreeding under

laboratory and semi-natural conditions. Proceedings Of The Royal Society B-Biological Sciences **275**:2055-2061.

Kristensen, T. N., C. Pertoldi, D. H. Andersen, and V. Loeschcke. 2003a. The use of fluctuating asymmetry and phenotypic variability as indicators of developmental instability: a test of a new method employing clonal organisms and high temperature stress. Evolutionary Ecology Research **5**:53-68.

Kristensen, T. N., J. G. Sorensen, and V. Loeschcke. 2003b. Mild heat stress at a young age in Drosophila melanogaster leads to increased Hsp70 synthesis after stress exposure later in life. Journal of Genetics **82**:89-94.

Kristensen, T. N., P. Sorensen, K. S. Pedersen, M. Kruhoffer, and V. Loeschcke. 2006. Inbreeding by environmental interactions affect gene expression in Drosophila melanogaster. Genetics **173**:1329-1336.

Marr, A. B., P. Arcese, W. M. Hochachka, J. M. Reid, and L. F. Keller. 2006. Interactive effects of environmental stress and inbreeding on reproductive traits in a wild bird population. Journal Of Animal Ecology **75**:1406-1415.

Moller, A. P. 1992. Patterns of fluctuating asymmetry in weapons - Evidence for reliable signaling of quality in beetle horns and bird spurs. Proceedings Of The Royal Society Of London Series B-Biological Sciences **248**:199-206.

Moller, A. P. and J. Hoglund. 1991. Patterns of fluctutaing asymmetry in avian feather ornaments - Implications for models of sexual selection. Proceedings Of The Royal Society Of London Series B-Biological Sciences **245**:1-5.

Reed, D. H. 2007. Natural selection and genetic diversity. Heredity **99**:1-2.

Reed, D. H., A. C. Nicholas, and G. E. Stratton. 2007. Genetic quality of individuals impacts population dynamics. Animal Conservation **10**:275-283.

Relyea, R. A. 2002. Local population differences in phenotypic plasticity: predator-induced changes in wood frog tadpoles. Ecological Monographs **72**:77-93.

Relyea, R. A. 2004. Fine-tuned phenotypes: Tadpole plasticity under 16 combinations of predators and competitors. Ecology **85**:172-179.

Relyea, R. A. and J. R. Auld. 2005. Predator- and competitor-induced plasticity: how changes in foraging morphology affect phenotypic trade-offs. Ecology **86**:1723-1729.

Relyea, R. A. and N. Mills. 2001. Predator-induced stress makes the pesticide carbaryl more deadly to gray treefrog tadpoles (*Hyla versicolor*). Proceedings Of The National Academy Of Sciences Of The United States Of America **98**:2491-2496.

Riley, S. P. D., J. P. Pollinger, R. M. Sauvajot, E. C. York, C. Bromley, T. K. Fuller, and R. K. Wayne. 2006. A southern California freeway is a physical and social barrier to gene flow in carnivores. Molecular Ecology **15**:1733-1741.

Romero, L. M. and M. Wikelski. 2010. Stress physiology as a predictor of survival in Galapagos marine iguanas. Proceedings Of The Royal Society B-Biological Sciences **277**:3157-3162.

Sapolsky, R. M., L. M. Romero, and A. U. Munck. 2000. How do glucocorticoids influence stress responses? Integrating permissive, suppressive, stimulatory, and preparative actions. Endocrine Reviews **21**:55-89.

Sih, A., A. M. Bell, and J. L. Kerby. 2004. Two stressors are far deadlier than one. Trends In Ecology & Evolution **19**:274-276.

Soderman, F., S. van Dongen, S. Pakkasmaa, and J. Merila. 2007. Environmental stress increases skeletal fluctuating asymmetry in the moor frog *Rana arvalis*. Oecologia **151**:593-604.

Szulkin, M. and B. C. Sheldon. 2007. The Environmental Dependence of Inbreeding Depression in a Wild Bird Population. Plos One **2**.

Van Buskirk, J. 2000. The costs of an inducible defense in anuran larvae. Ecology **81**:2813-2821.

Van Buskirk, J. and S. A. McCollum. 2000. Functional mechanisms of an inducible defence in tadpoles: morphology and behaviour influence mortality risk from predation. Journal of Evolutionary Biology **13**:336-347.

Van Buskirk, J., S. A. McCollum, and E. E. Werner. 1997. Natural selection for environmentally induced phenotypes in tadpoles. Evolution **51**:1983-1992.

Vandergast, A. G., A. J. Bohonak, D. B. Weissman, and R. N. Fisher. 2007. Understanding the genetic effects of recent habitat fragmentation in the context of evolutionary history: phylogeography and landscape genetics of a southern California endemic Jerusalem cricket (Orthoptera : Stenopelmatidae : Stenopelmatus). Molecular Ecology **16**:977-992.

Vasconcelos, D. and A. J. K. Calhoun. 2004. Movement patterns of adult and juvenile *Rana sylvatica* (LeConte) and *Ambystoma maculatum* (Shaw) in three restored seasonal pools in Maine. Journal of Herpetology **38**:551-561.

Vogt, G., M. Huber, M. Thiemann, G. van den Boogaart, O. J. Schmitz, and C. D. Schubart. 2008. Production of different phenotypes from the same genotype in the

same environment by developmental variation. Journal Of Experimental Biology **211**:510-523.

Waller, D. M., J. Dole, and A. J. Bersch. 2008. Effects of stress and phenotypic variation on inbreeding depression in Brassica rapa. Evolution **62**:917-931.

Wells, J. C. K., P. C. Hallal, J. T. Manning, and C. G. Victora. 2006. A trade-off between early growth rate and fluctuating asymmetry in Brazilian boys. Annals Of Human Biology **33**:112-124.

Werner, E. E., D. K. Skelly, R. A. Relyea, and K. L. Yurewicz. 2007. Amphibian species richness across environmental gradients. Oikos **116**:1697-1712.

Wingfield, J. C. and A. S. Kitaysky. 2002. Endocrine responses to unpredictable environmental events: Stress or anti-stress hormones? Integrative and Comparative Biology **42**:600-609.

Wingfield, J. C., D. L. Maney, C. W. Breuner, J. D. Jacobs, S. Lynn, M. Ramenofsky, and R. D. Richardson. 1998. Ecological bases of hormone-behavior interactions: The "emergency life history stage". American Zoologist **38**:191-206.

Wingfield, J. C. and L. M. Romero. 2001. Andrenocortical responses to stress and their modulation in free-living vertebrates. Pages 211-234 *in* B. S. McEwen and H. M. Goodman, editors. Handbook of Physiology; Section 7: The Endocrine System; Volume IV: Coping with the Environment: Neural and Endocrine Mechanisms. Oxford University Press.

Zellmer, A. J. 2010. Ecological and evolutionary consequences of population connectivity in an amphibian with local adaptation. Dissertation. University of Michigan, Ann Arbor, MI.

Zellmer, A. J. and L. L. Knowles. 2009. Disentangling the effects of historic vs. contemporary landscape structure on population genetic divergence. Molecular Ecology **18**:3593-3602.

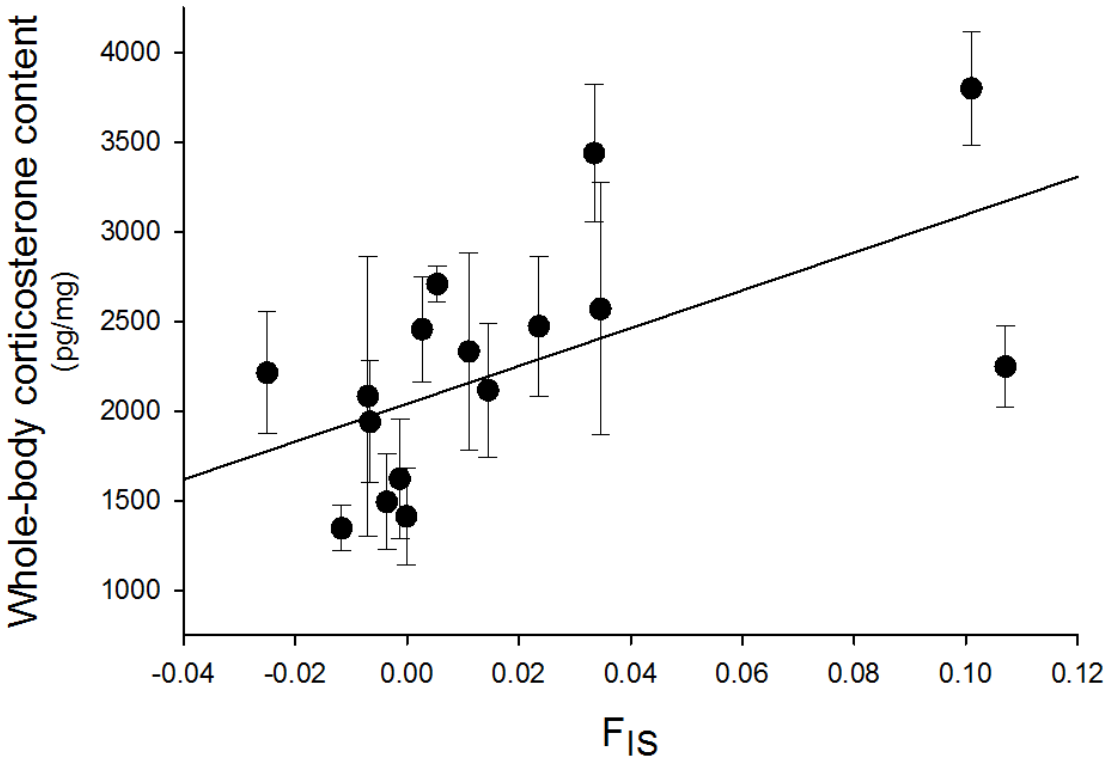


Figure 4.1. Baseline corticosterone content of tadpoles from 16 populations raised in a common garden in Study 1. Points represent population means and error bars +/-2 standard errors. Corticosterone content significantly increased with population inbreeding coefficient (F_{IS}; $R^2 = 0.348$, $p = 0.016$).

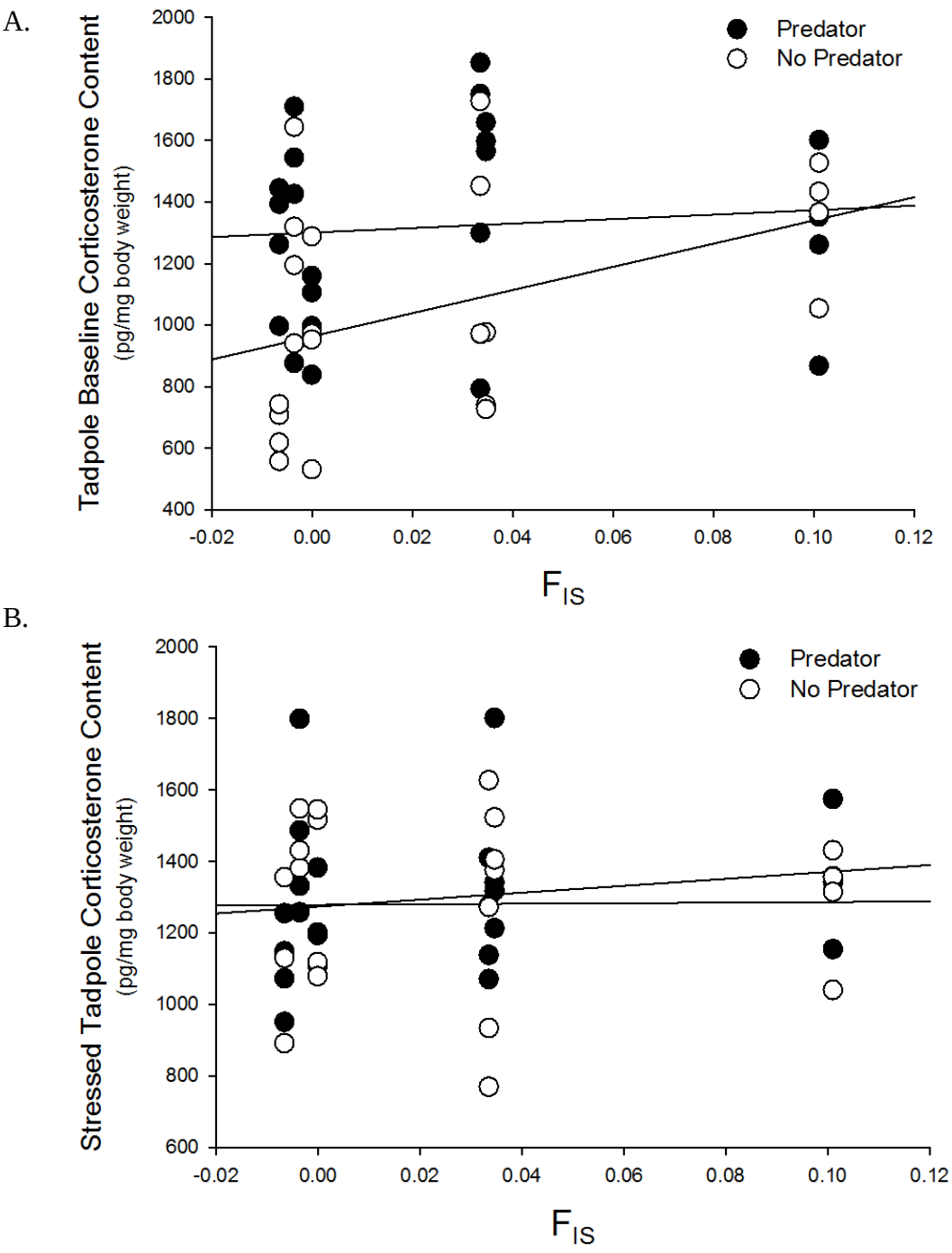


Figure 4.2. Baseline and stressed corticosterone production in tadpoles from 6 populations in Study 2. Each point represents one mesocosm. A. Baseline corticosterone was significantly affected by predator presence ($F = 8.266$, $p = 0.006$) and population inbreeding coefficient (F_{IS}; $F = 7.243$, $p = 0.010$), and the predator x F_{IS} interaction was marginally nonsignificant ($F = 2.916$, $p = 0.095$). B. When subjected to a novel confinement stressor, corticosterone content did not differ with respect to predator treatment ($F = 0.937$, $p = 0.339$), F_{IS} ($F = 0.635$, $p = 0.430$), or the predator x F_{IS} interaction ($F = 0.028$, $p = 0.869$).

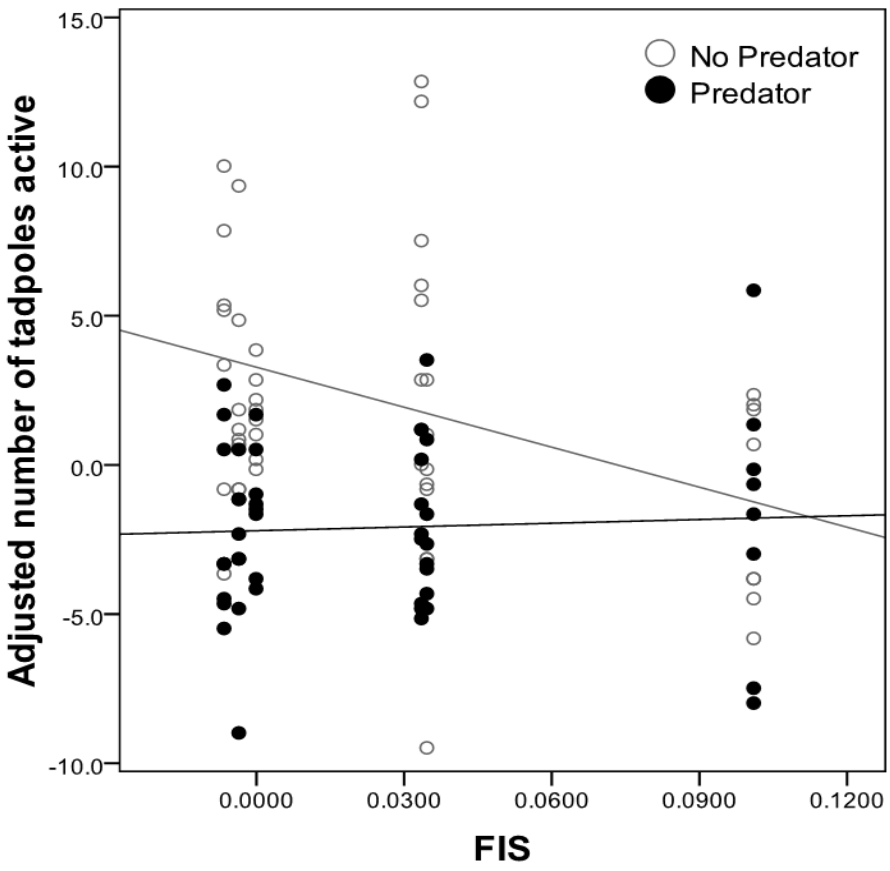


Figure 4.3. Number of tadpoles active in Study 2. To present data from both observation dates in the same figure, total number of active tadpoles was adjusted by calculating the daily mean number of active tadpoles and then subtracting that mean from the number of tadpoles active in each mesocosm. Therefore, points below 0 represent mesocosms with less-than-average tadpole activity, and points above 0 represents mesocosms with more-than-average activity within each observation day. Predator treatment significant decreased tadpole activity across populations ($t = 5.08$, $p < 0.0001$) and inbred tadpoles of both treatments showed low activity (predator x F_{IS}; $t = -2.21$, $p = 0.029$).

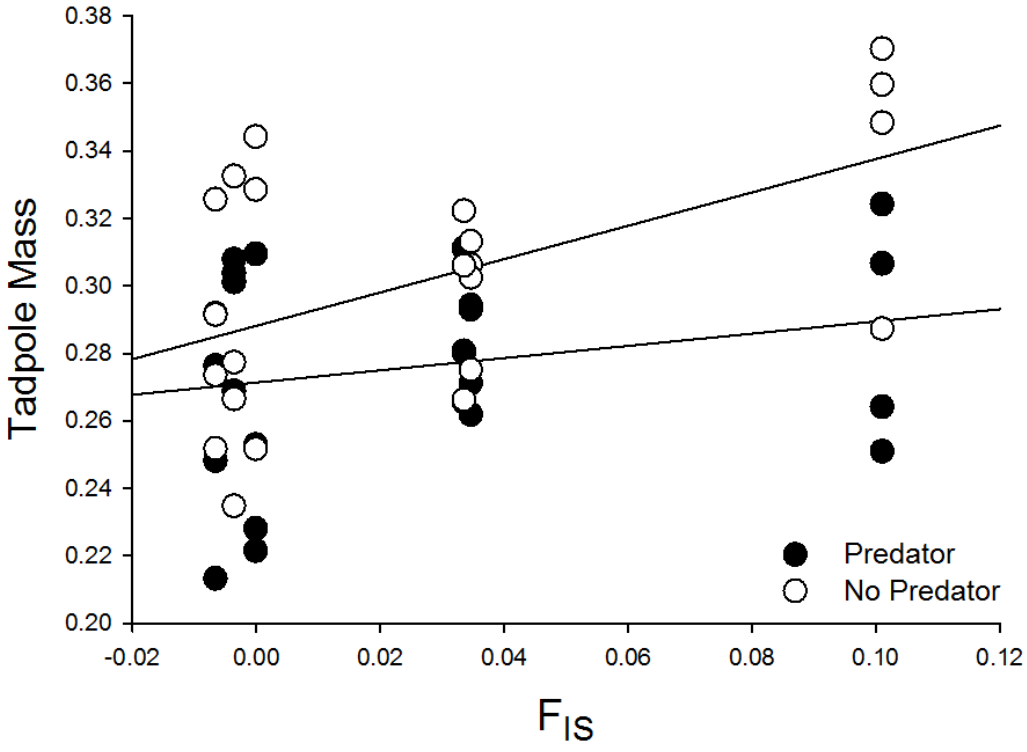
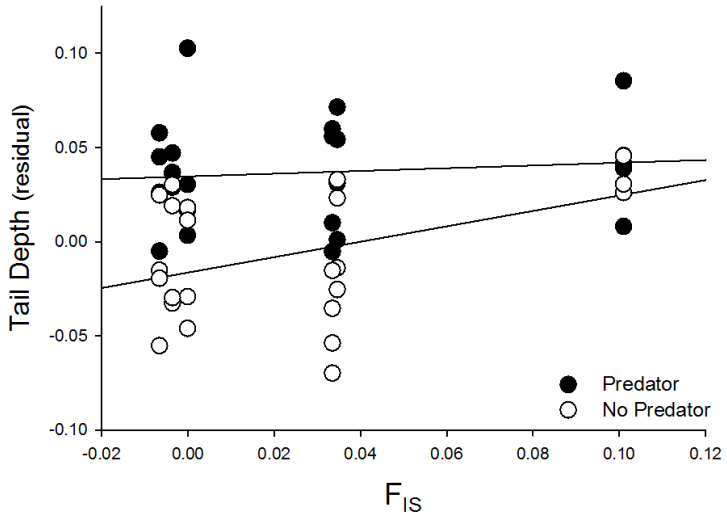
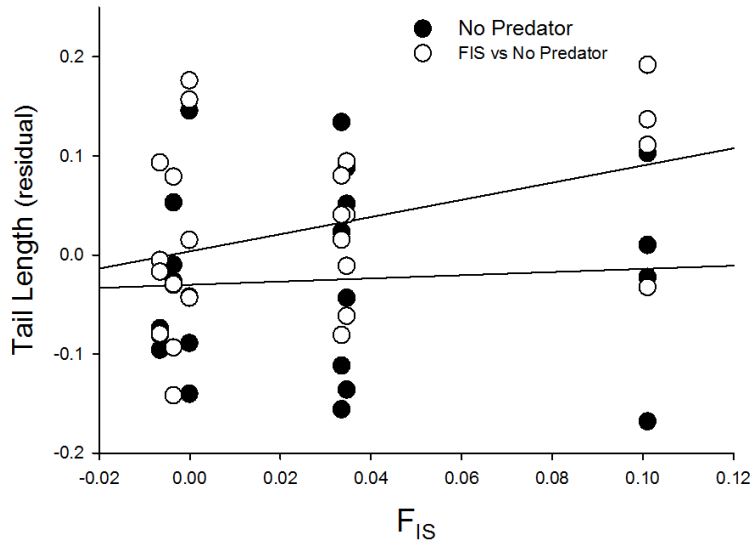


Figure 4.4. Tadpole mass in Study 2. Points represent mesocosm means. Mass of tadpoles significantly increased with population inbreeding coefficient (F_{IS}; F = 8.835, p = 0.005) but was not affected by predator treatment (F = 2.206, p = 0.145) nor was there an interaction effect (F = 1.974, p = 0.168).

A.


Tail Depth (residual)
0.10
0.05
0.00
-0.05
-0.10
-0.02
0.00
0.02
0.04
0.06
0.08
0.10
0.12
Predator
No Predator
F_{IS}

B.


No Predator
FIS vs No Predator
Tail Length (residual)
0.2
0.1
0.0
-0.1
-0.2
-0.02
0.00
0.02
0.04
0.06
0.08
0.10
0.12
F_{IS}

C.

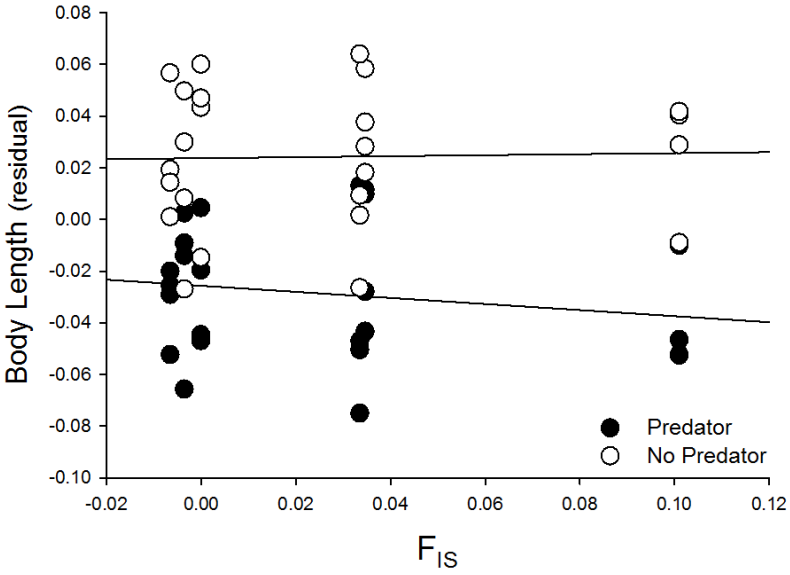

Body Length (residual)
0.08
0.06
0.04
0.02
0.00
-0.02
-0.04
-0.06
-0.08
-0.10
-0.02
0.00
0.02
0.04
0.06
0.08
0.10
0.12
Predator
No Predator
F_{IS}

Figure 4.5. Tadpole morphological measurements in Study 2. Points represent mesocosm means. A. Tadpole tails were significantly deeper in predator treatments ($F = 24.692$, $p < 0.0001$) and also increased with population inbreeding coefficient (F_{IS}; $F = 4.608$, $p = 0.038$), but the interaction term was not significant ($F = 2.248$, $p = 0.141$). B. Tail length showed a nonsignificant trend to increase with inbreeding ($F = 2.497$, $p = 0.122$) but there was no predator effect ($F = 1.287$, $p = 0.263$) and no interaction effect ($F = 1.185$, $p = 0.283$). C. Predator-exposed tadpoles had significantly shorter bodies across populations ($F = 26.713$, $p < 0.0001$; Fig 4.5C), but there was no significant effect of F_{IS} ($F = 0.221$, $p = 0.641$) and no interaction effect ($F = 0.429$, $p = 0.516$) on body length.

CHAPTER 5

THE COMBINED EFFECTS OF PREDATION RISK AND FOOD RESOURCES ON THE STRESS RESPONSE AND GROWTH IN LARVAL WOOD FROGS

Introduction

The trade-off between foraging for food and avoiding predators is arguably one of the most prevalent in nature, and has been investigated in a wide variety of taxa (e.g. Lima and Dill 1990, Anholt and Werner 1998, Anholt et al. 2000, Clinchy et al. 2004, McPeek 2004, Schiesari et al. 2006, Grol et al. 2011, Strobbe et al. 2011). In general, theory predicts that the strength of the anti-predator response should decrease as food resources or energetic reserves decline (Houston et al. 1993, Werner and Anholt 1993, McNamara and Houston 1994) and a number of studies have demonstrated this finding empirically (Dill and Fraser 1984, Horat and Semlitsch 1994, Anholt and Werner 1998, Hazlett 2003, Holker and Stief 2005, Fraker 2008). However, predator presence (Chapter 2, Scheuerlein et al. 2001, Clinchy et al. 2004) and low food availability (Kitaysky et al. 1999, Glennemeier and Denver 2002) are also both potential stressors that may trigger an increase in circulating glucocorticoid stress hormones. For example, in song sparrows (*Melospiza melodia*), birds at sites with high predator pressure and without artificial food supplementation showed an additive effect of each stressor on corticosterone (CORT) production (Clinchy et al. 2004).

When individuals must trade off acquiring food resources and avoiding predation, it is possible that the stress response to predators may manifest differently as individuals lose energy reserves and starvation becomes imminent. Additionally, individuals may respond to low food availability as an added or competing stressor, and modify their stress response accordingly. In this experiment, I capitalized on the well-developed model system of larval anurans, specifically wood frog tadpoles (*Rana sylvatica*) to investigate the combined consequences of food availability and predator presence on both CORT production and growth. I wished to determine whether 1) wood frog tadpoles respond to reduced food availability with increased stress hormone production, and if so 2) whether there is an additive response when low food is combined with predator presence. Furthermore, I asked whether 3) tadpoles reduce their stress response to predators when energetic reserves have declined (a trade-off), and 4) the various interaction effects above influence tadpole growth. The results demonstrate how tadpoles respond to predator presence and food availability alone and in combination.

Methods

Wood frog tadpoles for this experiment were collected as ten egg masses from Southeast Marsh in the University of Michigan's E. S. George Reserve. Eggs were hatched in outdoor wading pools and the resulting tadpoles were fed rabbit chow *ad libitum*. Approximately three weeks after hatching, when tadpoles had reached Gosner stage 27, eight tadpoles were haphazardly counted out, weighed, and placed into each of 80 plastic tanks filled with 8-L of aged well water and set up in a randomized pattern on shelves in the lab. Tanks were then assigned to treatments in a 2 x 2 factorial design of

high (HF) or low food (LF) and predator cue (P) or no predator cue (NP), in which each combination of food and predator treatment was replicated 20 times. Tadpole food was prepared by pulverizing a 3:1 mixture of rabbit chow and fish flakes; HF treatments received food at a daily rate of 20% of original body mass, and LF treatments received 2% of original body mass (based on previous work by (Anholt et al. 2000, Schiesari 2004).

Predator cue was produced by providing individually-housed penultimate instar dragonfly larvae (*Anax* spp.) with three wood frog tadpoles and leaving them for 2-3 hours to feed. Water was then poured off from the containers of *Anax* who had fed, and combined to make a cue solution from which each P tank received an approximately 200 ml aliquot; NP tanks received a similar-sized aliquot of well water as a control. Food and predator treatments were administered daily following a partial water change to remove uneaten food and waste matter. To compare the effects of treatments over time, half of the replicates were assigned to be sampled at 30 hrs after the experiment was initiated, and the other half at 8 days. At the assigned time, all tadpoles were quickly netted from their tank, pooled, and flash-frozen for corticosterone analysis. Tadpole mass was also measured for assessment of growth.

Analysis

Samples for corticosterone analysis were extracted and assayed as described in previous chapters. To determine whether treatments and sample time affected tadpole growth and CORT content, full-factorial ANOVAs were used with tank as the experimental unit and sample time, predator treatment, and food level as fixed effects. Analyses were done in PASW 18.0 (SPSS Inc., Chicago, IL, USA).

Results

No mortality occurred over the course of the experiment. Tadpole growth, as measured by group mass at sampling time, occurred in all treatments over the 8-day time period, amounting to approximately 7.5% of body mass per day in both low food treatments, 16.5% per day in the high food-predator treatment, and 24% of body mass per day in the high food-no predator treatment (Fig 5.1). Thus food level ($F = 74.104$, $p < 0.0001$), predator treatment ($F = 8.525$, $p = 0.005$), and time sampled ($F = 347.592$, $p < 0.0001$) all had significant main effects on growth. The two-way interactions, food level x time sampled ($F = 74.489$, $p < 0.0001$) and predator treatment x time sampled ($F = 6.117$, $p = 0.016$), were also significant, while there was a nonsignificant trend towards a predator treatment x food level interaction ($F = 3.284$, $p = 0.075$). The three-way interaction, food level x predator treatment x time sampled, also had a significant effect on growth ($F = 7.316$, $p = 0.009$).

Tadpole corticosterone content differed among some treatments and showed trends towards interaction effects over time (Fig 5.2). Predator treatment significantly increased CORT ($F = 55.618$, $p < 0.001$), but food level ($F = 0.002$, $p = 0.968$) and time sampled ($F = 0.054$, $p = 0.817$) did not have any effect. The food level x predator treatment interaction, however, was significant ($F = 4.237$, $p = 0.044$), although the other two-way interactions, predator treatment x time sampled ($F = 0.089$, $p = 0.776$) and food level x time sampled ($F = 0.003$, $p = 0.958$) had no effect. There was a nonsignificant trend towards a three-way interaction (food level x predator treatment x time sampled) on CORT content ($F = 2.771$, $p = 0.101$). Due to the trend in the three-way interaction,

separate ANOVA analyses were performed on the two data sets from different time periods in order to better elucidate the patterns among food and predator treatments across time. In this analysis, only predator treatment had significant effects at the 30 hr sample time ($F = 24.341$, $p < 0.0001$), but at 8 days both predator treatment ($F = 35.32$, $p < 0.0001$) and the food level x predator treatment interaction ($F = 9.552$, $p = 0.004$) showed significant effects on CORT production.

Discussion

Results from this study support the role of food availability on governing corticosterone production and a food availability-predator presence trade-off effect on growth rate in wood frog tadpoles. Specifically, although growth occurred in all treatments, predator presence had a significantly negative impact on growth after 8 days, as evidenced by the three-way interaction effect (Fig 5.1). Tadpoles that were fed at a high rate of 20% of body mass daily gained weight at only 16.5% daily when exposed to predator cue, whereas unexposed tadpoles gained 24% body mass per day. Reduction of feeding rate, and resulting growth, under predation risk is predicted by theory (Houston et al. 1993, Werner and Anholt 1993) and has been shown empirically in this and other systems (Anholt et al. 2000, Schiesari et al. 2006, Strobbe et al. 2011). In addition, tadpoles from both predator and no-predator treatments fed a low level of food did not differ in growth after 8 days, suggesting that tadpoles at low resource levels may be able to forage at the same efficiency even under predation risk and subsequent reduced activity (McPeek 2004, Peacor and Werner 2004). It is also possible that hungry tadpoles are ignoring predator cues to remain at no-predator activity level and foraging rate

(Fraker 2008). Under either mechanism, however, it is clear that a foraging gain-predation risk trade-off affects growth rate at high resource levels but erodes when resources are scarce, likely due either to increased foraging efficiency or reduction in anti-predator behavior.

Analysis of corticosterone production across treatments showed a robust increase in CORT content in tadpoles exposed to predator cues over both time periods. This supports earlier demonstrations of a similar pattern in chronic predator exposure (see previous chapters). We did not see a significant overall increase in CORT production in tadpoles fed low food, although the significant predator treatment x food level interaction in the 8-day samples suggest a trend in that direction (see Fig 5.2). The fact that this result does not appear until 8 days into the experiment is important, and suggests that there is a temporal component to the development of the stress response to reduced resource levels. Specifically, it appears as though low food availability may only become stressful once energetic reserves have had time to decline. The induction of a stress response to reduced food availability has been demonstrated in other larval anuran systems as well. Glennemeier and Denver (2002) showed an increase in CORT under either high tadpole density or low food availability in *Rana pipiens*, and suggested that CORT may mediate the growth suppression seen when organisms are under competition. Similarly, Crespi and Denver (2005) showed that *Spea hammondii* tadpoles increased CORT under food deprivation, while juveniles decreased CORT production, presumably to save energy under low resource conditions.

In this study, a low-resource effect on endocrine response may take time to develop particularly due to the species and feeding rate employed. Wood frogs are the

only anuran species in my study location that can consistently and successfully utilize, and even attain some of its highest densities in, low-productivity closed-canopy ponds (Skelly et al. 1999), and as our results show, can still show considerable growth over the 8 days of being fed only 2% of their body weight (see Fig 5.1). In addition, when choosing feeding levels for this experiment, I specifically did not want to induce mortality in low-food treatments since cannibalism of corpses would make interpretation of actual food availability in each tank difficult. The daily feeding rate of 2% of body weight provides enough energy for maintenance and growth in wood frogs, but significantly stunts potential growth rate (Schiesari 2004). Previously mentioned studies in larval anurans have shown an increase in CORT production in response to complete food deprivation or feeding rates at a maintenance level only (Glennemeier and Denver 2002, Crespi and Denver 2005). To produce a comparable feeding rate, which may be necessary to elicit the stronger stress response shown in those studies, we would have needed to feed less than 0.5% of body weight daily (Schiesari 2004).

However, the trend in the three-way interaction in the full analysis and the significant predator treatment x food level interaction at 8 days indicates that tadpoles may be responding to a lack of energy stores even at a feeding rate that allows for growth, particularly after an extended time with low food. Tadpole growth rate determines size at metamorphosis, which has strong effects on dispersal capability, juvenile survival, and adult reproductive success (Berven 1988, Newman and Dunham 1994, Scott 1994, Altwegg and Reyer 2003, Chelgren et al. 2006). Due to the pressure to metamorphose at a large size before the larval pond dries, wood frog tadpoles may

experience food stress even when growth rate is just reduced, rather than completely arrested, but only after a time lag during which stored energy reserves are depleted.

Although there was a strong effect of predator presence on CORT production, the trend towards increasing CORT under low food conditions removed the potential for a trade-off between hormonal response to predation risk and food availability. Previous demonstrations of the interaction between predation risk and foraging gain have focused on trade-offs mediated through activity. Tadpoles of several species have been demonstrated to have a blunted anti-predator behavioral response when food is scarce (Horat and Semlitsch 1994, Anholt et al. 2000) and increased hunger causes tadpoles to return to no-predator activity levels more quickly following detection of a predation event (Fraker 2008). Since CORT is involved in the mobilization of stored fuels (Sapolsky et al. 2000), tadpoles for whom foraging activity is risky (due to predators) and resources are scarce may increase CORT production in order to better take advantage of stored energy reserves, and therefore maintain a positive growth rate even under increased predation risk.

In summary, I have shown here that tadpoles respond to both predation risk and food availability with changes in growth rate and endocrine activity. When facing with the conflicting demands of predation survival and food acquisition, tadpoles appear to protect their growth potential as best as possible, potentially modifying their predator response and using hormonal mediation of metabolism to achieve a positive growth rate even under limiting resources. However, when predation risk and low resources coincide for long periods of time, the effects of chronic stress may have long-term detrimental

consequences to individual fitness and population demography (Krebs et al. 1995, Hubbs and Boonstra 1997, Karels et al. 2000, Zanette et al. 2006).

References

Altwegg, R. and H. U. Reyer. 2003. Patterns of natural selection on size at metamorphosis in water frogs. Evolution **57**:872-882.

Anholt, B. R., E. Werner, and D. K. Skelly. 2000. Effect of food and predators on the activity of four larval ranid frogs. Ecology **81**:3509-3521.

Anholt, B. R. and E. E. Werner. 1998. Predictable changes in predation mortality as a consequence of changes in food availability and predation risk. Evolutionary Ecology **12**:729-738.

Berven, K. A. 1988. Factors affecting variation in reproductive traits within a population of wood frogs (*Rana sylvatica*). Copeia **3**:605-615.

Chelgren, N. D., D. K. Rosenberg, S. S. Heppell, and A. I. Gitelman. 2006. Carryover aquatic effects on survival of metamorphic frogs during pond emigration. Ecological Applications **16**:250-261.

Clinchy, M., L. Zanette, R. Boonstra, J. C. Wingfield, and J. N. M. Smith. 2004. Balancing food and predator pressure induces chronic stress in songbirds. Proceedings Of The Royal Society Of London Series B-Biological Sciences **271**:2473-2479.

Crespi, E. J. and R. J. Denver. 2005. Roles of stress hormones in food intake regulation in anuran amphibians throughout the life cycle. Comparative Biochemistry And Physiology A-Molecular & Integrative Physiology **141**:381-390.

Dill, L. M. and A. H. G. Fraser. 1984. Risk of predation and the feeding behavior of juvenile Coho salmon (*Onchohynchus kisutch*). Behavioral Ecology And Sociobiology **16**:65-71.

Fraker, M. E. 2008. The effect of hunger on the strength and duration of the antipredator behavioral response of green frog (Rana clamitans) tadpoles. Behavioral Ecology And Sociobiology **62**:1201-1205.

Glennemeier, K. A. and R. J. Denver. 2002. Role for corticoids in mediating the response of *Rana pipiens* tadpoles to intraspecific competition. Journal Of Experimental Zoology **292**:32-40.

Grol, M. G. G., I. Nagelkerken, A. L. Rypel, and C. A. Layman. 2011. Simple ecological trade-offs give rise to emergent cross-ecosystem distributions of a coral reef fish. Oecologia **165**:79-88.

Hazlett, B. A. 2003. The effects of starvation on crayfish responses to alarm odor. Ethology **109**:587-592.

Holker, F. and P. Stief. 2005. Adaptive behaviour of chironomid larvae (Chironomus riparius) in response to chemical stimuli from predators and resource density. Behavioral Ecology And Sociobiology **58**:256-263.

Horat, P. and R. D. Semlitsch. 1994. Effects of predation risk and hunger on the behavior of two species of tadpoles. Behavioral Ecology And Sociobiology **34**:393-401.

Houston, A. I., J. M. McNamara, and J. M. C. Hutchinson. 1993. General results concerning the trade-off between gaining energy and avoiding predation. Philosophical Transactions of the Royal Society of London Series B-Biological Sciences **341**:375-397.

Hubbs, A. H. and R. Boonstra. 1997. Population limitation in Arctic ground squirrels: Effects of food and predation. Journal Of Animal Ecology **66**:527-541.

Karels, T. J., A. E. Byrom, R. Boonstra, and C. J. Krebs. 2000. The interactive effects of food and predators on reproduction and overwinter survival of arctic ground squirrels. Journal Of Animal Ecology **69**:235-247.

Kitaysky, A. S., J. C. Wingfield, and J. F. Piatt. 1999. Dynamics of food availability, body condition and physiological stress response in breeding Black-legged Kittiwakes. Functional Ecology **13**:577-584.

Krebs, C. J., S. Boutin, R. Boonstra, A. R. E. Sinclair, J. N. M. Smith, M. R. T. Dale, K. Martin, and R. Turkington. 1995. Impact of food and predation on the snowshoe hare cycle. Science **269**:1112-1115.

Lefcort, H., S. M. Thomson, E. E. Cowles, H. L. Harowicz, B. M. Livaudais, W. E. Roberts, and W. F. Ettinger. 1999. Ramifications of predator avoidance: Predator and heavy-metal-mediated competition between tadpoles and snails. Ecological Applications **9**:1477-1489.

Lima, S. L. and L. M. Dill. 1990. Behavioral decisions made under the risk of predation - A review and prospectus. Canadian Journal Of Zoology-Revue Canadienne De Zoologie **68**:619-640.

McNamara, J. M. and A. I. Houston. 1994. The effect of a change in foraging options on intake rate and predation rate. American Naturalist **144**:978-1000.

McPeek, M. A. 2004. The growth/predation risk trade-off: So what is the mechanism? American Naturalist **163**:E88-E111.

Newman, R. A. and A. E. Dunham. 1994. Size at metamorphosis and water-loss in a desert anuran (*Scaphiopus couchii*). Copeia:372-381.

Peacor, S. D. and E. E. Werner. 2004. Context dependence of nonlethal effects of a predator on prey growth. Israel Journal of Zoology **50**:139-167.

Sapolsky, R. M., L. M. Romero, and A. U. Munck. 2000. How do glucocorticoids influence stress responses? Integrating permissive, suppressive, stimulatory, and preparative actions. Endocrine Reviews **21**:55-89.

Scheuerlein, A., T. J. Van't Hof, and E. Gwinner. 2001. Predators as stressors? Physiological and reproductive consequences of predation risk in tropical stonechats (*Saxicola torquata axillaris*). Proceedings Of The Royal Society Of London Series B-Biological Sciences **268**:1575-1582.

Schiesari, L. 2004. Performance trade-offs across resource gradients in anuran larvae. Dissertation. University of Michigan, Ann Arbor, MI.

Schiesari, L., S. D. Peacor, and E. E. Werner. 2006. The growth-mortality tradeoff: evidence from anuran larvae and consequences for species distributions. Oecologia **149**:194-202.

Scott, D. E. 1994. The effect of larval density on adult demographic traits in *Ambystoma opacum*. Ecology **75**:1383-1396.

Skelly, D. K., E. E. Werner, and S. A. Cortwright. 1999. Long-term distributional dynamics of a Michigan amphibian assemblage. Ecology **80**:2326-2337.

Strobbe, F., M. A. McPeek, M. De Block, and R. Stoks. 2011. Fish predation selects for reduced foraging activity. Behavioral Ecology And Sociobiology **65**:241-247.

Werner, E. E. and B. R. Anholt. 1993. Ecological consequences of the trade-off between growth and mortality rates mediated by foraging activity American Naturalist **142**:242-272.

Zanette, L., M. Clinchy, and J. N. M. Smith. 2006. Combined food and predator effects on songbird nest survival and annual reproductive success: results from a bi-factorial experiment. Oecologia **147**:632-640.

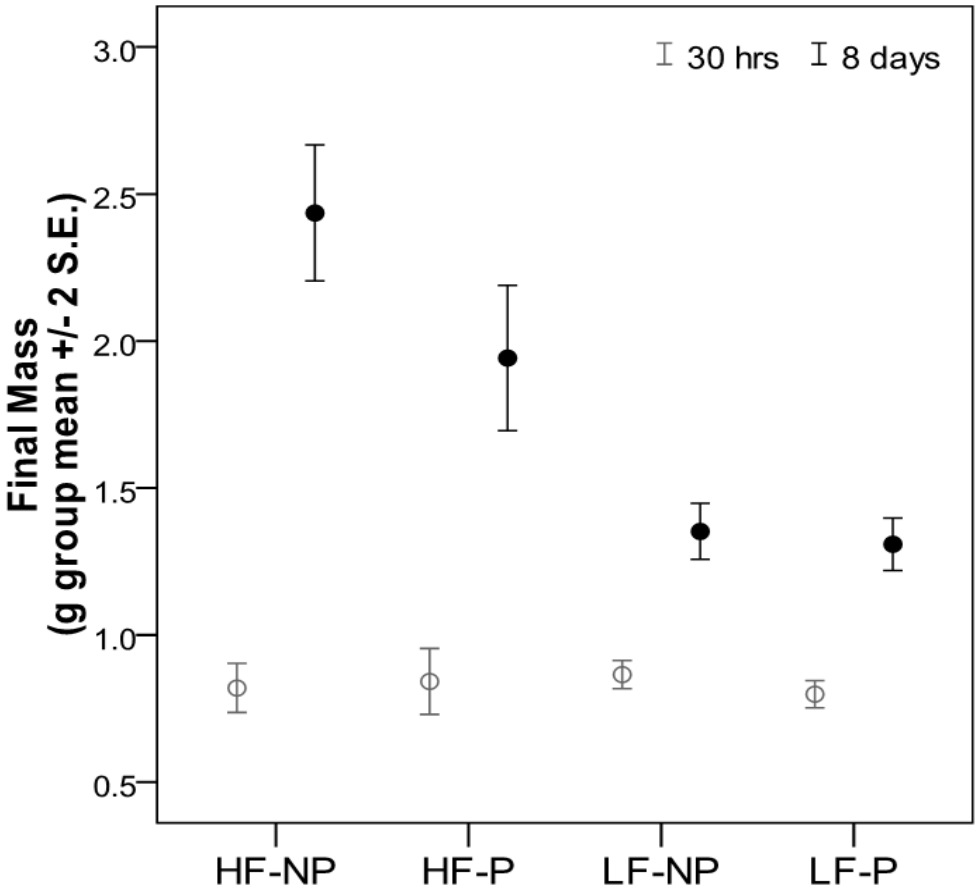


Figure 5.1. Growth in tadpoles over 30 hours (open circles, gray bars) and 8 days (closed circles, black bars) of treatment. Treatment combinations refer to food levels and predator cue exposure: high food (HF), which corresponded to a feeding rate of 20% body mass, versus low food (LF), which corresponded to a feeding rate of 2% of body mass, and predator cue (P) versus no predator cue (NP). Food level ($F = 74.104$, $p < 0.0001$), predator treatment ($F = 8.525$, $p = 0.005$), and time sampled ($F = 347.592$, $p < 0.0001$) all had significant main effects on growth. The two-way interactions, food level x time sampled ($F = 74.489$, $p < 0.0001$) and predator treatment x time sampled ($F = 6.117$, $p = 0.016$), were also significant, while there was a nonsignificant trend towards a predator treatment x food level interaction ($F = 3.284$, $p = 0.075$). The three-way interaction, food level x predator treatment x time sampled, also had a significant effect on growth ($F = 7.316$, $p = 0.009$). Bars represent +/- 2 SE.

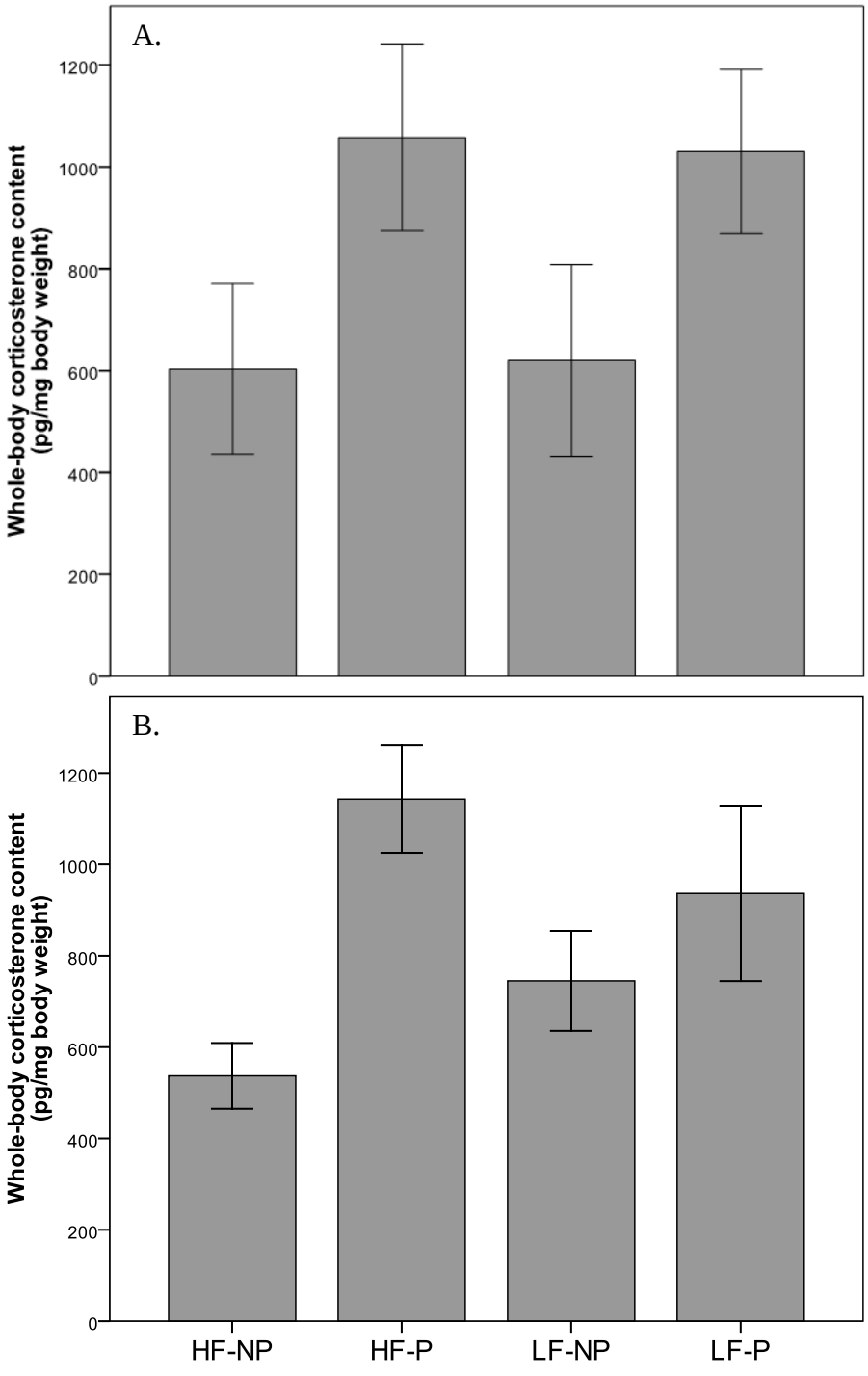


Figure 5.2. Tadpole corticosterone content at 30 hours (A) and 8 days (B). Treatment combinations refer to food levels and predator cue exposure: high food (HF), which corresponded to a feeding rate of 20% body mass, versus low food (LF), which corresponded to a feeding rate of 2% of body mass, and predator cue (P) versus no

predator cue (NP). A. Corticosterone content after 30 hours was affected only by predator treatment ($F = 24.341$, $p < 0.0001$). B. After 8 days, predator treatment significantly affected CORT ($F = 35.32$, $p < 0.0001$) and the food level x predator treatment interaction was also significant ($F = 9.552$, $p = 0.004$). Error bars represent +/- 2 SE.

CHAPTER 6

CONCLUSION

Describing and understanding the interactions between environment and phenotype, and the resulting consequences for populations and communities, is a central goal of ecological and evolutionary research and theory. The field of environmental endocrinology has recently made important advancements in describing how the pattern and reactivity of the stress response confer individual fitness and influence population demography (Boonstra et al. 1998, Creel 2001, Romero and Wikelski 2001, Hawlena and Schmitz 2010, Romero and Wikelski 2010). In addition, simultaneous co-occurrence of stressors, a situation often faced by wild populations, has been shown to have unpredictable and non-additive effects on phenotype and survival (Relyea and Mills 2001, Sih et al. 2004, Hayes et al. 2006). These contributions have been possible due to the incorporation of physiological concepts and tools in an ecological context (Romero 2004, Tracy et al. 2006, Wikelski and Cooke 2006, Teixeira et al. 2007). The research presented in this dissertation capitalized on this approach, treating the glucocorticoid stress response in tadpoles as a response variable that is both influenced by the environment and induces phenotypic changes with fitness effects.

Using an integrative approach that combined field surveys, mesocosm experiments, and exogenous hormone manipulations, I explored the complex interactions

among the predator stress response, behavior, morphology, growth, and fitness, with respect to other contemporary stressors and species evolutionary history. Overall, my research demonstrates that larval anurans respond robustly to predator presence, the hormonal consequence of which initiates the development of defense phenotypes but is also modulated by evolutionary history and genetic background. Below I review the results and implications of each chapter and then integrate those results in a discussion of the broader conclusions from my dissertation research and directions for future research.

Predators are clearly an important source of stress for many wild populations (Scheuerlein et al. 2001, Clinchy et al. 2004, Adamec et al. 2005, Barcellos et al. 2007, Mateo 2007, Roseboom et al. 2007), but the mechanistic connections between predator stress and phenotypic plasticity in larval anurans are not well understood. In Chapter 2, I address this gap by mapping the relationship between the predator stress response, phenotype, and fitness in wood frog tadpoles. In addition to demonstrating the predator presence induces an endocrine response, I also show a temporal pattern in the predator stress response which exhibits an initial down-regulation of corticosterone (CORT) production that reverses course within 48 hrs of predator exposure to produce the high levels of CORT observed in predator-rich ponds and mesocosms. This response pattern appears to improve tadpole survivorship, allowing for behavioral quiescence immediately following detection of predator presence (e.g. Fraker et al. 2009) while initiating long-term investment in predator defense morphology (Chapter 2). Thus, my research shows that tadpoles can adaptively modulate their stress response to predators, and provides a mechanistic understanding of phenotypic plasticity displayed by larval wood frogs.

Wood frog tadpoles sampled in natural ponds and mesocosms maintained high CORT production when co-occurring with high predator densities (Chapter 2), but such a chronic response could become damaging over time (Sapolsky et al. 2000, Rollins-Smith 2001). In a comparative study, I showed that anuran species that predictably experience high predator risk may mitigate the costs of maintaining a chronic glucocorticoid response by reducing the magnitude of the predator stress response (Chapter 3). These results support previous comparisons of the same species demonstrating similar patterns in expression of phenotypic plasticity in morphology and behavior (Skelly 1995, Relyea and Werner 2000). The next step in this line of research should determine whether this species-level modulation of stress activity is restricted to only the predator response, and whether it reduces the fitness costs of chronic activation of the stress axis while maintaining reactivity to novel stressors (Romero and Wikelski 2010).

Wild populations often experience more than one environmental stress in combination, from predator presence to low resource levels to gene x environment interactions imposed by differing individual genetic backgrounds. My research shows that both inbreeding and low food availability can cause wood frog tadpoles to respond with increased CORT production (Chapters 4 and 5), although the lack of additive effects or response to a novel stressor suggest the presence of a maximal response ceiling that could reduce survivorship during an acute environmental challenge (Wingfield and Kitaysky 2002, Romero and Wikelski 2010). In addition, I observed that some of the phenotypes exhibited by inbred individuals could be detrimental: under no-predator conditions, inbred tadpoles were less active than outbred individuals and produced

morphology similar to those induced by predators (Chapter 4). Both of these responses could pose a cost to tadpoles when predators are not a threat.

As a whole, my research demonstrates that larval anurans adaptively modulate their stress response to predators both within and among species. In addition, reduced genetic quality or resource levels also induce an endocrine response whose consequences may depend upon the presence of other environmental perturbations. I show that tadpole stress response modifies behavior, morphology, and predation survival, lending a mechanistic understanding of the ecological and evolutionary relevance of these traits in larval anurans (Skelly 1994, Peacor and Werner 1997, Van Buskirk et al. 1997, Relyea and Werner 2000, Relyea 2002, 2004). My research also demonstrates the utility of applying endocrine concepts and tools within an ecological context and considering the potential for the role of individual and evolutionary history in shaping hormone responses. By considering the glucocorticoid stress response as an important phenotypic mediator of organism-environment interactions, and then using experimental manipulations to determine the causative relationships between stress hormones and adaptive phenotypes, I present a comprehensive understanding of the relationships between environment, stress physiology, phenotypic plasticity, and fitness in larval anurans. This work contributes to a rapidly developing body of research that brings new and exciting insights to the ecological and evolutionary outcomes of organism-environment interactions.

Future Research Directions

The integration of environmental endocrinology into existing ecological and evolutionary conceptual models of organism-environment interactions opens new

windows to our understanding of phenotypic and life history responses to environmental perturbations. As this field progresses, studies should increasingly consider the effects of individual history, and thus individual variation, on an organism's response to environmental conditions. An individual's response to its environmental is based on not only evolutionary history and genetic background, but is often plastic over the individual's lifetime due to the effects of developmental history and physical condition (Tibbetts and Izzo 2009). Thus, the analysis of relationships between environmental input and organism response output would benefit from focusing on the effects of variation in individual sensitivity to environmental perturbations and the consequences of developmental history on the relationship between the endocrine stress response and fitness.

An organism's response to its environment depends on a combination of internal and external factors that trade-off or accumulate to determine the magnitude of the response (Astheimer et al. 1995, Wingfield et al. 1995). Sensitivity to a stressful event is governed in part by evolutionary history and ecological constraints (as demonstrated in Chapter 3), which may differ among individuals or populations. For example, when organisms initiate an endocrine stress response, the result is investment in self-maintenance strategies at the expense of non-essential but often fitness-enhancing activities such as reproduction (Sapolsky et al. 2000, Wingfield and Sapolsky 2003). Therefore, in inherently challenging environments, individuals may develop or populations may evolve decreased sensitivity to stress in order to prevent fitness loss from chronic stress activity; this concept was tested in Chapter 3 and had been demonstrated in systems such as Arctic-breeding birds (Wingfield et al. 1995).

However, we are far from understanding the entire cost/benefit analysis that determines such a plastic or adaptive outcome. Furthermore, as human-induced climate changes cause organisms to increasingly experience mismatches between their life history and the environment, individual perception and response to the environment will fall under renewed selection pressure.

Differences in reactivity set-points not only vary among groups occupying different habitats or across seasons, but individual condition and life history demands also change optimal sensitivity and magnitude of the stress response. For example, fat reserves or breeding investment may change how an individual reacts to an environmental perturbation (Astheimer et al. 1995, Sorato and Kotrschal 2006). Therefore, future research should focus on understanding the internal and external inputs that determine an appropriate endocrine response in individuals, as well as follow the ability of individuals to successfully meet those conflicting demands through responses in hormone production, receptor density, or thresholds for inducing phenotypic change. These data may then be integrated with conceptual models of allostasis or homeostasis, and over-load of those systems, to improve our predictions regarding the cost and benefits of the glucocorticoid stress response (McEwen and Wingfield 2003, Romero et al. 2009, McEwen and Wingfield 2010) and consequences for fitness (Bonier et al. 2009, Angelier et al. 2010).

Some aspects of individual variation, unlike body condition or reproductive status, are not reversible and instead accumulate over the lifetime. In particular, the effects of developmental history deserve a strong research focus, in order to determine how environmental perturbations compound and interact throughout an individual's life.

The consequences of stressful events during early life are not only relevant to phenotype and fitness of developing individuals, but also may continue to manifest long after the stressor is removed, a phenomenon known as "developmental programming" (Matthews 2002, Hu et al. 2008, Denver and Middlemis Maher 2010). For example, laboratory experiments in birds and rodents have shown that stressed juveniles develop into adults that exhibit altered stress activity, either through increasing reactivity to stressful stimuli or failure to mount a normal stress response (Anisman et al. 1998, Meaney 1999, Hayward and Wingfield 2004, Parfitt et al. 2004). In anurans, exposure to elevated glucocorticoids during the tadpole stage can lead to long-term changes in behavior, life history, and gene expression in juvenile frogs (Hu et al. 2008). Therefore, the relationship between glucorticoid activity and fitness (Blas et al. 2007, Bonier et al. 2009, Angelier et al. 2010) may be mediated through developmental history.

Furthermore, the endocrine stress response is not the only phenotype to demonstrate "carry-over effects" across stages of the life cycle. Carry-over has been observed in phenotypic responses among wide range of organisms: arachnids (Hebets 2003), lepidopterans (Mevi-Schutz and Erhardt 2003, Bauerfeind and Fischer 2005, Boggs and Freeman 2005), marine invertebrates (Pechenik et al. 1998, Phillips 2002, Hg and Keough 2003, Gimenez et al. 2004), dipterans (Ray 1999), odonates (De Block and Stoks 2005), rodents (Anisman et al. 1998, Meaney 1999, Parfitt et al. 2004), birds (Hayward and Wingfield 2004, Naguib and Gil 2005, Blas et al. 2007), and of course anurans (Berven 1990, Goater 1994, Scott 1994, Beck and Congdon 2000, Alvarez and Nicieza 2002, Altwegg and Reyer 2003, Relyea and Hoverman 2003, Chelgren et al. 2006, Niehaus et al. 2006), with consequences on morphology, behavior, physiological

performance, and ultimately reproductive fitness. Humans are also not immune to the programming effects of prenatal environment, including changes in risk of diabetes and heart disease (Barker 1995, Ellison 2005, McMillen and Robinson 2005, Plagemann 2005, Reyes and Manalich 2005). Endocrine responses may mediate many of these carry-over effects; in particular, calibration of hormonal responses acquired during development likely help match individual phenotype to the environment, with the consequence of reduced fitness when mismatches occur (Monaghan 2008).

The consequences of carry-over effects are especially interesting in species that exhibit a complex life cycle, where life stages are ecologically distinct and thus early developmental experiences are unlikely to be predictive of future environmental conditions (Wilbur 1980). As a result, the adaptive value of many documented carry-over effects in species with complex life cycles is uncertain. On the one hand, some carry-over traits may prove adaptive for dispersing to better habitats before reproducing; for example, predator presence during development induces longer thoraces, which are typical of species that move longer distances, in three *Libellula* dragonfly species (McCauley 2005). Often, though, the fitness outcome appears to be context-dependent; for example, in western toads (*Bufo borealis*), juveniles that developed under larval predation risk produced a higher concentration of toxic bufadienolides, but at the cost of reduced handling time by a toxin-resistant predator (Benard and Fordyce 2003). The diversity in causes and consequences of carry-over effects in complex life cycles requires further research in order to develop a predictive understanding of how individual developmental history can shape phenotype and fitness. Since carry-over among stages of a life cycle may be mediated through the diverse actions of glucocorticoids (Denver

2009), tracing the interactions between environmental perturbations and phenotypic response will elucidate how developmental conditions may impact fitness under varying environmental contexts.

To progress our understanding of the connection between glucocorticoid expression and fitness (Bonier et al. 2009, Angelier et al. 2010), future work should focus on the complex relationships among developmental environment and individual reactivity variation in organism-environment interactions, as well as diversity of responses individuals may employ to maximize fitness. To that end, my research and the future directions that I have outlined here emphasize the utility and increasing importance of integrative approaches to biological questions. By developing collaborative teams with a shared goal, we can investigate a problem from molecular mechanisms to community and ecosystem outcomes. Combining the tools and concepts of field ecology, physiology, and evolutionary biology allows us to develop a much deeper and richer understanding of how organisms interact with their environments, and should be the focal approach of future research.

References

Adamec, R. E., J. Blundell, and P. Burton. 2005. Neural circuit changes mediating lasting brain and behavioral response to predator stress. Neuroscience And Biobehavioral Reviews **29**:1225-1241.

Altwegg, R. and H.-U. Reyer. 2003. Patterns of natural selection on size at metamorphosis in water frogs. Evolution **57**:872-882.

Alvarez, D. and A. G. Nicieza. 2002. Effects of induced variation in anuran larval development on postmetamorphic energy reserves and locomotion. Oecologia **131**:186-195.

Angelier, F., J. C. Wingfield, H. Weimerskirch, and O. Chastel. 2010. Hormonal correlates of individual quality in a long-lived bird: a test of the 'corticosterone-fitness hypothesis'. Biology Letters **6**:846-849.

Anisman, H., M. D. Zaharia, M. J. Meaney, and Z. Merali. 1998. Do early-life events permanently alter behavioral and hormonal responses to stressors? International Journal Of Developmental Neuroscience **16**:149-164.

Astheimer, L. B., W. A. Buttemer, and J. C. Wingfield. 1995. Seasonal and acute changes in adrenocortical responsiveness in an arctic-breeding bird. Hormones And Behavior **29**:442-457.

Barcellos, L. J. G., F. Ritter, L. C. Kreutz, R. M. Quevedo, L. B. da Silva, A. C. Bedin, J. Finco, and L. Cericato. 2007. Whole-body cortisol increases after direct and visual contact with a predator in zebrafish, Danio rerio. Aquaculture **272**:774-778.

Barker, D. J. P. 1995. The Wellcome Foundation Lecture, 1994 - The fetal origins of adult disease. Proceedings Of The Royal Society Of London Series B-Biological Sciences **262**:37-43.

Bauerfeind, S. S. and K. Fischer. 2005. Effects of food stress and density in different life stages on reproduction in a butterfly. Oikos **111**:514-524.

Beck, C. W. and J. D. Congdon. 2000. Effects of age and size at metamorphosis on performance and metabolic rates of southern toad, *Bufo terrestris*, metamorphs. Functional Ecology **14**:32-38.

Benard, M. F. and J. A. Fordyce. 2003. Are induced defenses costly? Consequences of predator-induced defenses in western toads, *Bufo boreas*. Ecology **84**:68-78.

Berven, K. A. 1990. Factors affecting population fluctuations in larval and adult stages of the wood frog (*Rana sylvatica*). Ecology **71**:1599-1608.

Blas, J., G. R. Bortolotti, J. L. Tella, R. Baos, and T. A. Marchant. 2007. Stress response during development predicts fitness in a wild, long lived vertebrate. Proceedings Of The National Academy Of Sciences Of The United States Of America **104**:8880-8884.

Boggs, C. L. and K. D. Freeman. 2005. Larval food limitation in butterflies: effect on adult resource allocation and fitness. Oecologia **144**:353-361.

Bonier, F., P. R. Martin, I. T. Moore, and J. C. Wingfield. 2009. Do baseline glucocorticoids predict fitness? Trends In Ecology & Evolution **24**:634-642.

Boonstra, R., D. Hik, G. R. Singleton, and A. Tinnikov. 1998. The impacts of predator-induced stress on the snowshoe hare cycle. Ecological Monographs **68**:371-394.

Chelgren, N. D., D. K. Rosenberg, S. S. Heppell, and A. I. Gitelman. 2006. Carryover aquatic effects on survival of metamorphic frogs during pond emigration. Ecological Applications **16**:250-261.

Clinchy, M., L. Zanette, R. Boonstra, J. C. Wingfield, and J. N. M. Smith. 2004. Balancing food and predator pressure induces chronic stress in songbirds. Proceedings Of The Royal Society Of London Series B-Biological Sciences **271**:2473-2479.

Creel, S. 2001. Social dominance and stress hormones. Trends In Ecology & Evolution **16**:491-497.

De Block, M. and R. Stoks. 2005. Fitness effects from egg to reproduction: bridging the life history transition. Ecology **86**:185-197.

Denver, R. J. 2009. Stress hormones mediate environment-genotype interactions during amphibian development. General And Comparative Endocrinology **164**:20-31.

Denver, R. J. and J. Middlemis Maher. 2010. Lessons from evolution: developmental plasticity in vertebrates with complex life cycles. Journal of Developmental Origins of Health and Disease **1**:282-291.

Ellison, P. T. 2005. Evolutionary perspectives on the fetal origins hypothesis. American Journal Of Human Biology **17**:113-118.

Fraker, M. E., F. Hu, V. Cuddapah, S. A. McCollum, R. A. Relyea, J. Hempel, and R. J. Denver. 2009. Characterization of an alarm pheromone secreted by amphibian tadpoles that induces behavioral inhibition and suppression of the neuroendocrine stress axis. Hormones And Behavior **55**:520-529.

Gimenez, L., K. Anger, and G. Torres. 2004. Linking life history traits in successive phases of a complex life cycle. Oikos **104**:570-580.

Goater, C. P. 1994. Growth and survival of postmetamorphic toads: interactions among larval history, density, and parasitism. Ecology **75**:2264-2274.

Hawlena, D. and O. J. Schmitz. 2010. Physiological stress as a fundamental mechanism linking predation to ecosystem functioning. American Naturalist **176**:537-556.

Hayes, T. B., P. Case, S. Chui, D. Chung, C. Haeffele, K. Haston, M. Lee, V. P. Mai, Y. Marjuoa, J. Parker, and M. Tsui. 2006. Pesticide mixtures, endocrine disruption, and amphibian declines: Are we underestimating the impact? Environmental Health Perspectives **114**:40-50.

Hayward, L. S. and J. C. Wingfield. 2004. Maternal corticosterone is transferred to avian yolk and may alter offspring growth and adult phenotype. General And Comparative Endocrinology **135**:365-371.

Hebets, E. 2003. Subadult experience influences adult mate choice in an arthropod: exposed female wolf spiders prefer males of a familiar phenotype. Proceedings of the National Academy of Sciences **100**:13390-13395.

Hg, T. Y.-T. and M. J. Keough. 2003. Delayed effects of larval exposure to Cu in the bryozoan Watersipora subtorquata. Marine Ecology Progress Series **257**:77-85.

Hu, F., E. J. Crespi, and R. J. Denver. 2008. Programming neuroendocrine stress axis activity by exposure to glucocorticoids during postembryonic development of the frog, *Xenopus laevis*. Endocrinology **149**:5470-5481.

Mateo, J. M. 2007. Ecological and hormonal correlates of antipredator behavior in adult Belding's ground squirrels (*Spermophilus beldingi*). Behavioral Ecology And Sociobiology **62**:37-49.

Matthews, S. G. 2002. Early programming of the hypothalamo-pituitary-adrenal axis. Trends In Endocrinology And Metabolism **13**:373-380.

McCauley, S. J. 2005. Relationship between habitat distribution, growth rate, and plasticity in congeneric larval dragonflies. Canadian Journal Of Zoology **83**:1128-1133.

McEwen, B. S. and J. C. Wingfield. 2003. The concept of allostasis in biology and biomedicine. Hormones And Behavior **43**:2-15.

McEwen, B. S. and J. C. Wingfield. 2010. What is in a name? Integrating homeostasis, allostasis and stress. Hormones And Behavior **57**:105-111.

McMillen, I. C. and J. S. Robinson. 2005. Developmental origins of the metabolic syndrome: Prediction, plasticity, and programming. Physiological Reviews **85**:571-633.

Meaney, M. J. 1999. Early environmental experience and lifelong changes in behavior and in gene expression. Biological Psychiatry **45**:44S-44S.

Mevi-Schutz, J. and A. Erhardt. 2003. Larval nutrition affects female nectar amino acid preference in the map butterfly (*Araschnia levana*). Ecology **84**:2788-2794.

Monaghan, P. 2008. Early growth conditions, phenotypic development and environmental change. Philosophical Transactions Of The Royal Society B-Biological Sciences **363**:1635-1645.

Naguib, M. and D. Gil. 2005. Transgenerational effects on body size caused by early developmental stress in zebra finches. Biology Letters **1**:95-97.

Niehaus, A. C., R. S. Wilson, and C. E. Franklin. 2006. Short- and long-term consequences of thermal variation in the larval environment of anurans. Journal Of Animal Ecology **75**:686-692.

Parfitt, D. B., J. K. Levin, K. P. Saltstein, A. S. Klayman, L. M. Greer, and D. L. Helmreich. 2004. Differential early rearing environments can accentuate or attenuate the responses to stress in male C57BL/6 mice. Brain Research **1016**:111-118.

Peacor, S. D. and E. E. Werner. 1997. Trait-mediated indirect interactions in a simple aquatic food web. Ecology **78**:1146-1156.

Pechenik, J. A., D. E. Wendt, and J. N. Jarrett. 1998. Metamorphosis is not a new beginning. Bioscience **48**:901-910.

Phillips, N. E. 2002. Effects of nutrition-mediated larval condition on juvenile performance in a marine mussel. Ecology **83**:2562-2574.

Plagemann, A. 2005. Perinatal programming and functional teratogenesis: Impact on body weight regulation and obesity. Physiology & Behavior **86**:661-668.

Ray, S. 1999. Survival of olfactory memory through metamorphosis in the fly *Musca domestica*. Neuroscience Letters **259**:37-40.

Relyea, R. A. 2002. Local population differences in phenotypic plasticity: predator-induced changes in wood frog tadpoles. Ecological Monographs **72**:77-93.

Relyea, R. A. 2004. Fine-tuned phenotypes: Tadpole plasticity under 16 combinations of predators and competitors. Ecology **85**:172-179.

Relyea, R. A. and J. T. Hoverman. 2003. The impact of larval predators and competitors on the morphology and fitness of juvenile treefrogs. Oecologia **134**:596-604.

Relyea, R. A. and N. Mills. 2001. Predator-induced stress makes the pesticide carbaryl more deadly to gray treefrog tadpoles (*Hyla versicolor*). Proceedings Of The National Academy Of Sciences Of The United States Of America **98**:2491-2496.

Relyea, R. A. and E. E. Werner. 2000. Morphological plasticity in four larval anurans distributed along an environmental gradient. Copeia:178-190.

Reyes, L. and R. Manalich. 2005. Long-term consequences of low birth weight. Kidney International **68**:S107-S111.

Rollins-Smith, L. A. 2001. Neuroendocrine-immune system interactions in amphibians - Implications for understanding global amphibian declines. Immunologic Research **23**:273-280.

Romero, L. M. 2004. Physiological stress in ecology: lessons from biomedical research. Trends In Ecology & Evolution **19**:249-255.

Romero, L. M., M. J. Dickens, and N. E. Cyr. 2009. The reactive scope model - A new model integrating homeostasis, allostasis, and stress. Hormones And Behavior **55**:375-389.

Romero, L. M. and M. Wikelski. 2001. Corticosterone levels predict survival probabilities of Galapagos marine iguanas during El Nino events. Proceedings Of The National Academy Of Sciences Of The United States Of America **98**:7366-7370.

Romero, L. M. and M. Wikelski. 2010. Stress physiology as a predictor of survival in Galapagos marine iguanas. Proceedings Of The Royal Society B-Biological Sciences **277**:3157-3162.

Roseboom, P. H., S. A. Nanda, V. P. Bakshi, A. Trentani, S. M. Newman, and N. H. Kalin. 2007. Predator threat induces behavioral inhibition, pituitary-adrenal activation and changes in amygdala CRF-binding protein gene expression. Psychoneuroendocrinology **32**:44-55.

Sapolsky, R. M., L. M. Romero, and A. U. Munck. 2000. How do glucocorticoids influence stress responses? Integrating permissive, suppressive, stimulatory, and preparative actions. Endocrine Reviews **21**:55-89.

Scheuerlein, A., T. J. Van't Hof, and E. Gwinner. 2001. Predators as stressors? Physiological and reproductive consequences of predation risk in tropical stonechats (*Saxicola torquata axillaris*). Proceedings Of The Royal Society Of London Series B-Biological Sciences **268**:1575-1582.

Scott, D. E. 1994. The effect of larval density on adult demographic traits in *Ambystoma opacum*. Ecology **75**:1383-1396.

Sih, A., A. M. Bell, and J. L. Kerby. 2004. Two stressors are far deadlier than one. Trends In Ecology & Evolution **19**:274-276.

Skelly, D. K. 1994. Activity level and the susceptibility of anuran larvae to predation. Animal Behaviour **47**:465-468.

Skelly, D. K. 1995. A behavioral trade-off and its consequences for the distribution of Pseudacris treefrog larvae. Ecology **76**:150-164.

Sorato, E. and K. Kotrschal. 2006. Hormonal and behavioural symmetries between the sexes in the Northern bald ibis. General And Comparative Endocrinology **146**:265-274.

Teixeira, C. P., C. S. De Azevedo, M. Mendl, C. F. Cipreste, and R. J. Young. 2007. Revisiting translocation and reintroduction programmes: the importance of considering stress. Animal Behaviour **73**:1-13.

Tibbetts, E. A. and A. S. Izzo. 2009. Endocrine mediated phenotypic plasticity: Condition-dependent effects of juvenile hormone on dominance and fertility of wasp queens. Hormones And Behavior **56**:527-531.

Tracy, C. R., K. E. Nussear, T. C. Esque, K. Dean-Bradley, C. R. Tracy, L. A. DeFalco, K. T. Castle, L. C. Zimmerman, R. E. Espinoza, and A. M. Barber. 2006. The importance of physiological ecology in conservation biology. Integrative and Comparative Biology **46**:1191-1205.

Van Buskirk, J., S. A. McCollum, and E. E. Werner. 1997. Natural selection for environmentally induced phenotypes in tadpoles. Evolution **51**:1983-1992.

Wikelski, M. and S. J. Cooke. 2006. Conservation physiology. Trends In Ecology & Evolution **21**:38-46.

Wilbur, H. M. 1980. Complex life-cycles. Annual Review Of Ecology And Systematics **11**:67-93.

Wingfield, J. C. and A. S. Kitaysky. 2002. Endocrine responses to unpredictable environmental events: Stress or anti-stress hormones? Integrative and Comparative Biology **42**:600-609.

Wingfield, J. C., K. M. O'Reilly, and L. B. Astheimer. 1995. Modulation of the adrenocortical responses to acute stress in arctic birds - a possible ecological basis. American Zoologist **35**:285-294.

Wingfield, J. C. and R. M. Sapolsky. 2003. Reproduction and resistance to stress: When and how. Journal Of Neuroendocrinology **15**:711-724.