



4-2005

## Bufadienolides In The Chemical Defenses Of The Toads, *Bufo Americanus* and *Bufo Fowleri*

Catherine E. Merovich  
*Western Michigan University*

Follow this and additional works at: <https://scholarworks.wmich.edu/dissertations>



Part of the Animal Sciences Commons, Biochemistry, Biophysics, and Structural Biology Commons, and the Biology Commons

---

### Recommended Citation

Merovich, Catherine E., "Bufadienolides In The Chemical Defenses Of The Toads, *Bufo Americanus* and *Bufo Fowleri*" (2005). *Dissertations*. 1049.

<https://scholarworks.wmich.edu/dissertations/1049>

This Dissertation-Open Access is brought to you for free and open access by the Graduate College at ScholarWorks at WMU. It has been accepted for inclusion in Dissertations by an authorized administrator of ScholarWorks at WMU. For more information, please contact [wmu-scholarworks@wmich.edu](mailto:wmu-scholarworks@wmich.edu).



**BUFADIENOLIDES IN THE CHEMICAL DEFENSES OF THE TOADS,  
*BUFO AMERICANUS* AND *BUFO FOWLERI***

**by**

**Catherine E. Merovich**

**A Dissertation  
Submitted to the  
Faculty of The Graduate College  
in partial fulfillment of the  
requirements for the  
Degree of Doctor of Philosophy  
Department of Biological Sciences**

**Western Michigan University  
Kalamazoo, Michigan  
April 2005**

BUFADIENOLIDES IN THE CHEMICAL DEFENSES OF THE TOADS,  
*BUFO AMERICANUS* AND *BUFO FOWLERI*

Catherine E. Merovich, Ph.D.

Western Michigan University, 2005

I investigated the steroidal chemical defenses (bufadienolides) of *Bufo americanus* and *Bufo fowleri*. By the nature of their complex, biphasic life cycles, toads, like other amphibians are important components of aquatic and terrestrial habitats and are prey to numerous invertebrates and vertebrates. Bufadienolides are presumed to be important anti-predatory compounds although much of their chemical ecology is poorly understood. I investigated (1) ontogenetic variation in bufadienolides, (2) bufadienolides from adult parotoid secretion, (3) effectiveness of bufadienolides against a terrestrial predator, and (4) effectiveness of bufadienolides against an aquatic predator. I hypothesized that *B. americanus* would have a more extensive bufadienolide profile and a more effective suite of chemical defenses than *B. fowleri* and that this could account for distributional differences in these toads. Results showed variability in numbers and concentrations of bufadienolides among toad developmental stages, but cumulatively no difference in total mean

concentrations. Bufadienolide concentrations across developmental stages appeared to fit Brodie and Formanowicz's (1987) model, but were more pronounced in eggs. Bufadienolides did not appear to be inducible following metamorphosis. More bufadienolides were detected in adult *B. americanus* but their mean total concentrations were not larger than in *B. fowleri* suggesting that distributional differences might be explained by variation in bufadienolide types rather than by bufadienolide concentrations. However, seven bufadienolides were statistically different between toad species. In *B. americanus* there was much overlap in bufadienolide types and concentrations from all collection sites. Repeated expressions of parotoid glands revealed highly variable individual responses among toads. In tongue-flick bioassays, terrestrial snake predators responded to chemical stimuli from both *B. americanus* and *B. fowleri* with more tongue-flicks and greater tongue-flick attack scores than snakes exposed to distilled water. Because snakes showed elevated tongue-flick rates with parotoid secretions than with toad skin stimuli, parotoid chemicals may present a more concentrated toad stimulant and are not necessarily deterrents to predation. Also, aquatic Dytiscid beetle predators equally consumed both *B. americanus* and *B. fowleri* suggesting no discrimination between species or between toad developmental stages.

# NOTE TO USERS

This reproduction is the best copy available.

**UMI<sup>®</sup>**



UMI Number: 3164169

### INFORMATION TO USERS

The quality of this reproduction is dependent upon the quality of the copy submitted. Broken or indistinct print, colored or poor quality illustrations and photographs, print bleed-through, substandard margins, and improper alignment can adversely affect reproduction.

In the unlikely event that the author did not send a complete manuscript and there are missing pages, these will be noted. Also, if unauthorized copyright material had to be removed, a note will indicate the deletion.

**UMI<sup>®</sup>**

---

UMI Microform 3164169

Copyright 2005 by ProQuest Information and Learning Company.

All rights reserved. This microform edition is protected against unauthorized copying under Title 17, United States Code.

ProQuest Information and Learning Company  
300 North Zeeb Road  
P.O. Box 1346  
Ann Arbor, MI 48106-1346

© 2005 Catherine E. Merovich



## ACKNOWLEDGMENTS

West-Eberhard (2001) wrote that a truly taxon-centered biologist is a person who possesses several important attitudes that include (1) recognition of the organism as an individual, (2) proprietary recognition of the species or group, (3) respect for the organism as a living thing, (4) recognition of the organism as a beautiful and aesthetically pleasing entity, and (5) a taxon-centered biologist has a genuine feeling for the organism. For me, anuran amphibians are the reason I became a biologist. I have found myself immersed in an amphibian bias that urges me to learn all I can about my taxon. As West-Eberhard also wrote, a taxon-centered biologist is a person "who lets the organism suggest the important questions." In my dissertation, I have examined several important questions about toad chemical defenses and I certainly have many people to thank for helping me get to this point on my journey.

I first wish to acknowledge and express my sincere appreciation to the Department of Biological Sciences and to the Graduate College at Western Michigan University for support as a Doctoral Associate and for the grants that I received in support of my research. Being a Doctoral Associate in the

#### Acknowledgments—continued

Department of Biological Sciences at Western Michigan University also helped me to learn how to become a good teacher through experiences as a lab instructor as well as a lecturer. I especially thank Dr. Alexander Enyedi for giving me such wonderful teaching tools and for remaining a source of valuable advice.

Many have encouraged me throughout my academic pursuits and helped to shape my development as a taxon-centered biologist—whether they realized it or not. Dr. Stephen B. Malcolm excited me about ecology while I was an undergraduate so much so that I came back to become his Ph.D. student. I owe him tremendous thanks for his advice, guidance, wisdom, and especially for his patience. In staying true to the attitudes of a taxon-centered biologist, with my desire to learn all about frogs, Steve always had a knack of reminding me about the nature of food webs and the place of amphibians in them—namely as prey. It's no wonder that I choose to focus my dissertation on toad anti-predator defenses. I am also sincerely grateful to the other members of my committee, Dr. David Cowan, Dr. Charles Ide, Dr. David Karowe, and Dr. Steven Kohler.

I also received tremendous help with field collections and kindly thank all those involved, especially to Mike Franz, Steve Malcolm, George

#### Acknowledgments—continued

Merovich, Guy Link, Heather Link, and a young, budding herpetologist, Miss Katherine Schwallier. Sandy Taverna also permitted access to her property rich in wetlands and encouraged me to study hard and “save the frogs.”

Tammi Roberts and Jim Fordyce assisted me with HPLC methods and I owe them many thanks. Also, Dr. Robert Eversole kindly used his contacts at MSU to prepare and stain my parotoid gland tissues. Kurtis Hunsberger was a tremendous help to me in this regard. Thanks also to Dr. Carlos Jared for his valuable comments on the parotoid gland histology of my toads and to Dr. Christy Foran and Dr. Sarah Farris for their comments on microscopy and for the use of their laboratories at West Virginia University. Dr. Steve Kohler and Julie Ryan helped with insect identification. I received wonderful support from many graduate students and friends and especially thank Marla Fisher, Julie Stahlhut, Eric Bushrow, Bobbie Heard, Daryl Arkwright-Keeler, and Celene and Chris Jackson.

Finally, I thank my family for their continuous love and support, especially my mom, Suzanne Link, and my sister, Heather, who aided me in many ways—especially by proof reading my document over and over again. My dad, Guy Link, gave me a lot of support in many ways. Thanks also to my brother Guy, and sister-in-law, Liz, my husband, George, and my

Acknowledgments—continued

daughter Emily who reminds me what it is like to discover the natural world for the first time. I also thank my mother- and father-in-law, Vicky and George Merovich, for their encouragement and patience. Dr. Hillary Cressey gave me much needed encouragement and Art Fiser gave helpful statistical comments. I am also grateful to my grandparents, especially to Art Fiser, Sr., for giving me motivation and being with me in spirit through this time in my life.

Catherine E. Merovich

West-Eberhard, M. J. 2001. The importance of taxon-centered research in biology. *In*: M. J. Ryan (Ed.). *Anuran Communication*. Smithsonian Institution Press, Washington and London.

## TABLE OF CONTENTS

ACKNOWLEDGMENTS.....	ii
LIST OF TABLES.....	x
LIST OF FIGURES.....	xiii
CHAPTER	
I. INTRODUCTION AND REVIEW OF THE POISONS IN TOADS OF THE GENUS <i>BUFO</i> .....	1
Introduction.....	1
Granular glands and their compounds .....	8
Steroids – bufadienolides and bufotoxins.....	11
Biological activity of bufadienolides.....	15
Study ethics and toad conservation .....	16
Study objectives .....	17
Ontogenetic variation in bufadienolides – Chapter II.....	18
Parotoid gland secretions of adult toads – Chapter III .....	20
Responses of a vertebrate predator to toad secretions – Chapter IV .....	23
Responses of an invertebrate predator to toadpoles – Chapter V .....	24
II. ONTOGENETIC VARIATION IN THE BUFADIENOLIDES OF <i>BUFO AMERICANUS</i> AND <i>BUFO FOWLERI</i> .....	26
Introduction.....	26

## Table of Contents—continued

Methods.....	32
Collection and maintenance of <i>Bufo</i> .....	32
Eggs and tadpoles.....	32
Metamorphs and transformed toads.....	33
The influence of predation on bufadienolide content of developing toads.....	33
Sample preparation and bufadienolide extraction .....	36
HPLC of toad bufadienolides .....	37
Statistical analyses .....	38
Results.....	39
Ontogenetic variation in bufadienolides.....	39
Bufadienolide induction .....	44
Discussion .....	50
III. A COMPARISON OF BUFADIENOLIDES IN THE CUTANEOUS PAROTOID GLAND SECRETIONS OF ADULT <i>BUFO AMERICANUS</i> AND <i>BUFO FOWLERI</i> .....	57
Introduction.....	57
Methods.....	63
Parotoid gland secretions .....	63
HPLC of toad bufadienolides .....	64
Inducibility of bufadienolides.....	67
Histology of toad parotoid glands .....	68

# Table of Contents—continued

Results.....	69
Parotoid gland secretions — bufadienolide composition.....	69
Variation in bufadienolides between toad species .....	78
Histology of toad parotoid glands .....	84
Bufadienolide induction .....	90
Discussion .....	94
IV. RESPONSE OF A VERTEBRATE PREDATOR TO THE PAROTOID SECRETIONS OF <i>BUFO AMERICANUS</i> AND <i>BUFO FOWLERI</i> .....	99
Introduction.....	99
Methods.....	103
Collection and maintenance of <i>Bufo</i> and <i>Thamnophis</i> .....	103
Tongue-flick bioassays .....	104
Data analyses .....	107
Results.....	107
Tongue-flick responses of <i>Thamnophis sirtalis</i> .....	108
Tongue-flick attack scores for repeated measures [TFAS(R)s] .....	112
Discussion .....	120
V. RESPONSE OF AN INVERTEBRATE PREDATOR TO THE LARVAE OF <i>BUFO AMERICANUS</i> AND <i>BUFO FOWLERI</i> .....	125
Introduction.....	125
Methods.....	131

Table of Contents—continued

Animal collection .....	131
Experimental design.....	132
Data analyses .....	133
Results.....	134
Predator response—attack and consumption .....	134
Predator response—survival.....	136
Discussion .....	137
BIBLIOGRAPHY .....	140
APPENDICES	
A. IACUC Protocol For the Use of Vertebrate Animals in Research ....	157
B. Permission To Use Published Materials .....	172



## LIST OF TABLES

1.	Pharmacologically active substances from skin extracts of <i>B. americanus</i> and <i>B. fowleri</i> .....	14
2.	Bufadienolide analyses of <i>B. americanus</i> and <i>B. fowleri</i> in each of the following developmental stage ranges (after Gosner 1960).....	34
3.	Mean mass and total length (TL) of all stages of <i>Bufo americanus</i> individuals analyzed for their bufadienolide composition .....	41
4.	Mean mass and total length (TL) of all stages of <i>Bufo fowleri</i> individuals analyzed for their bufadienolide composition .....	42
5.	Number of individuals with each of the ten identified bufadienolides across all developmental stages. Percentages are in parentheses.....	43
6.	Mean number of bufadienolides identified in <i>B. americanus</i> and <i>B. fowleri</i> across all developmental stages .....	44
7.	Number of <i>Bufo americanus</i> individuals with each of the ten identified bufadienolides by developmental stage. Percentages are in parentheses .....	45
8.	Number of <i>Bufo fowleri</i> individuals with each of the ten identified bufadienolides by developmental stage. Percentages are in parentheses .....	46
9.	Mean mass and snout-vent length (SVL) of toads whose parotoid gland secretions were analyzed.....	70
10.	Number of individuals ( <i>B. americanus</i> n = 63 and <i>B. fowleri</i> n = 31) that possessed each of the ten identified bufadienolides (B1-B30) .....	74

List of Tables—continued

11. Univariate ANOVAs on bufadienolide concentrations (ng/ $\mu$ g dry weight of parotoid secretions) from <i>B. americanus</i> and <i>B. fowleri</i> .....	80
12. Eigen analysis of bufadienolide concentrations in adult <i>B. americanus</i> and <i>B. fowleri</i> parotoid gland secretions.....	81
13. Eigen analysis of bufadienolide concentrations in adult <i>B. americanus</i> parotoid secretions by habitat .....	83
14. Measurements taken on toads used in the examination of parotoid glands .....	88
15. Bufadienolides present in <i>B. fowleri</i> parotoid secretion and their average concentrations during three sampling periods spaced at one month intervals.....	91
16. Adult <i>Thamnophis sirtalis</i> (n = 9) responses (number of tongue-flicks directed toward cotton applicators in 60-seconds) to toad skin stimuli, toad secretion stimuli, and distilled water .....	109
17. Litter A neonatal <i>Thamnophis sirtalis</i> (n = 13) responses (number of tongue-flicks directed toward cotton applicators in 60 seconds) to toad skin stimuli, toad secretion stimuli, and distilled water .....	109
18. Litter B neonatal <i>Thamnophis sirtalis</i> (n = 5) responses (number of tongue-flicks directed toward cotton applicators in 60-seconds) to toad skin stimuli, toad secretion stimuli, and distilled water .....	110
19. Litter C neonatal <i>Thamnophis sirtalis</i> (n = 15) responses (number of tongue-flicks directed toward cotton applicators in 60-seconds) to toad skin stimuli, toad secretion stimuli, and distilled water .....	110
20. Adult <i>Thamnophis sirtalis</i> (n = 9) responses [TFAS(R)s] to toad skin stimuli, toad secretion stimuli, and distilled water .....	116

List of Tables—continued

21.	Litter A neonatal <i>Thamnophis sirtalis</i> (n = 13) responses [TFAS(R)s] to toad skin stimuli, toad secretion stimuli, and distilled water.....	116
22.	Litter B neonatal <i>Thamnophis sirtalis</i> (n = 5) responses [TFAS(R)s] to toad skin stimuli, toad secretion stimuli, and distilled water .....	117
23.	Litter C neonatal <i>Thamnophis sirtalis</i> (n = 15) responses [TFAS(R)s] to toad skin stimuli, toad secretion stimuli, and distilled water.....	117
24.	Mean lengths and masses ( $\pm$ SE) for predators and prey used in the experiment. Starting masses were measured prior to the experiment and the ending masses were measured at 72 hours .....	135

## LIST OF FIGURES

1.	(a) Illustration of the typical location and shape of the anuran parotoid glands and (b) morphological differences in placement of parotoid glands in <i>Bufo americanus</i> and <i>Bufo fowleri</i> .....	10
2.	Photograph of <i>B. americanus</i> showing the enlarged kidney-shaped parotoid glands (PG). Photograph by G. Merovich.....	11
3.	The chemical structures of bufadienolides (a) bufalin and bufotoxins (b) bufotoxin (bufotalin-3-O-suberylarginine).....	13
4.	The geographic ranges of (a) <i>Bufo americanus</i> and (b) <i>Bufo fowleri</i> (Conant and Collins 1998) .....	22
5.	A model of palatability and ability to escape as they affect stage-specific survivorship in anuran larvae .....	30
6.	Schematic of rearing tanks for tadpoles exposed to predator cues during development.....	35
7.	Mean number ( $\pm$ SE) of bufadienolides across all developmental stages in <i>B. americanus</i> .....	47
8.	Mean number ( $\pm$ SE) of bufadienolides across all developmental stages in <i>B. fowleri</i> .....	47
9.	Mean ( $\pm$ SE) total bufadienolide concentration across all developmental stages in <i>B. americanus</i> and <i>B. fowleri</i> .....	48
10.	Mean ( $\pm$ SE) total bufadienolide concentration (ng bufadienolide/ $\mu$ g dry weight) for both toad species across all developmental stages.....	48

List of Figures—continued

11.	Mean ( $\pm$ SE) total bufadienolide concentration (ng bufadienolide/ $\mu$ g dry weight) for <i>B. americanus</i> across all developmental stages .....	49
12.	Mean ( $\pm$ SE) bufadienolide concentration (ng bufadienolide/ $\mu$ g dry weight) for <i>B. fowleri</i> across all developmental stages .....	49
13.	Mean ( $\pm$ SE) bufadienolide concentration (ng bufadienolide/ $\mu$ g dry weight) for <i>B. americanus</i> and <i>B. fowleri</i> across all developmental stages. Concentrations were significantly different only between eggs of <i>B. americanus</i> and <i>B. fowleri</i> .....	50
14.	Mean ( $\pm$ SE) total bufadienolide concentration (ng bufadienolide/ $\mu$ g dry weight) in transformed individuals of <i>B. americanus</i> and <i>B. fowleri</i> raised from eggs with and without predatory cues.....	51
15.	Mean total bufadienolide concentrations (ng bufadienolide/ $\mu$ g dry weight) for each developmental stage joined by a curved line. This may represent the contribution of bufadienolides to the unpalatability of certain developmental stages .....	53
16.	(a) Bufonid toads possess large aggregations of granular glands that form two protuberances, the parotoid glands, on both sides of the body. Shown here is <i>B. americanus</i> with enlarged kidney-shaped parotoid glands (PG) and (b) the morphology and placement of the parotoids differs slightly between <i>B. americanus</i> and <i>B. fowleri</i> (Conant 1975) .....	61
17.	Mean mass ( $\pm$ SE) of adult <i>B. americanus</i> and <i>B. fowleri</i> .....	70
18.	Mean ( $\pm$ SE) total bufadienolide concentration as a function of toad mass. Units are in ng bufadienolide/g toad .....	72
19.	(a) The mean number ( $\pm$ SE) of bufadienolides identified in <i>B. americanus</i> and <i>B. fowleri</i> and (b) the mean total concentration ( $\pm$ SE) of bufadienolides in <i>B. americanus</i> and <i>B. fowleri</i> . Units are in ng bufadienolide/ $\mu$ g dry parotoid secretion.....	73

# List of Figures—continued

20.	Mean concentrations ( $\pm$ SE) of each of twelve common bufadienolides in <i>B. americanus</i> .....	76
21.	Mean concentrations ( $\pm$ SE) of each of twelve common bufadienolides in <i>B. fowleri</i> .....	77
22.	HPLC chromatograms for (a) one <i>B. americanus</i> individual (Ba26FF) collected at Franz Farm (Van Buren County) and (b) one <i>B. fowleri</i> individual (Bf5BT) collected along Belmont Trail (Kent County). These bufadienolides were typically found in each species respectively .....	78
23.	Scatterplot of PCA scores for <i>B. americanus</i> (open squares) and <i>B. fowleri</i> (filled circles) with respect to concentrations of twelve bufadienolides.....	82
24.	Scatterplot of PCA scores for the concentrations of twelve bufadienolides in <i>B. americanus</i> collected from six locations.....	84
25.	Parotoid section from the left parotoid of <i>B. americanus</i> showing non-compressed granular alveoli (g) full of darkly stained secretion and arranged in a honey-comb fashion.....	86
26.	Parotoid section from the left parotoid of <i>B. fowleri</i> showing non-compressed granular alveoli (g) full of darkly stained secretion and arranged in a honey-comb fashion.....	87
27.	Parotoid section from the right parotoid of <i>B. americanus</i> showing compressed granular alveoli including some in the non-compressed state.....	89
28.	Parotoid section from the right parotoid of <i>B. fowleri</i> showing compressed granular alveoli including some in the non-compressed state.....	90
29.	Mean total concentration ( $\pm$ SE) of bufadienolides collected from four <i>B. fowleri</i> individuals over a three month period .....	92
30.	Mean bufadienolide concentrations ( $\pm$ SE) in <i>B. fowleri</i> over a three month period.....	93

# List of Figures—continued

31.	Mean number ( $\pm$ SE) of tongue-flicks directed toward cotton applicators by adult garter snakes from experiment one .....	112
32.	Mean number ( $\pm$ SE) of tongue-flicks directed toward cotton applicators by neonatal snakes (Litter A).....	113
33.	Mean number ( $\pm$ SE) of tongue-flicks directed toward cotton applicators by neonatal snakes (Litter B) .....	114
34.	Mean number ( $\pm$ SE) of tongue-flicks directed toward cotton applicators by neonatal snakes (Litter C).....	114
35.	Mean number ( $\pm$ SE) of tongue-flicks directed toward cotton applicators by all neonatal snakes combined (Litters A, B, and C).....	115
36.	Mean ( $\pm$ SE) tongue-flick attack scores for repeated measures [TFAS(R)s] generated from adult snakes .....	118
37.	Mean ( $\pm$ SE) tongue-flick attack scores for repeated measures [TFAS(R)s] generated from neonatal snakes from Litter A.....	118
38.	Mean ( $\pm$ SE) tongue-flick attack scores for repeated measures [TFAS(R)s] generated from neonatal snakes from Litter B .....	119
39.	Mean ( $\pm$ SE) tongue-flick attack scores for repeated measures [TFAS(R)s] generated from neonatal snakes (Litter C).....	120
40.	Mean ( $\pm$ SE) tongue-flick attack scores for repeated measures [TFAS(R)s] generated from all neonatal snakes combined (Litters A, B, and C).....	121

## CHAPTER I

### INTRODUCTION WITH A REVIEW OF THE POISONS IN TOADS OF THE GENUS *BUFO*

#### Introduction

Amphibians are important components of both terrestrial and aquatic ecosystems. Worldwide, amphibians link diverse environments by the nature of their complex, largely biphasic life cycles and by possessing life histories that vary from almost completely aquatic to almost completely terrestrial (Duellman and Trueb 1986). Foraging modes of natural enemies in these diverse environments influences the evolution of anti-predatory mechanisms and behaviors in amphibians and affects other life history traits that enhance defensive functions. Most amphibia appear to rely primarily on crypsis and anachoresis (hiding in holes) for defense against natural enemies (Cott 1941; Edmunds 1974), yet defensive strategies employed by amphibians are diverse, involving various morphological, physiological, and behavioral attributes that can act alone or in combination to increase an amphibian's chance of surviving encounters with predators (Duellman and Trueb 1986). In many organisms, including amphibians, the evolution of chemical defenses



against natural enemies may promote more conspicuous life styles, enhance distributions, and increase abundances. The toads are well known for their chemical defenses, abundance, and wide distributions and in some cases for their aposematic coloration (Cott 1941) (e.g., schools of darkly-colored tadpoles) and/or conspicuous behaviors (e.g., aggregation and metamorphic synchrony).

Here, I describe my research into the steroidal chemical defenses of two North American toad species, the American toad, *Bufo americanus* (Holbrook), and Fowler's toad, *Bufo fowleri* (Hinkley). *Bufo americanus* is an abundant and widely distributed toad in North America and in some locations it occurs with the considerably less abundant toad, *B. fowleri*. I tested the null hypotheses that there are no differences between either the presence or the functioning of steroidal chemical defenses between *B. americanus* and *B. fowleri*. Alternatively, I hypothesized that the chemical defenses of *B. fowleri* are less effective and less abundant than those of *B. americanus* and that this difference may account for the abundance of *B. americanus* and the rarity of *B. fowleri*.

Amphibians of the Anuran family Bufonidae include members of twenty-five genera, only one of which, *Bufo*, contains representative species found in North America. Bufonid toads, like other amphibians, possess many

defensive mechanisms and behaviors that are important in predator-prey interactions. These defensive strategies include various morphological, physiological, and behavioral attributes that can act alone or in combination to increase a toad's chance of surviving an encounter with a predator (Duellman and Trueb 1986). Such defensive behaviors and responsiveness have also been shown to vary across individuals and populations (Ducey and Brodie 1991).

Transformed or post-metamorphic amphibians are well defended chemically. Their skin secretions contain various compounds, depending on the species, such as biogenic amines, steroids (bufadienolides and bufotoxins), both water-soluble and lipid-soluble alkaloids, as well as peptides and proteins (Daly 1995). The defensive chemicals of all amphibian species are predominantly concentrated in cutaneous granular (poison or serous) glands (Duellman and Trueb 1986) but are also found in other locations in the animals and are not limited to the skin. For example, chemicals have been isolated from other tissues and organs such as stomach, ovaries, eggs, and bile (Matsukawa et al. 1996; Meyer 1966). Butler et al. (1996) found compounds similar to bufotoxins in fresh plasma of *Bufo marinus*. Some of these chemicals (the steroidal bufadienolides) may be involved with endogenous regulation of the membrane enzyme  $\text{Na}^+/\text{K}^+$

ATPase (Lichtstein et al. 1986), as has recently been suggested for mammals including humans (Schoner 2002), as well as other important physiological roles such as water and electrolyte homeostasis (Flier et al. 1980; Matsukawa et al. 1997), or hypertension (Blaustein et al. 1998; Doris and Bagrov 1998).

Chemicals have also been isolated from early amphibian developmental stages—in eggs, tadpoles, and juveniles (e.g., bufadienolides found in *B. marinus* tadpoles and juveniles by Flier et al. (1980)). Akizawa et al. (1994) reported the structures of nine compounds of bufadienolides isolated from the eggs of *Bufo marinus*. The fact that these early developmental stages have these chemicals is interesting since they completely lack or do not have fully developed cutaneous granular glands (Delfino et al. 1995; Hayes and Gill 1995; Trong 1973). The two main groups of compounds found in Bufonid toads are the amines and steroids (Clarke 1997), with especially high titers of steroids occurring in toads (Flier et al. 1980). In this dissertation, I focus on the chemical ecology of the steroidal bufadienolides found in two North American toad species, *Bufo americanus* and *Bufo fowleri*.

Amphibian chemicals can be noxious and sometimes toxic defenses against predators (Brodie et al. 1978, Brodie and Formanowicz 1987, Brodie and Tumbarello 1978). Frogs in the family Dendrobatidae are typically

brightly colored and this aposematism is indicative of the diverse array of poisonous alkaloids that they possess (Edwards et al. 1988). Presumably, amphibians have a vast array of chemicals in response to a diversity of predators (Clarke 1997). It is interesting to point out that in one known instance another animal can exploit amphibian chemicals for its own defensive purposes. For example, hedgehogs coat their spines with toad secretions, termed "self anointing behavior," to augment their own anti-predator defenses (Brodie 1977). Not only are amphibians endowed with chemicals as deterrents to predation, but they also possess efficient anti-microbial and anti-fungal defense systems (Bradford et al. 1996, Clarke 1997). All compounds, except the alkaloids, appear to be synthesized *de novo* by the animals themselves from cholesterol and other precursors such as bile acids (Chen and Osuch 1969; Clarke 1997; Daly 1995; Doull et al. 1951; Garraffo and Gros 1986; Santa Coloma et al. 1984; Siperstein et al. 1957; Tschesche 1972).

Although a defensive function for bufadienolides in toads is an intuitively appealing idea, very little is known about the ecology of chemical defenses in toads and whether bufadienolides are in fact an effective defense against natural enemies such as snakes, birds, or mammals. Indeed, some predators, such as garter (*Thamnophis sirtalis*) and hognose (*Heterodon platirhinos*) snakes have been reported to prey on toads with no apparent ill

effects (Smith and White 1955). Smith and Green (2002) reported the predation of an adult Fowler's toad (*Bufo fowleri*) by a bullfrog, *Rana catesbeiana*, showing that among numerous aquatic and terrestrial toad predators, other, less recognized or known predators may feed on toads.

Compounds isolated from toads have been used for medicinal purposes for the past 3000 years in traditional Oriental medicine (Clarke 1997). For example, Ch'an Su in Liu-Shen-Wan (called Senso in Japanese) contains bufadienolides (bufalin, cinobufagin and resibufogenin) obtained from the toads *Bufo melanostictus* or *Bufo bufo gargarizans* (Hong et al. 1992; Kamano et al. 1998). Toad chemicals were introduced and used medicinally in Europe beginning in the 1600's and replaced by digitalis about 200 years later (Clarke 1997).

Just over a century ago, Phisalix and Bertrand (1902) investigated the chemicals found in the European common toad, *Bufo vulgaris* (*Bufo bufo* Linne) and their research promoted interest in isolating pharmacologically active chemicals from amphibians (Chen and Kovaříková 1967). For example, Abel and Macht (1912) isolated epinephrine and bufagin from secretions of *Bufo marinus*. The chemistry of amphibian secretions has received considerable attention for the last 70 years primarily for pharmacological reasons because the cardiac-active bufadienolides are a major constituent of

chemical secretions in toads (Barry et al. 1996; Chen and Kovaříková 1967; Clarke 1997; Deulofeu and Rúveda 1971; Cei et al. 1972; Low 1972; Daly and Witkop 1971; Hong et al. 1992; Meyer 1966; Meyer and Linde 1971; Okada 1966). Today, the bufadienolide resibufogenin is used as a cardiac-active drug (Kamano et al. 1998).

It was clear to researchers in the late 1960's that "the chemical composition of each species of *Bufo* differs from one another, although there are many similarities" (Chen and Kovaříková 1967). Chemical diversity in secretions from toad parotoid glands (large clusters of poison-producing glands found in the skin) has also been used to infer evolutionary relationships among toad species (Low 1972; Porter and Porter 1967; Wittliff 1962). The bufadienolides, however, show no apparent phylogenetic pattern suggesting that bufadienolides are a primitive trait "that is retained at detectable levels on a rather random basis" or that bufadienolides "are physiological regulators of  $\text{Na}^+/\text{K}^+$  ATPase, whose levels are correlated with unknown physiological or ecological factors" (Flier et al. 1980). Differences in proteins from the parotoid secretions of *Bufo americanus* and *Bufo fowleri* have also been found and are thought to be potentially important for understanding the evolution of bufonid species (Mahan and Biggers 1977).

### *Granular glands and their compounds*

The poison-producing granular glands of amphibian skin have been studied for many years (e.g., Muhse 1909). Anatomically, these glands may reach into the stratum compactum and may have one or two types of myoepithelial cells (Deullman and Trueb 1986). Amphibian granular glands are considered to be the source and/or storage site of most of the active compounds important for chemical defenses against predators (Clarke 1997); while some alkaloids are sequestered from the diet as in Dendrobatid and other frogs (Daly 1995), yet others (tetrodotoxin and similar compounds) may be produced by symbiotic microorganisms such as bacteria (Daly et al. 1997).

Granular glands can be either evenly distributed or concentrated in certain parts of the body (Duellman and Trueb 1986). The concentration of granular glands that form the large parotoid glands in toads is an example of such clustering. The term parotid gland (as in salivary gland) should not be confused with the chemical-producing parotoid glands of amphibians. Erroneously, these two terms are often used synonymously in the literature and other misspellings such as paratoid (referring to glands in salamanders) also occur (Cannon and Palkuti 1976; Tyler et al. 2001).

Clusters of granular glands can occur elsewhere (e.g., on the head, body, or limbs) and their names are based on location (e.g., rostral, labial,

coccygeal, femoral, tibial, mental) (Tyler 1987 as cited by Tyler et al. 2001). Granular glands can also be found in clusters in lateral warts and as enlarged middorsal granular glands that are recessed into modified epaxial musculature as in *Salamandra salamandra* (Brodie and Smatresk 1990). Typically, toad parotoid glands extend from the tympanum onto the shoulders or sides of the body (Wright and Wright 1967) (Figures 1a and 2). In *B. americanus*, the parotoid glands are separated from the cranial ridge behind the eyes or connected to the ridge by a short spur, while in *B. fowleri*, the parotoid glands touch the ridge behind the eyes (Conant 1975) (Figure 1b). As an antipredator defense, toads and other frogs orient their parotoid glands toward a potential threat and exhibit 'head-butting' behavior (Deullman and Trueb 1986; Green 1988; Smith et al. 2003). When the parotoid glands are stimulated, the chemical secretions discharge through multiple pores as a white or yellowish, "milky" substance (Toledo et al. 1992). Meyer and Linde (1971) reported that the approximate amount of dried venom obtained from *Bufo americanus* (between 54-110 mm in length) is 16 mg/animal and in *Bufo fowleri* (between 51-82 mm in length) is 14 mg/animal.



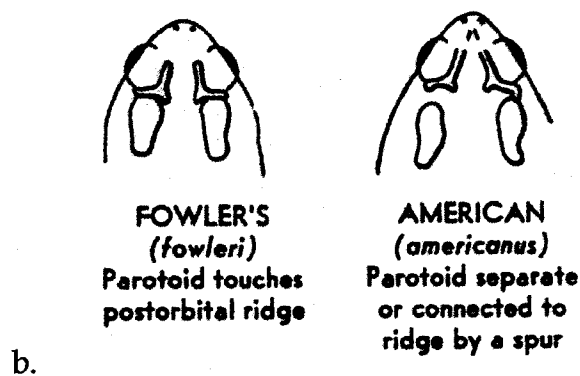
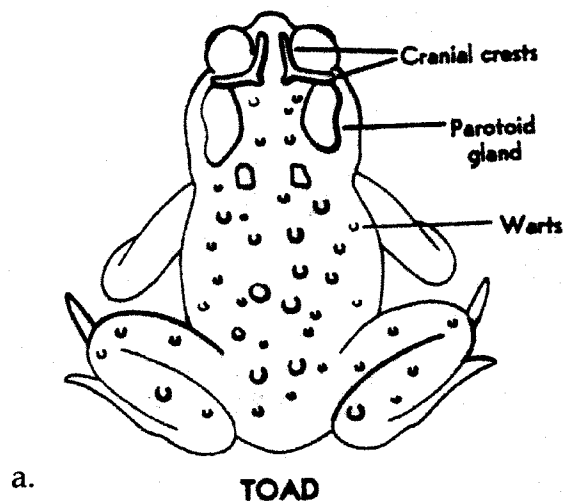


Figure 1. (a) Illustration of the typical location and shape of anuran parotoid glands and (b) morphological differences in placement of parotoid glands in *Bufo americanus* and *B. fowleri* (Conant 1975).

Source: Conant, R. 1975. A Field Guide to Reptiles and Amphibians. Houghton Mifflin Company. Boston, Massachusetts. Used with permission of Ronald Hussey, Permissions Manager/Serial Rights Manager, Houghton Mifflin Company, 9-22-04.

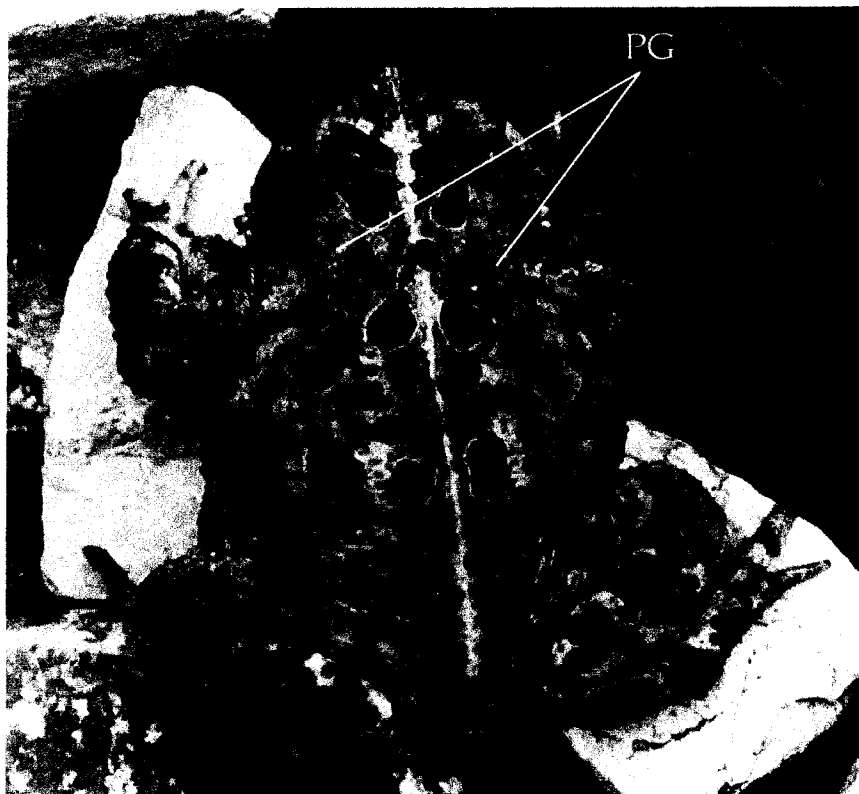


Figure 2. Photograph of *B. americanus* showing the enlarged kidney-shaped parotoid glands (PG). Photograph by G. Merovich.

*Steroids – bufadienolides and bufotoxins*

Bufadienolides (bufogenins or bufagins) and their conjugates with suberyl-arginine, suberyl-histidine, or suberyl-glutamine (bufotoxins) are steroidal compounds derived from cholesterol (Erspamer 1994). The first bufotoxin was isolated from the European common toad, *Bufo vulgaris* (*Bufo bufo*), by Wieland and Alles (1922). Bufadienolides are similar in biological activity to plant cardenolides (inhibitors of the membrane enzyme  $\text{Na}^+/\text{K}^+$

adenosine triphosphatase) and together these compounds are referred to as cardiac glycosides (Malcolm 1991, Steyn and van Heerden 1998) but none of the bufadienolides from toads conjugates with a carbohydrate to form a glycoside (Chen and Kovaříková 1967). Bufadienolides found in plants, however, usually do possess sugar moieties.

Many plant species in the families Liliaceae, Ranunculaceae and Crassulaceae contain bufadienolides (Kren et al. 2000; Kopp et al. 1996; Majinda et al. 1997). Bufadienolides are  $C_{24}$  steroids with a 6-membered lactone ring ( $\alpha$ -pyrone type) attached to the steroid nucleus (Klyne 1957; Meyer and Linde 1971; Clarke 1997) (Figure 3). The lactone ring of bufadienolides contains an ultraviolet chromophore with an absorption minimum at about 254 nm and maximum at 300 nm (Meyer and Linde 1971). Bufalin shown in Figure 3 is typical of many bufadienolides, however many differ substantially (e.g., the rotation of the lactone ring about the  $C_{17}$ - $C_{20}$  bond) (Argay and Kálmán 1992). Bufadienolides are "abundantly present in variable combinations in the skin glands, especially parotoid glands, of the bufonid genera *Atelopus*, *Dendrophryniscus*, and *Melanophryniscus* as well as in all of the more than 30 species of *Bufo* examined" (Erspamer 1994). Table 1 (adapted from Daly and Witkop 1971 and Meyer and Linde 1971) shows the occurrence of pharmacologically active substances in skin extracts from *B.*

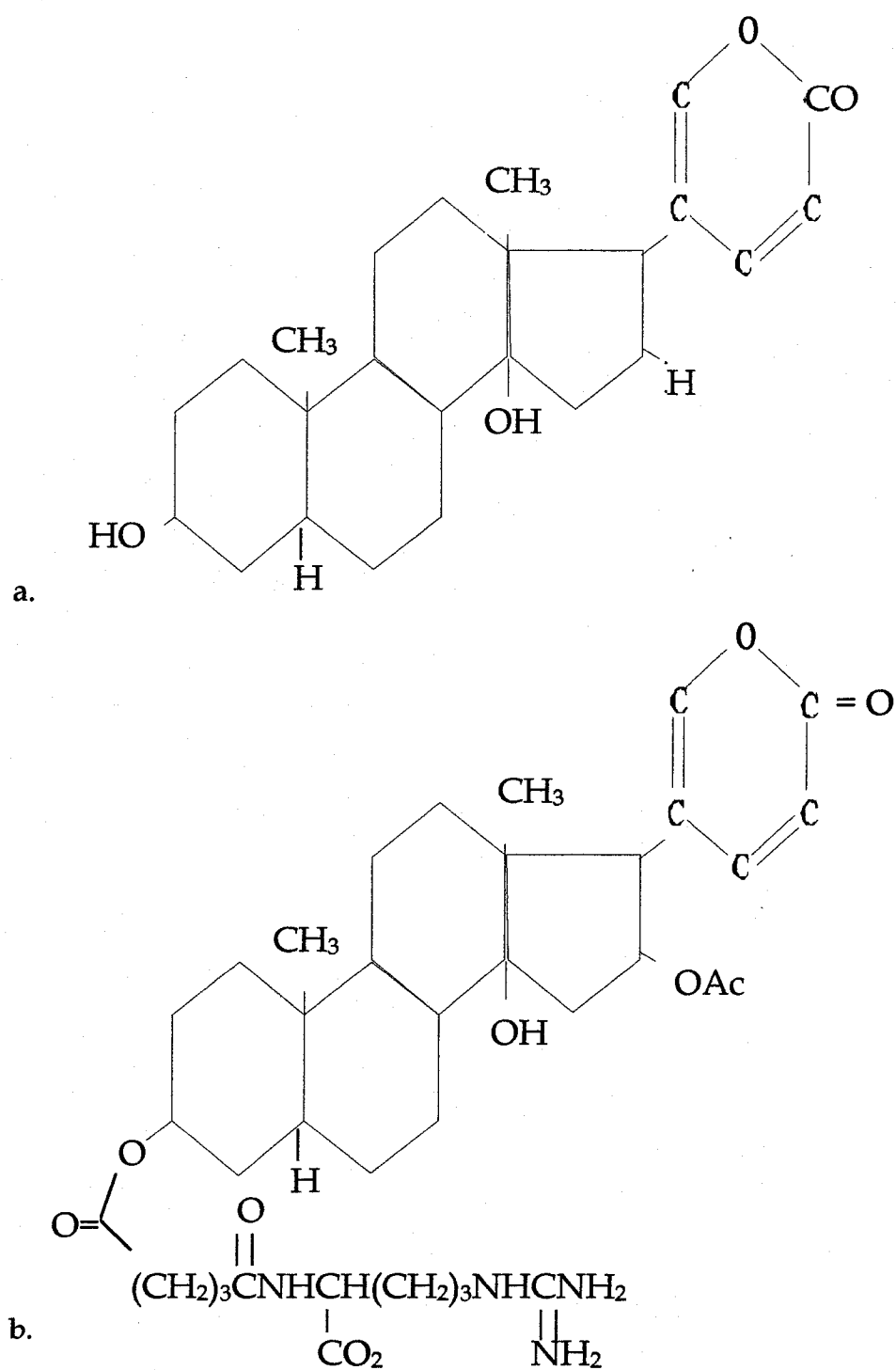


Figure 3. The chemical structures of bufadienolides (a) bufalin and bufotoxins (b) bufotoxin (bufotalin-3-O-suberylarginine).

Table 1

Pharmacologically active substances from skin extracts of *B. americanus* and *B. fowleri* (from Daly and Witkop 1971; Meyer and Linde 1971).

General Class	Compound Name	<i>B. fowleri</i>	<i>B. americanus</i>
Biogenic Amines	1. serotonin	+	+
	2. N-methyl serotonin	+	+
	3. bufotenine	+	+
	4. bufotenidine	+	+
	5. bufoviridine	□	□
	6. dehydrobufotenine	+	+
	7. bufothionine	□	□
	8. leptodactyline	0	0
	9. epinephrine	□	□
	10. histamine	0	0
Peptides/Proteins	11. active peptides	0	0
	12. hemolytic proteins	0	0
Alkaloids	13. steroidal alkaloids	0	0
Bufadienolides	14. arenobufagin	■	0
	15. bufalin	■	0
	16. gamabufotalin	■	0

+ = 1-100 µg/gm

□ = not present or less than 1 µg/gm

0 = no data available

■ = present but no amount reported

*americanus* and *B. fowleri*. Steyn and van Heerden (1998) reviewed compounds of bufadienolides present in species of *Bufo* overall but did not report which compounds were isolated from particular species. A novel bufotoxin was isolated from *B. americanus* by Shimada and Nambara (1980) and its chemical characteristics were given, however, the compound was unnamed. The skin of *Bufo viridis* was found to contain a bufadienolide derivative upon HPLC analysis (Lichtstein et al. 1986).

#### *Biological activity of bufadienolides*

Both the bufadienolides and the bufotoxins can increase the strength of the heartbeat and can decrease heart rate (Clarke 1997). The cardiotonic properties of cardiac glycosides are due to the inhibition of the membrane enzyme, sodium/potassium adenosine triphosphatase ( $\text{Na}^+/\text{K}^+$  ATPase or the sodium pump) (Daly 1995). Akizawa et al. (1994) were the first to show that bufadienolides in the eggs of *Bufo marinus* inhibited  $\text{Na}^+/\text{K}^+$  ATPase activity typical of cardiac steroids. Sodium/potassium ATPase is the only receptor for the cardiac glycosides (Steyn and van Heerden 1998) and a sensitive test for small amounts of bufadienolides is to assess an antagonism of radioactive ouabain binding to  $\text{Na}^+/\text{K}^+$  ATPase (Erspamer 1994). At low doses, bufadienolides can act as regulators of cardiac rhythm, however at

higher doses, bufadienolides can be highly toxic and are thought to provide a formidable chemical defense against natural enemies in toads (Daly 1995). Because bufadienolides have different chemical structures, their cardiac activities vary and they have different potencies (Chen and Kovaříková 1967). Several recent human deaths attributed to bufadienolides were reported by Barry et al. (1996), but toad chemicals have been used since Roman times for nefarious purposes. Brubacher et al. (1999) describe a method for treating poisoning by toad secretions. Interestingly, there is widespread occurrence of skin compounds that interact with the ouabain site of  $\text{Na}^+/\text{K}^+$  ATPase in frogs and toads and the highest levels of bufadienolides have been found in those species that migrate between dry and aquatic environments (Flier et al. 1980). This finding implicates bufadienolides as possible regulators of salt and water balance. It might also implicate a wider range of natural toad enemies and a greater need for more potent chemical defenses.

#### *Study ethics and toad conservation*

I chose to pay particular attention to the bufadienolides of toads because they are thought to act as both toxins as well as signals of their own toxicity, much like the related cardenolides in plants (Malcolm 1991). Further, the killing of large numbers of adult toads is minimized or made

completely unnecessary because the chemical secretions are readily obtained by 'milking' the parotoid glands. As Clarke (1997) wrote, "In my estimation, research on amphibian skin secretions should be compatible with their welfare and the use of their secretions as a sustainable resource. The aims of such research may be achieved by working on species with enlarged compact glands. There is no need to harm, let alone kill amphibians for this work." Throughout my research on the bufadienolides in toads, I have paid particular attention to the concerns for amphibian populations and purposefully considered ways to minimize the number of animals used in this project. I killed five adult toads of each species for the histology section of Chapter III and although early developmental stages (eggs, tadpoles, and metamorphs) were used in much larger numbers (Chapter II), overall their mortality during these stages is naturally very high.

### Study objectives

In my research, I have investigated several aspects of the biology and chemical ecology of toad bufadienolides. In Chapter II, I addressed (a) ontogenetic variation in bufadienolides, (b) whether the bufadienolide



composition of *B. americanus* and *B. fowleri* differs, and (c) whether bufadienolide biosynthesis is inducible in newly metamorphosed toads by predatory cues during development. In Chapter III, I addressed (a) whether the bufadienolide composition of *B. americanus* and *B. fowleri* differs, (b) whether there is individual and geographic variation in bufadienolides among adult toad populations, and (c) whether bufadienolide biosynthesis is inducible in adult toads by simulated encounters with predators. And in Chapters IV and V, I addressed whether bufadienolides are effective against terrestrial and aquatic toad predators respectively. Below I discuss the hypotheses of my research and describe in more detail the goals of each of these chapters.

#### *Ontogenetic variation in bufadienolides—Chapter II*

Early amphibian life history stages (eggs and tadpoles) and newly metamorphosed juveniles have been shown to have defensive characteristics attributed to toxic and noxious chemicals based on palatability studies (Brodie et al. 1978, Brodie and Formanowicz 1987, Brodie and Tumbarello 1978). Changes in chemical defenses may have important implications for an amphibian confronted with many different predators throughout their aquatic development and eventual growth to adulthood on dry land. As far

as I am aware, there have been no studies addressing chemical defenses in amphibians that consider the entire life history (egg to adult). Crossland (1998) investigated whether the toxicity of larval *Bufo marinus* to native aquatic invertebrate predators changes during ontogeny. He showed that the toxicity of *B. marinus* increases during development, but not all predators are affected by this increase.

In Chapter II, I describe how the chemical defenses (specifically the bufadienolides) of *Bufo americanus* and *Bufo fowleri* change during ontogeny by paying particular attention to investigating multiple stages of development spanning the entire life history of both species. Earlier palatability studies led to the prediction that there are certain times during development when an amphibian is likely to possess chemical defenses. Brodie and Formanowicz (1987) developed a model that illustrates the presumed presence of chemical defenses early in the life history of amphibians (beginning at time of hatching) and again during metamorphic climax just prior to transformation. The times between hatching and metamorphosis are believed to be devoid of chemical defenses based on palatability studies. I hypothesized that *B. americanus* and *B. fowleri* will have bufadienolides at the times that correlate with unpalatability by the Brodie and Formanowicz (1987) model. Also, I hypothesized that both toad species

would have variation in the amounts and types of bufadienolides that they possess. Specifically, I predicted that *B. fowleri* would possess fewer and less concentrated bufadienolides than *B. americanus* and that different populations of toads would have different bufadienolide profiles.

In addition to these hypotheses, I investigated whether raising toads with predatory cues would result in a change in the chemical phenotypes (of bufadienolides) produced by newly metamorphosed toads. It is well established that amphibians show considerable phenotypic plasticity in morphology, life history, and behavior. Predator-induced changes in behavior also occur (Laurila et al. 2002; Eklöv 2000). Different predatory pressures, in part, may explain potential differences in the composition of defensive toad chemicals.

### *Parotoid gland secretions of adult toads – Chapter III*

The geographic distributions of *B. americanus* and *B. fowleri* are sympatric over much of the eastern United States, but the range of *B. fowleri* is less extensive than that of *B. americanus* in Michigan (Conant 1975; Conant and Collins 1998; Harding 1997) (Figures 4a and 4b). As a result of these distributional differences, *B. fowleri* may not encounter the same diversity of predatory species as does *B. americanus*. Thus, differences in the chemical

phenotypes of adult toads could also be attributed to natural selective differences in the amount and type of predation that they experience.

To investigate the potential induction of bufadienolides in adult toads subjected to simulated encounters with predators, toads were maintained in the laboratory and parotoid secretions were obtained once a month for three months. Chen and Kovariková (1967) reported that "The process of regeneration of the venom is slow. If the pair of parotoid glands (*B. marinus*) are manually expressed, it takes about 11 weeks for the toad to restore two-thirds of the original amount. It would be impossible for the animal to furnish large quantities of the poison repeatedly for defensive purposes." However, large quantities may not be necessary to provide a toad with adequate defense since bufadienolides can be highly toxic at low doses (Clarke 1997). In doing this experiment with *B. americanus* and *B. fowleri*, I was interested to know if non-lethal predator encounters (repeated expressions of parotoid glands) would cause an induction of bufadienolide biosynthesis and the production of different and/or more bufadienolides without regard to whether this would in fact be an effective response.

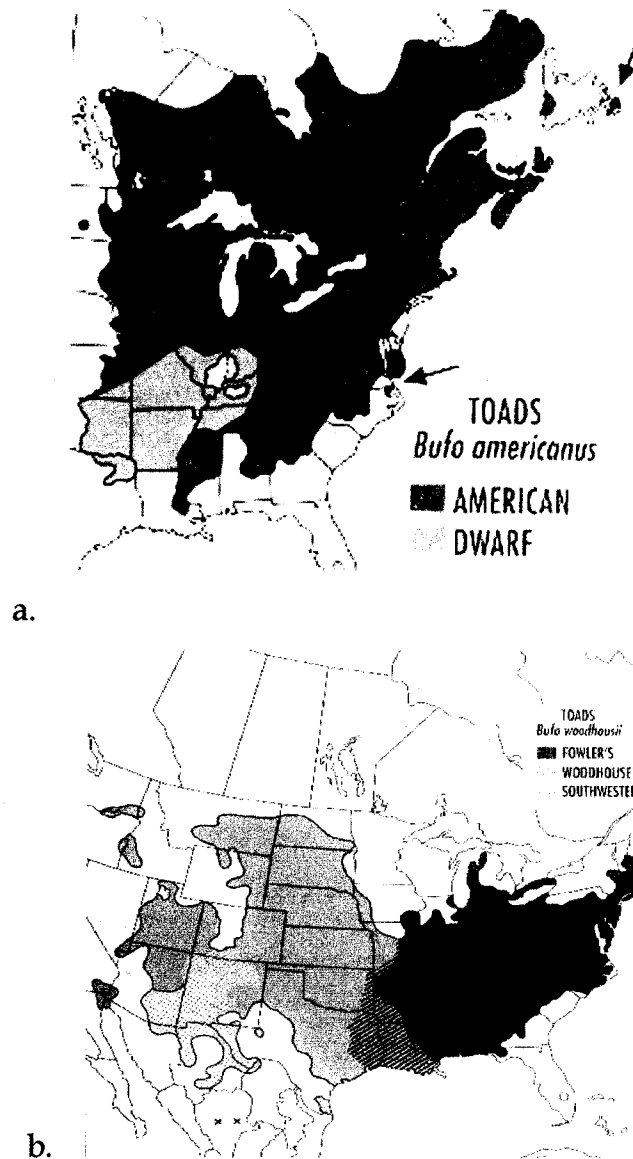


Figure 4. The geographic ranges of (a) *Bufo americanus* and (b) *Bufo fowleri* (Conant and Collins 1998).

Source: Conant, R. and J.T. Collins. 1998. A Field Guide to Reptiles and Amphibians of Eastern and Central North America. Third Edition, Expanded. Houghton Mifflin Company. Boston, Massachusetts. Used with permission of Ronald Hussey, Permissions Manager/Serial Rights Manager, Houghton Mifflin Company, 9-22-04.

*Responses of a vertebrate predator to toad secretions – Chapter IV*

*Thamnophis sirtalis*, the Eastern garter snake, is considered to have a very broad diet (Seigel 1996). This snake species relies heavily upon chemoreception to recognize and find prey. Garter snakes are known to prey heavily upon earthworms (Harding 1997), to prefer fish and small amphibians (Wright and Wright 1967), to be major toad predators (Lagler and Salyer 1945, Licht and Low 1968), and especially to prefer newly metamorphosed frogs and toads (Harding 1997). In Michigan, toads have been found to make up 25% of the diet of garter snakes living near natural waters (Lagler and Salyer 1945). Despite the fact that toads possess a variety of compounds in their cutaneous poison glands, garter snakes can consume toads with no apparent ill effects. However, some studies have shown that garter snakes exhibit selective responsiveness to chemical stimuli of preferred prey and that geographic variation in responsiveness is correlated with prey preference (Burghardt 1969, 1970; Arnold 1977, 1981).

I tested the responses of *T. sirtalis* to chemical stimuli from *B. americanus* and *B. fowleri* with tongue-flick bioassays to investigate potential differences in prey preference and to determine if toad parotoid secretions would deter predation. I hypothesized that snakes would potentially recognize one toad species over the other as prey and that this difference

could be attributed to distributional differences between *B. americanus* and *B. fowleri* populations and/or to differences in the bufadienolides between toad species. I also hypothesized that toad parotoid secretions would deter predation.

*Responses of an invertebrate predator to tadpoles – Chapter V*

Aquatic insects such as dragonfly larvae (*Anax* and *Pantala*), predaceous diving beetle larvae (*Acilius* and *Dytiscus*), and giant water bugs (*Belostoma*) are among the most important predators of amphibian larvae (Brockelman 1969; Heyer et al. 1975). Larval Dytiscid beetles, in particular, are important predators of *Bufo americanus* tadpoles (Brokelman 1969; Brodie et al. 1978). Chapter V describes an experimental assay to test the responses of predaceous diving beetle larvae (*Acilius* sp. and *Dytiscus* sp.) to the larvae of American toad (*Bufo americanus*) and Fowler's toad (*Bufo fowleri*). My interest was to determine if these aquatic insect larvae would respond differently (by attack, consumption, and survival) when exposed to tadpoles in intermediate stages of development and in metamorphic climax. Based on the Brodie and Formanowicz (1987) model of stage-specific survival of anuran larvae, chemical defenses are predicted only during early and late developmental stages and not during intermediate stages when larvae are

palatable. Thus, my goal was to relate the presence and/or absence of bufadienolides to this variability in palatability during toad development. My other interest was to determine if Dytiscid larvae would discriminate between *B. americanus* and *B. fowleri* as prey. Again, a difference in prey preference could be attributed to variation in chemical defenses between these toad species.



## CHAPTER II

### ONTOGENETIC VARIATION IN THE BUFADIENOLIDES OF *BUFO AMERICANUS* AND *BUFO FOWLERI*

#### Introduction

The ability to defend against predation is especially important for amphibians during their early life history stages (eggs and larvae) when predation risks are large and come from a wide range of invertebrate and vertebrate predators. Predation pressures can affect various behavioral characteristics involving habitat preferences, use of refugia, reductions in mobility, aggregation, and schooling behavior (Caldwell 1989; DeVito 2003; Eklöv 2000; Heyer et. al 1975; Kiesecker et. al 1996; Laurila et. al 1997; Relyea and Yurewicz 2002; Semlitsch and Reyer 1992) and can affect numerous morphological and physiological traits involving alterations in body size and coloration, timing of hatching, onset of metamorphosis, size at metamorphosis, and metamorphic synchrony (Arnold and Wassersug 1978; Laurila et. al 2002; Moore et. al 2004; McCollum and Leimberger 1997; Van Bushkirk 2002; Van Bushkirk and Saxer 2001; Werner 1986; Wilbur et. al 1983). Indeed, considerable attention has been given to understanding

predator-induced phenotypic plasticity in amphibians, however, only one study (Benard and Fordyce 2003) has examined the plasticity of chemical phenotypes in amphibians and the influence of predation during development on the production of defensive chemicals. Furthermore, a quantitative understanding of how chemical defenses vary throughout the amphibian life cycle is largely lacking. More attention to these aspects of larval development and the production of defensive compounds is especially important given that vulnerability to predation is influenced by chemicals that render individuals distasteful or toxic to their predators. The main categories of amphibian compounds used in chemical defense include water-soluble and lipid-soluble alkaloids, alkaline biogenic amines, steroidal bufadienolides and bufotoxins, as well as peptides and proteins (Daly 1995). I examined one group of amphibian chemicals (the steroidal bufadienolides) in individuals of *Bufo americanus* and *Bufo fowleri* from southwest Michigan to determine (a) if bufadienolide composition varies during toad development, (b) if bufadienolide composition is different between toad species, and (c) if predatory pressure during larval development induces bufadienolide production following metamorphosis.

Transformed amphibians (juveniles and adults) are well known for possessing a wide variety of chemicals in their skin secretions (Clarke 1997;

Daly 1995; Erspamer 1994) and it is during these largely terrestrial life history stages that a wealth of information exists on the diversity of cutaneous chemical secretions present in amphibian species. In comparison, less is known about the chemical defenses of pre-metamorphic amphibians (eggs and larvae) and of larval amphibians in the midst of metamorphic change (during metamorphic climax prior to transformation). Akizawa et al. (1994) reported the structures of nine bufadienolides isolated from the eggs of *Bufo marinus*. Bufadienolides have also been found in *B. marinus* tadpoles and juveniles (Flier et al. 1980). Other studies have addressed the development of granular glands during periods of larval growth and onset of metamorphosis (Delfino et al. 1995; Hayes and Gill 1995). Again, to my knowledge, no study has quantified the chemicals present spanning the entire life history of any amphibian species.

To understand predation on larval amphibians, many studies have assessed the palatability of tadpoles to various predators and have considered tadpole palatability to predators during different developmental stages (Brodie et. al 1978; Brodie and Formanowicz 1987; Cooke 1974; Crossland 1998; Formanowicz and Brodie 1982; Kats et al. 1988; Kruse and Stone 1984; Peterson and Blaustein 1991; Voris and Bacon 1966; Wassersug 1971). It has been suggested that the unpalatability of developmental stages

is correlated with the development of granular glands in the skin and hence the presumed presence of defensive chemicals (Brodie and Tumbarello 1978; Brodie et al. 1978; Brodie and Formanowicz 1987). Palatability of tadpoles to predators has been shown to change with ontogeny in some studies (Brodie et al. 1978; Formanowicz and Brodie 1982; Brodie and Formanowicz 1987; Denton and Beebee 1997; Crossland 1998; Crossland and Alford 1998) but Peterson and Blaustein (1992) did not find any developmental changes in palatability of *Bufo boreas*.

Brodie and Formanowicz (1987) described a graphical model that, in part, illustrates the change in tadpole palatability during ontogeny. This model shows that tadpoles garner protection from predation due to chemical defenses early in development and during metamorphic climax as depicted by lines B<sub>1</sub> and B<sub>2</sub> in Figure 5. Specifically, during the very early developmental stages (hatchlings, Gosner stages 20-25), individuals are unpalatable to predators perhaps as a result of chemical defenses present in the yolk of eggs. Protection during these early stages is followed by a drop in stage-specific survival when intermediate-stage tadpoles [within the range of Gosner stages 26-41; but the explicit stages of palatability examined by Brodie and Formanowicz (1987) were between Gosner stages 29-33] lose their chemical defenses and become palatable to predators. Then unpalatability

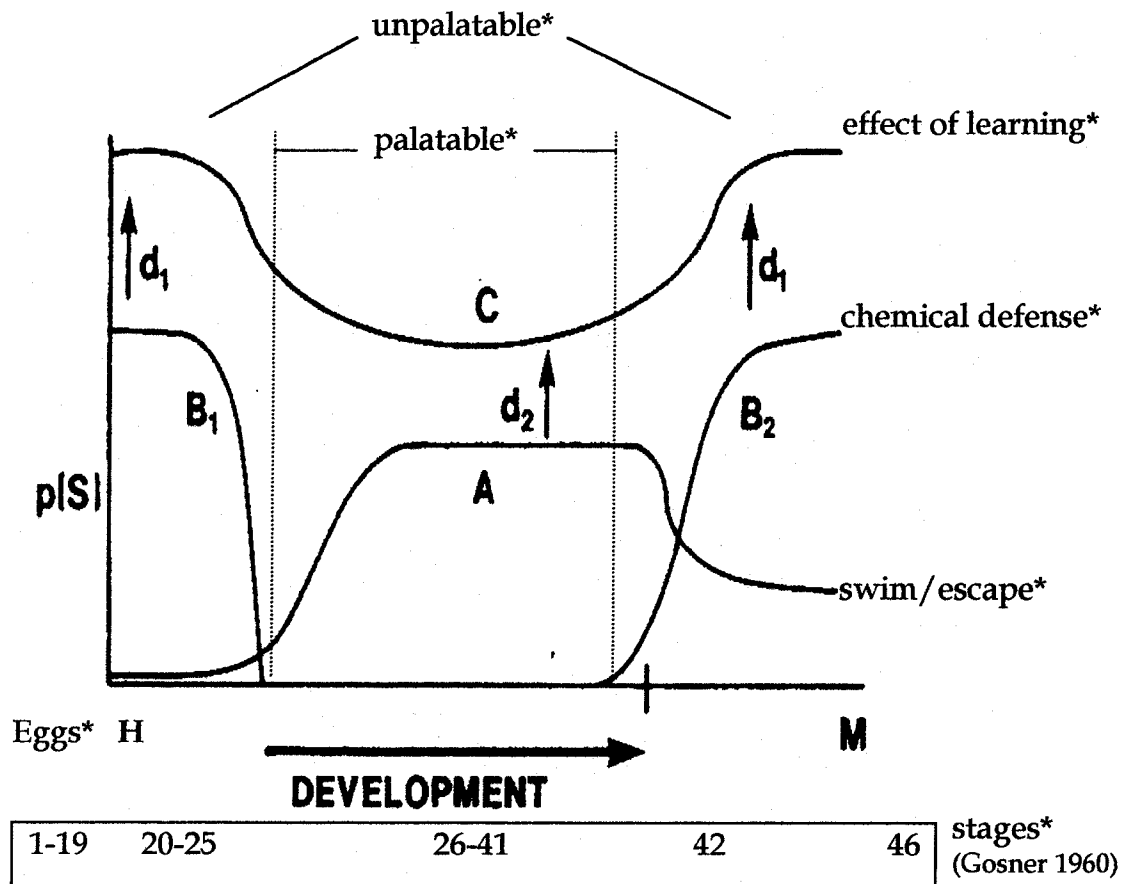


Figure 5. A model of palatability and ability to escape as they affect stage-specific survivorship of anuran larvae.  $p[S]$  = stage-specific probability of survival; H = hatching; M = metamorphosis; A = survival based on ability to escape;  $B_1$  and  $B_2$  = probability of survival based on chemical defenses in newly hatched and metamorphosing larvae, respectively; C = probability of survival based on the ability to escape augmented by predator avoidance after contact with unpalatable stages;  $d_1$  = survival benefit accrued to unpalatable stages;  $d_2$  = survival benefit accrued to palatable stages. The vertical line on the abscissa indicates the onset of metamorphic climax (Brodie and Formanowicz 1987). I have made some modifications as noted.\*

Source: Brodie, E. D. Jr. and D. R. Formanowicz. 1987. Anti-predator mechanisms of larval anurans: protection of palatable individuals. *Herpetologica* 43: 369-373. Used with permission of Edmund D. Brodie, Jr. 23 July 2004.

and survivorship increase again as the tadpoles approach metamorphosis (beginning with the emergence of forelimbs, Gosner stage 42) and reach a peak when metamorphosis is complete (Gosner stage 46) (Figure 5). The prediction of this stage-specific palatability model is that amphibian larvae should possess chemical defenses during their development when they are not palatable to predators. In one bufonid species, *Bufo calamita*, early developmental stages (just after hatching) suffer high mortality due to invertebrate predation and then the relative strength of predation falls as tadpoles become larger (Denton and Beebee 1997). Lawler and Hero (1997) showed an ontogenetic shift in palatability of *Bufo marinus* tadpoles to barramundi fish predators where later-staged tadpoles were less palatable. Thus, there are exceptions to this generalized survivorship model. My objective was to assess whether changes in bufadienolide concentrations in *B. americanus* and *B. fowleri* would agree with predictions from the Brodie and Formanowicz (1987) model. Not included in this model is the relationship between toad body size and risk of predation. Werner (1986) developed a model that shows that mortality risk from predators declines with increasing body size. Thus, a larger tadpole may have enhanced survival due to size rather than to chemical defense.

## Methods

### *Collection and maintenance of Bufo*

#### Eggs and tadpoles

*Bufo americanus* and *B. fowleri* eggs were collected from several locations in southwest Michigan after the first sign of reproduction in the springs of 1999-2003 and raised in the laboratory. *Bufo fowleri* eggs were found only in the spring of 2003. To maintain the developing toads, approximately 100 eggs of each species from the collection sites (different clutches of eggs were reared separately) were placed into separate 40 L glass aquaria containing store-bought aquarium gravel as the substrate and aged tap water aerated with an activated carbon filter. The aquaria were arranged on a laboratory bench top at room temperature and laboratory windows were uncovered allowing tadpoles to develop with both natural light and photoperiods. Aged tap water was used to replace water lost through evaporation and the tanks were periodically cleaned to remove extensive algal growth. Tadpoles were fed Frog Brittle (Nasco Scientific) *ad libitum* supplemented with goldfish flakes. At least five individuals from each developmental stage (shown in Table 2) were frozen and kept at -80 °C until chemical analyses were conducted. Developmental stages were divided into

the Gosner (1960) stage ranges as shown in Table 2. Tadpole stages were separated into three groups designated TadsA, TadsB, and TadsC (Table 2). Tadpoles not used for analysis were released at the site of egg collection. Additional field-collected tadpoles were also included in the analyses.

### Metamorphs and Transformed Toads

Laboratory raised toads were removed from their natal tanks upon the onset of metamorphosis (beginning with the eruption of forelimbs at Gosner stage 42) and placed into separate 40 L aquaria containing store bought aquarium gravel shaped into a depression. A small amount of water was added to the gravel depressions to permit successful metamorphosis and to keep transformed individuals moist. Metamorphs from different clutches were placed into separate 'metamorphic' aquaria. When metamorphosis was complete (ending with complete absorption of the tail at Gosner stage 46), flightless fruit flies were added and constantly available to toadlets.

### *The influence of predation on bufadienolide content of developing toads*

For this experiment, six 40 L aquaria per toad species (three controls without predators and three treatments with predators) for a total of 12 aquaria were divided by semi-transparent, porous dividers such that one end



Table 2

Bufadienolide analyses of *B. americanus* and *B. fowleri* in each of the following developmental stage ranges (after Gosner 1960).

Gosner Stages			
Stages	Early	Mid	Late
Eggs	1-19		
Hatchlings	20-25		
Tadpoles			
A		26-30	
B		31-35	
C		36-41	
Metamorphs			42-45
Transformed			46

of the aquaria comprised 1/3 and the other end comprised 2/3 of the total area (Figure 6). Aquaria were arranged randomly on a laboratory bench top, filled with store-bought aquarium gravel and aged tap water, and exposed to natural photoperiods from uncovered lab room windows. Approximately 300 *B. americanus* and 300 *B. fowleri* eggs were collected in the spring of 2003 for this experiment. *Bufo americanus* eggs were collected from a ditch near Goldsworth Valley Pond on the campus of Western Michigan University (Kalamazoo County) and *Bufo fowleri* eggs were collected from a constructed

pond near Covert Michigan (Van Buren County). Approximately 50 eggs were placed into each of the larger tank compartments. Six predators (larval *Ambystoma*) were also collected from a woodland pond (Steve's Pond) in Kalamazoo County and randomly assigned (one per tank) to each of the smaller tank compartments in the predator treatments. Each predator was offered 1-2 tadpole prey per day and all predators consumed tadpoles. Thus, developing tadpoles were exposed to multiple predatory stimuli (i.e., visual and chemical) as well as to cues from active consumption of conspecifics and to their alarm substances.

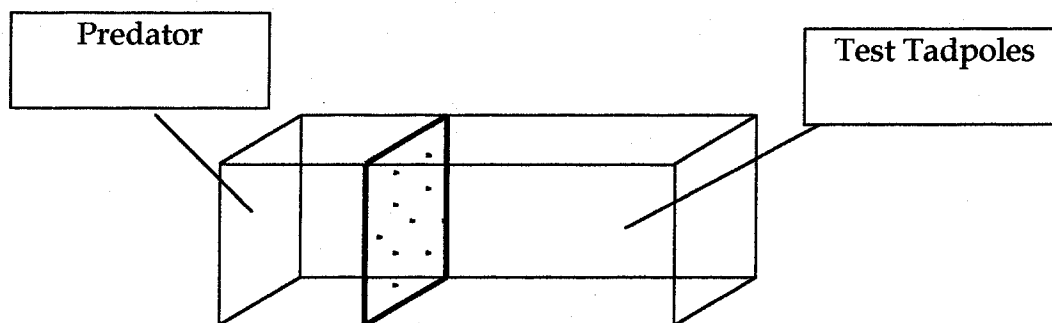


Figure 6. Schematic of rearing tanks for tadpoles exposed to predator cues during development.

Individual toads were randomly sampled from control and treatment tanks during metamorphic climax (between stage 42 and 45) and placed into 'metamorphic' aquaria as before. Once metamorphosis was complete, toads were frozen and prepared for HPLC as before.

*Sample preparation and bufadienolide extraction*

Toads (eggs, larvae, metamorphs, and transformed individuals) were weighed (to the nearest 0.001 g) and then lyophilized. When applicable, total lengths (TL) of individuals were recorded to the nearest (0.01 cm) with Vernier calipers. Once dried, samples were weighed again (to the nearest 0.001 g) and crushed with a glass rod in a test tube. Each sample received five milliliters of methanol and was sonicated for 30 minutes. Following sonication, the samples were centrifuged at high speed for 5 minutes. The supernatant from each sample was removed and placed into a new test tube and dried under nitrogen in a water bath at 60 °C. The remaining residue from each tube was re-suspended in 1 ml of methanol and passed through a 0.45- $\mu$ m filter into a 1 ml autosampler vial for HPLC. Fifty  $\mu$ g samples of a known bufadienolide standard, bufalin (obtained from Sigma Scientific), were run in separate vials as an external standard.

*HPLC of toad bufadienolides*

Chemical analyses of toad secretion samples by HPLC were performed on a Waters gradient HPLC system with WISP autosampler, 600E pump, 996 photodiode array detector (at 298 nm) ranging between 270-330 nm using Millennium 2010™ chromatography manager software. Injection volumes for individual samples were 30 µl. Samples were eluted isocratically with a mixture of water and acetonitrile (60:40) at 1.25 ml/min on a 250-4 LiChroCART RP-18 column packed with 5 µl LiChrospher 100 (E. Merck) and a guard column packed with the same material, following the methods of Gella et al. (1995) and Benard and Fordyce (2003). Run durations were 20 minutes per sample.

I identified bufadienolides by comparing the absorption spectra of unknown peaks to that of bufalin. I quantified the bufadienolide content of each sample by using a calibration curve obtained from running a bufalin dilution series (between 10 ng and 100 µg). Peaks identified as bufadienolides were constrained within the purity values (purity angle and purity threshold) quantified by the lowest concentration of bufalin. If the purity angle and purity threshold of an unknown peak (with a characteristic absorption spectra) was equal to or less than 9.017 and 12.750 respectively, then the peak was considered a bufadienolide. If the absorption spectra of an unknown

peak fit the characteristics of a bufadienolide but did not fall within these limits of purity, it was not considered a bufadienolide.

#### *Statistical analyses*

The data from all experiments were not normally distributed and could not be transformed, therefore I used Mann Whitney U tests to determine whether the mean total numbers of bufadienolides and the mean total concentration of bufadienolides was different in *B. americanus* and *B. fowleri*. A Mann Whitney U test was also conducted on the mean total concentration of bufadienolides in toads raised with and without predatory cues. To determine if mean total bufadienolide concentrations differed among developmental stages in *B. americanus* and *B. fowleri*, I conducted Kruskal Wallis tests. In the case of significant tests, the nonparametric Q test (Zar 1999) was performed for unequal sample sizes to determine where the differences existed. Analyses were conducted with JMP statistical software version 5.0.1 and Statistica software version 4.5.

## Results

### *Ontogenetic variation in bufadienolides*

A total of 222 individuals of both toad species, *Bufo americanus* and *Bufo fowleri*, in seven developmental stage ranges (Table 2) were analyzed for their bufadienolide composition (Tables 3 and 4). Across all stages and species, ten bufadienolides were identified with average retention times (RTs) between 2.69 and 17.68 minutes (Tables 5, 6, 7 and 8). Nine bufadienolides were found in *B. americanus* and five were found in *B. fowleri*, only one of which was not found in *B. americanus* (ave RT = 10.34). The vast majority (83.3%) of individuals (both species combined) possessed no detectable bufadienolides. All developmental stages possessed some individuals with no bufadienolides. Hatchlings and tadpoles (TadsA, TadsB, and TadsC) collectively accounted for the majority of these bufadienolide-devoid individuals in *B. americanus* and *B. fowleri* (21.1%, 16.2%, 26.0%, and 16.8%) but there was variation between species (Tables 7 and 8). Of all sampled toads, 16.7% of individuals possessed detectable bufadienolides and were from six of the seven developmental stages. Individuals with bufadienolides were most frequently from the egg, transformed, and hatchling stages (37.8%, 21.6%, and 18.9%) while no bufadienolides were detected in TadsC tadpoles and only one TadsB individual possessed bufadienolides. One bufadienolide

(ave RT = 3.4) was found more frequently in individuals of both species of toads. This bufadienolide was present in 7% and 13% of *B. americanus* and *B. fowleri* respectively (Table 5). The average concentration of this bufadienolide in *B. americanus* and *B. fowleri* was between 0.4 – 1.0 ng bufadienolide/ $\mu$ g dry secretion sample respectively.

Results indicated that the mean number of bufadienolides among developmental stages differed significantly in both *B. americanus* and *B. fowleri* ( $H = 26.8$ ,  $P = 0.0002$  and  $H = 41.5$ ,  $P < 0.0001$  respectively) (Figures 7 and 8). In *B. americanus*, differences among developmental stages were between transformed individuals and tadpoles (TadsB,  $Q = 3.4$ ,  $P < 0.01$  and Tads C,  $Q = 3.29$ ,  $P < 0.02$ ) (Figure 7). Developmental stages that were different from each other in *B. fowleri* were between eggs and all other stages except transformed individuals (hatchlings,  $Q = 4.71$ ,  $P < 0.001$ ; TadsA,  $Q = 4.9$ ,  $P < 0.001$ ; TadsB,  $Q = 5.0$ ,  $P < 0.001$ ; TadsC,  $Q = 5.1$ ,  $P < 0.001$ ; and metamorphs,  $Q = 3.63$ ,  $P < 0.005$ ) (Figure 8).

The average total concentrations of bufadienolides in *B. americanus* and *B. fowleri* were 1.19 and 1.44 ng bufadienolide/ $\mu$ g dry weight respectively and not statistically different ( $U = 5971$ ;  $P = 0.85$ ) (Figure 9). For both species

Table 3

Mean mass and total length (TL) of all stages of *Bufo americanus* individuals analyzed for their bufadienolide composition.

Toad	Stage	Mean +/- SE	Mean +/- SE	Mean +/- SE
		Wet Mass (g)	Dry Mass (g)	TL (cm)
<i>B. americanus</i> (n = 125)	Eggs (n = 15)	0.093 ± 0.029	0.001 ± 0.0002	NA
	Hatchlings (n = 32)	0.035 ± 0.007	0.001 ± 0.00015	0.62 ± 0.02
	Tadpoles			
	A (n = 13)	0.029 ± 0.007	0.001 ± 0.0002	0.87 ± 0.08
	B (n = 32)	0.106 ± .008	0.015 ± 0.003	1.89 ± 0.05
	C (n = 18)	0.214 ± 0.013	0.033 ± 0.004	2.19 ± 0.04
	Metamorphs (n = 7)	0.255 ± 0.014	0.058 ± 0.009	1.83 ± 0.17
	Transformed (n = 8)	0.089 ± 0.011	0.022 ± 0.004	0.94 ± 0.03

combined, the mean total bufadienolide concentrations among developmental stages were statistically significant ( $H = 49.8$ ;  $P < 0.0001$ ). Non-parametric post-hoc tests revealed that differences existed between eggs and four other stages (TadsA,  $Q = 4.8$ ,  $P < 0.001$ ; TadsB,  $Q = 6.2$ ,  $P < 0.001$ ; TadsC,  $Q = 5.9$ ,  $P < 0.001$  and hatchlings,  $Q = 4.6$ ,  $P < 0.001$ ) (Figure 10).

In *B. americanus*, mean total bufadienolide concentrations were not the same among toad developmental stages ( $H = 26.8$ ;  $P = 0.0002$ ) (Figure 11). This was also true for *B. fowleri* ( $H = 41.5$ ;  $P < 0.0001$ ) (Figure 11).



Table 4

Mean mass and total length (TL) of all stages of *Bufo fowleri* individuals analyzed for their bufadienolide composition.

Toad	Stage	Mean +/- SE	Mean +/- SE	Mean +/- SE
		Wet Mass (g)	Dry Mass (g)	TL (cm)
<i>B. fowleri</i> (n = 97)	Eggs (n = 10)	0.036 ± 0.007	0.01 ± 0.0004	NA
	Hatchlings (n = 14)	0.0200 ± 0.004	0.001 ± 0.00002	0.55 ± 0.03
	Tadpoles			
	A (n = 21)	0.040 ± 0.006	0.01 ± 0.0004	1.17 ± 0.06
	B (n = 17)	0.158 ± 0.020	0.012 ± 0.002	1.87 ± 0.08
	C (n = 13)	0.219 ± 0.018	0.018 ± 0.002	2.23 ± 0.06
	Metamorphs (n = 4)	0.189 ± 0.041	0.021 ± 0.005	1.59 ± 0.21
	Transformed (n = 18)	0.128 ± 0.007	0.017 ± 0.001	1.00 ± 0.03

Nonparametric post-hoc tests revealed that in *B. americanus*, mean total bufadienolide concentrations were different between eggs and tadpoles (TadsA) ( $Q = 7.13$ ,  $P < 0.001$ ), (TadsB) ( $Q = 6.11$ ,  $P < 0.001$ ), (TadsC) ( $Q = 7.13$ ,  $P < 0.001$ ), eggs and metamorphs ( $Q = 6.45$ ,  $P < 0.001$ ) and between tadpoles (TadsB) and transformed individuals ( $Q = 3.68$ ,  $P < 0.01$ ) (Figure 11). In *B. fowleri*, post-hoc tests revealed similar differences among developmental stages. *Bufo fowleri* eggs were different from hatchlings ( $Q = 3.72$ ,  $P < 0.005$ ), tadpoles (TadsA) ( $Q = 3.90$ ,  $P < 0.005$ ), (TadsB) ( $Q = 3.97$ ,  $P < 0.002$ ), and

Table 5

Number of individuals with each of the ten identified bufadienolides across all developmental stages. Percentages are in parentheses.

Species	Bufadienolide Average Retention Time (min)									
	2.69	3.40	3.89	4.29	5.27	6.86	8.41	10.34	12.70	17.68
<i>Bufo americanus</i> (n = 125)	1 (0.79)	9 (7.09)	4 (3.15)	3 (2.36)	2 (1.57)	1 (0.79)	3 (2.36)	0 (0.00)	7 (5.51)	1 (0.79)
<i>Bufo fowleri</i> (n = 97)	1 (1.03)	13 (13.40)	1 (1.03)	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)	1 (1.03)	1 (1.03)	0 (0.00)

Table 6

Mean number of bufadienolides identified in *B. americanus* and *B. fowleri* across all developmental stages.

	N	Mean $\pm$ SE	Min	Max
<i>B. americanus</i> and <i>B. fowleri</i>	222	0.20 $\pm$ 0.04	0	5
<i>B. americanus</i>	125	0.22 $\pm$ 0.06	0	5
<i>B. fowleri</i>	97	0.18 $\pm$ 0.04	0	2

(TadsC) ( $Q = 4.05$ ,  $P < 0.002$ ), and transformed individuals ( $Q = 3.59$ ,  $P < 0.01$ ) (Figure 12). Comparisons between species revealed that *B. fowleri* eggs had higher concentrations of bufadienolides than did *B. americanus* eggs (Figure 13).

#### *Bufadienolide Induction*

The mean total concentration of bufadienolides in *B. americanus* and *B. fowleri* raised from eggs with and without predatory cues were not statistically different (*B. americanus*:  $U = 77.5$ ;  $P = 0.44$  and *B. fowleri*:  $U = 19.0$ ;  $P = 0.52$ ) (Figure 14).

Table 7

Number of *Bufo americanus* individuals with each of the ten identified bufadienolides by developmental stage. Percentages are in parentheses.

Stage	Bufadienolide Average Retention Time (min)									
	2.69	3.40	3.89	4.29	5.27	6.86	8.41	10.34	12.70	17.68
Eggs (n = 15)	0 (0.00)	2 (12.50)	3 (18.75)	1 (6.25)	0 (0.00)	0 (0.00)	1 (6.25)	0 (0.00)	2 (12.50)	1 (6.25)
Hatchlings (n = 32)	0 (0.00)	3 (9.09)	0 (0.00)	0 (0.00)	1 (3.03)	0 (0.00)	0 (0.00)	0 (0.00)	3 (9.09)	0 (0.00)
Tadpoles										
A (n = 13)	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)	2 (15.38)	0 (0.00)
B (n = 32)	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)
C (n = 18)	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)
Metamorphs (n = 7)	0 (0.00)	2 (28.57)	0 (0.00)	0 (0.00)	1 (14.29)	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)

Table 8

Number of *Bufo fowleri* individuals with each of the ten identified bufadienolides by developmental stage. Percentages are in parentheses.

Stage	Bufadienolide Average Retention Time (min)									
	2.69	3.40	3.89	4.29	5.27	6.86	8.41	10.34	12.70	17.68
Eggs (n = 10)	0 (0.00)	8 (80.00)	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)
Hatchlings (n = 14)	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)	1 (7.14)	0 (0.00)
Tadpoles										
A (n = 21)	0 (0.00)	2 (9.52)	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)	1 (4.76)	0 (0.00)	0 (0.00)
B (n = 17)	0 (0.00)	1 (5.88)	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)
C (n = 13)	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)
Metamorphs (n = 4)	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)
Transformed (n = 18)	1 (5.56)	2 (11.11)	1 (5.56)	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)

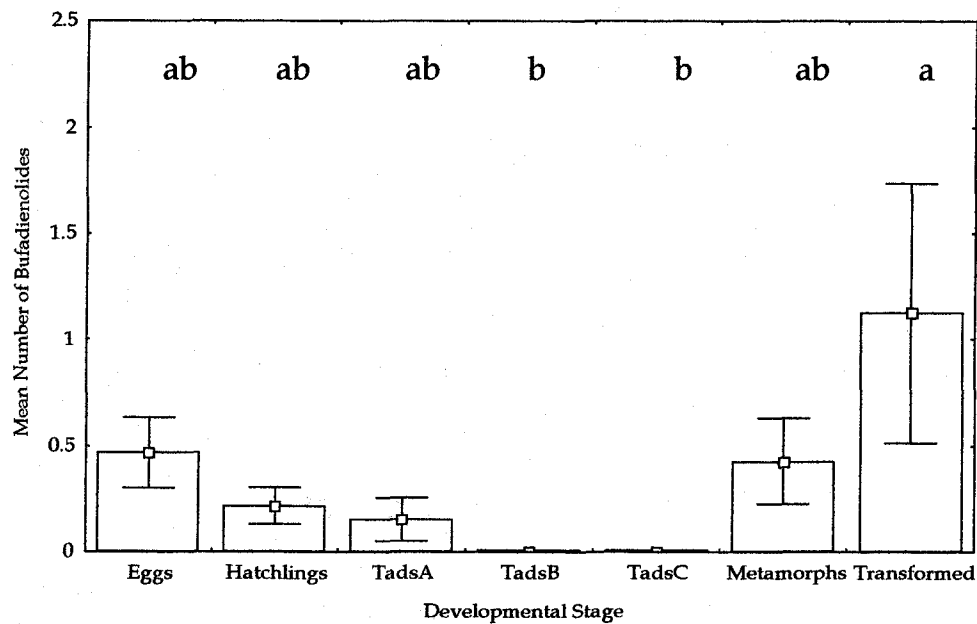


Figure 7. Mean number ( $\pm$  SE) of bufadienolides across all developmental stages in *B. americanus*. Different letters denote significant differences.

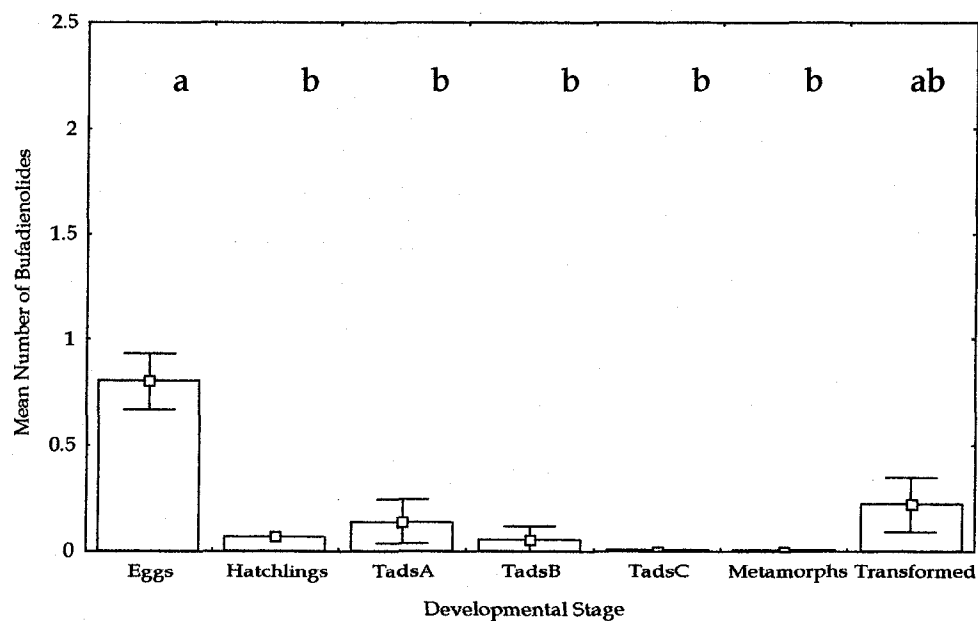


Figure 8. Mean number ( $\pm$  SE) of bufadienolides across all developmental stages in *B. fowleri*. Different letters denote significant differences.

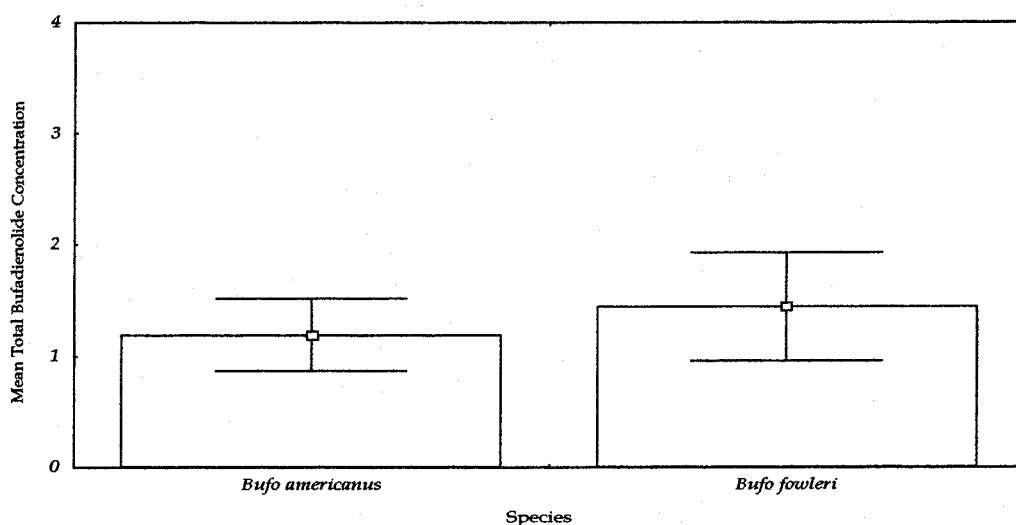


Figure 9. Mean ( $\pm$  SE) total bufadienolide concentration across all developmental stages in *B. americanus* and *B. fowleri*. Concentrations are in ng/ $\mu$ g dry weight.

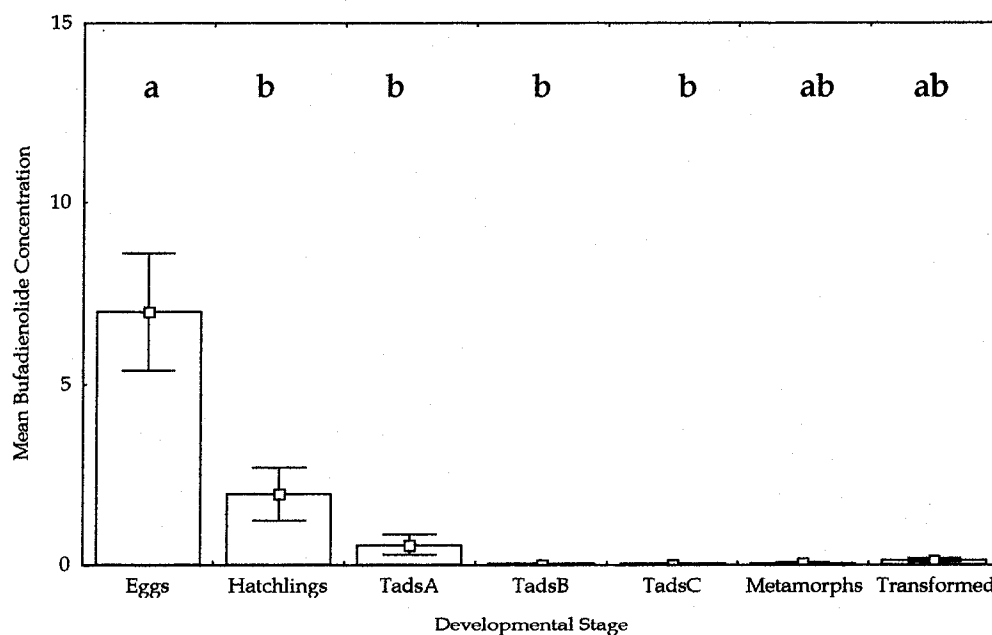


Figure 10. Mean ( $\pm$  SE) total bufadienolide concentration (ng bufadienolide/ $\mu$ g dry weight) for both toad species across all developmental stages. Different letters denote significant differences.

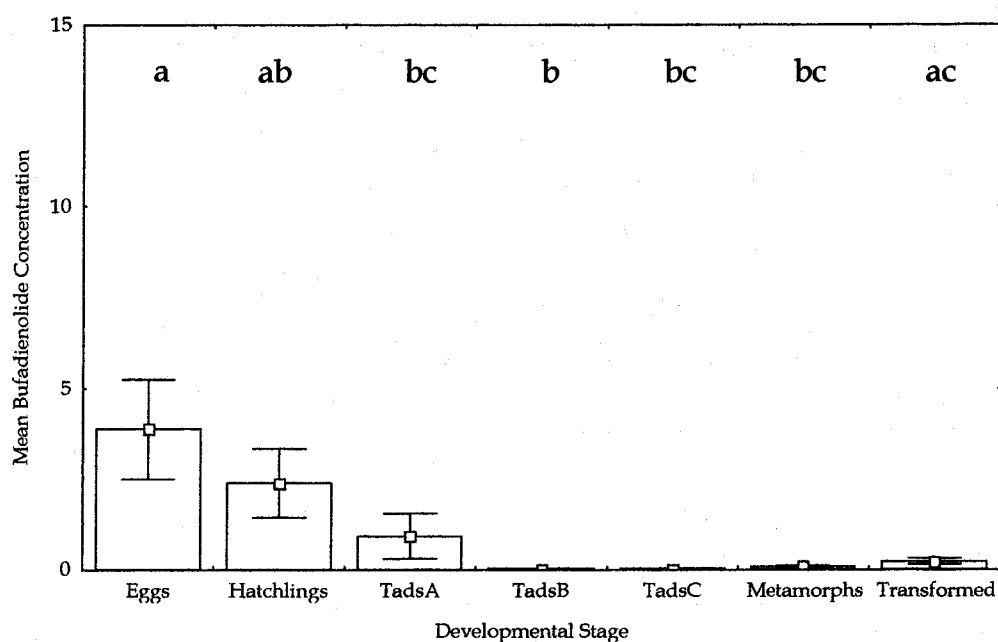


Figure 11. Mean ( $\pm$  SE) total bufadienolide concentration (ng bufadienolide/ $\mu$ g dry weight) for *B. americanus* across all developmental stages. Different letters denote significant differences.

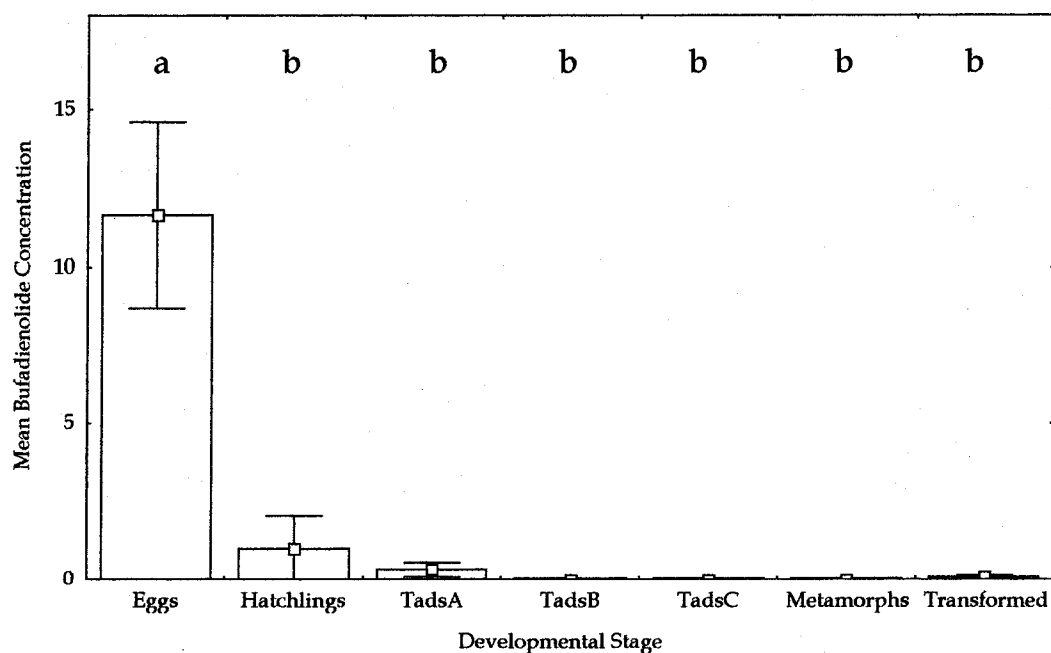


Figure 12. Mean ( $\pm$  SE) bufadienolide concentration (ng bufadienolide/ $\mu$ g dry weight) for *B. fowleri* across all developmental stages. Different letters denote significant differences.



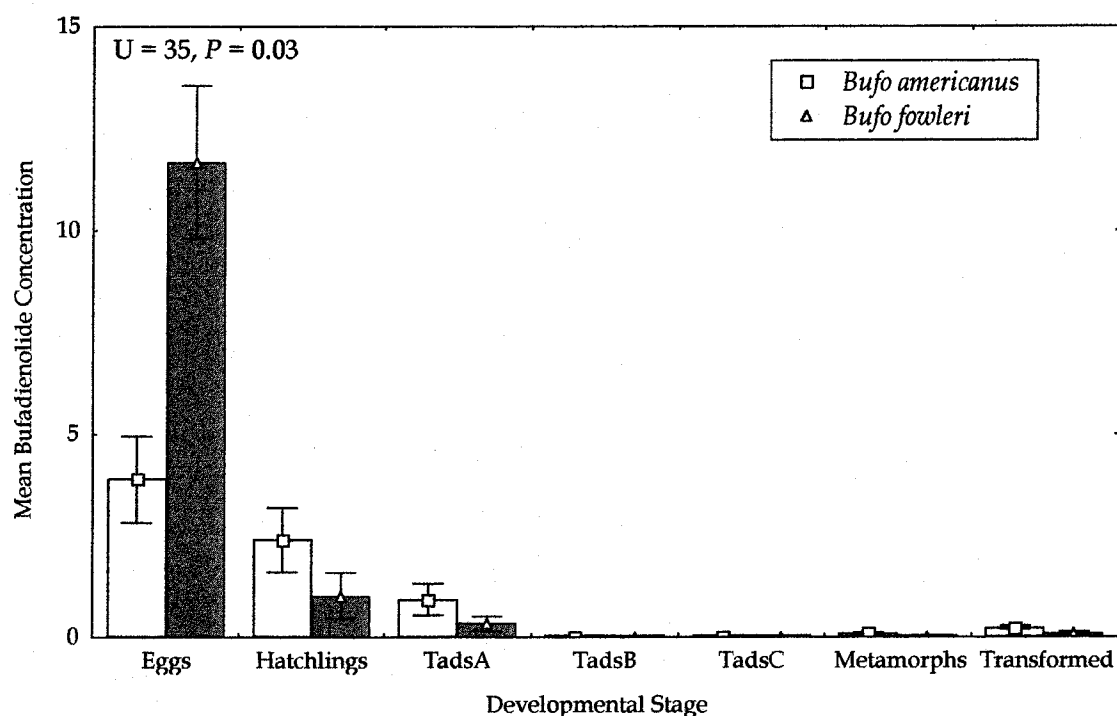


Figure 13. Mean ( $\pm$  SE) bufadienolide concentration (ng bufadienolide/ $\mu$ g dry weight) for *B. americanus* and *B. fowleri* across all developmental stages. Concentrations were significantly different only between eggs of *B. americanus* and *B. fowleri*.

## Discussion

The steroidal chemical defenses (bufadienolides) of the toads, *B. americanus* and *B. fowleri* varied ontogenetically. Although the vast majority of sampled toads possessed no detectable bufadienolides, similar patterns were observed in the differences among toad developmental stages. The fact

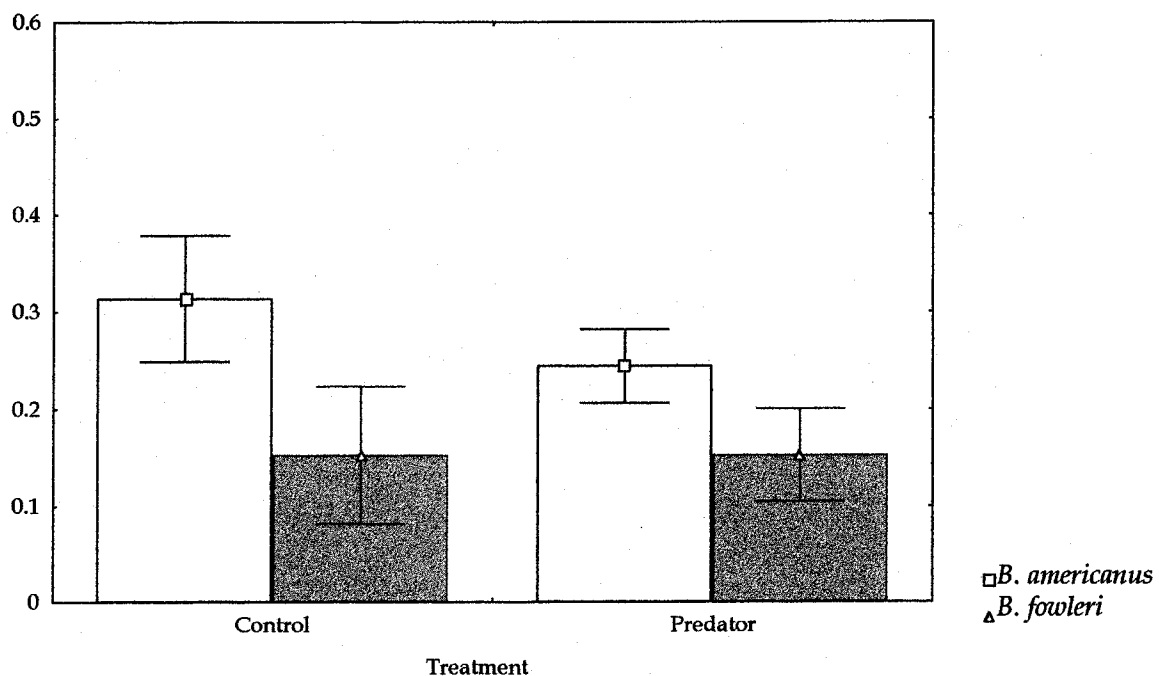


Figure 14. Mean ( $\pm$  SE) total bufadienolide concentration (ng bufadienolide/ $\mu$ g dry weight) in transformed individuals of *B. americanus* and *B. fowleri* raised from eggs with and without predatory cues.

that the vast majority of toads did not possess bufadienolides is not surprising given that the majority of sampled toads comprised the middle developmental stages (tadpoles) when anuran larvae are known to lack skin glands (such as the granular or poison glands) (Hayes and Gill 1995). This finding was consistent with the study by Benard and Fordyce (2003) where no bufadienolides were detected in *Bufo boreas* larvae (Gosner stages 31 and 35).

Differences in bufadienolide concentrations were found among developmental stages in both toad species. When all stages were considered collectively, there were no statistically significant differences in the mean numbers of bufadienolides nor in the mean total concentrations of bufadienolides between *B. americanus* and *B. fowleri*. Not only did the developmental stages differ with respect to the mean number of bufadienolides present, but they also differed with respect to the average concentrations of those bufadienolides as well. As Brodie and Formanowicz (1987) predicted by their palatability and stage-specific survivorship model, chemical defenses were present (in this case the bufadienolides) in early and late toad developmental stages, but the decrease and increase in bufadienolides, as predicted by lines B<sub>1</sub> and B<sub>2</sub> (Figure 5), do not appear to be as gradual. Not only this, but the concentrations of bufadienolides found in *B. americanus* and *B. fowleri* developmental stages are pronounced only in early stages (eggs and hatchlings) but not in late stages. Figure 15 shows the mean total bufadienolide concentrations (in both toad species) joined by a curve. This curve may represent the relative palatabilities of toad developmental stages where larger concentrations of chemicals may positively correlate with unpalatability. This remains a hypothesis to be tested. Unlike the Brodie and Formanowicz (1987) model where there is an

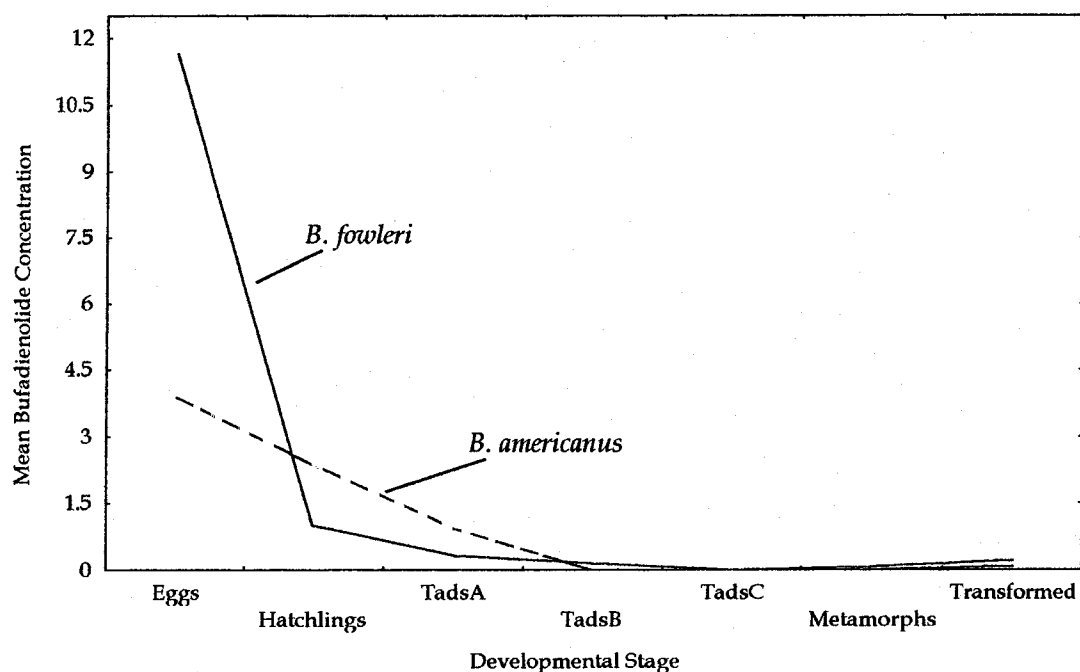


Figure 15. Mean total bufadienolide concentrations (ng bufadienolide/ $\mu$ g dry weight) for each developmental stage joined by a curved line. This may represent the contribution of bufadienolides to the unpalatability of certain developmental stages.

increase in unpalatability of metamorphs and transformed individuals, bufadienolide defenses do not match the model's predictions because only trace amounts were detected in these stages. This suggests that either the unpalatability of *Bufo* species in these later developmental stages may not be due to bufadienolides or that very small amounts are adequate defenses. It seems likely that the small amounts of bufadienolides found in both toads at this stage is a result of the time required for the maturation of the poison-

producing granular glands following metamorphosis (Delfino et al. 1995). However, this was not directly controlled in this study. Newly transformed individuals ranged in age from about one to ten days post metamorphosis. It may take longer for the glands to be fully functional and longer yet for the formation of the large clusters of granular glands that form the parotoids (Licht 1967). Delfino et al. (1995) reported the maturation process of granular glands in *Bufo bufo*. They found a consistent reduction in the amount of smooth endoplasmic reticulum following metamorphosis, indicating that steroid biosynthesis slows down considerably during toad development and that the immature poison 'cannot exert its full activity until the myoepithelium and secretory unit are fully mature following metamorphosis.'

The Brodie and Formanowicz model did not address the earliest Gosner (1960) stages (1-19) (eggs) but they were well defended chemically in these two toad species. In fact, *B. americanus* and *B. fowleri* eggs collectively possessed more bufadienolides than all tadpole stages including hatchlings but possessed similar numbers of bufadienolides as late staged-toads (metamorphs and transformed individuals). Considering each species separately, a striking difference was apparant. In *B. americanus*, the eggs did not possess more bufadienolides than any other developmental stage—only

transformed toads had more bufadienolides than two tadpole stage ranges (TadsB and TadsC) (Figure 7). In *B. fowleri*, however, eggs had more bufadienolides than all other stages except transformed individuals. This suggests that differences may exist between *B. americanus* and *B. fowleri* in terms of female investment in bufadienolide defenses for offspring. This should not mean necessarily that *B. fowleri* is better defended by the larger concentrations of bufadienolides found in their eggs than in those of *B. americanus* because *B. americanus* had a more diverse bufadienolide profile overall (Table 5).

With respect to bufadienolide concentrations, there were differences among developmental stages in both toad species but this time the patterns were quite similar. Both *B. americanus* and *B. fowleri* had larger concentrations of bufadienolides in eggs than in other developmental stages. Yet, *B. fowleri* eggs seem to be better defended than those of *B. americanus* because the concentrations of bufadienolides found in *B. fowleri* eggs was also larger than that found in transformed individuals whereas in *B. americanus* eggs the concentrations were not larger than in transformed individuals.

Benard and Fordyce (2003) showed that transformed *Bufo boreas* from predator cue larval environments had significantly higher concentrations of bufadienolides than those raised without predator cues. In contrast, I found

no predator-induced bufadienolide induction in either *B. americanus* or *B. fowleri*. This result is rather surprising given that plasticity in chemical defenses was demonstrated for a bufonid species and that multiple predatory cues were present during the duration of larval development (egg-metamorph). Although it is unclear why induction was not observed, the time of sampling following the completion of metamorphosis may be important for observing the bufadienolides if poison glands are still immature. Sampling individuals later (20 or more days post metamorphosis) rather than soon after metamorphosis may reveal possible induction of chemical defenses as was observed by Benard and Fordyce (2003).

## CHAPTER III

### A COMPARISON OF BUFADIENOLIDES IN THE CUTANEOUS PAROTOID GLAND SECRETIONS OF ADULT *BUFO AMERICANUS* AND *BUFO FOWLERI*

#### Introduction

Steroidal bufadienolides (bufagins or bufagenins) are present in the parotoid gland secretions of bufonid frogs particularly in species belonging to the genus *Bufo* (Meyer 1966) and in several plant families (e.g., Crassulaceae, Hyacinthaceae, Iridaceae, and Melianthaceae) (Steyn and van Heerden 1998). Bufadienolides are C<sub>24</sub> steroids that possess a six-membered, doubly unsaturated lactone ring and are biochemically similar to plant cardenolides (e.g., digitoxin and ouabain) (Fieser and Fieser 1959; Malcolm 1991). The biological activity of bufadienolides and cardenolides targets inhibition of the membrane enzyme, sodium/potassium adenosine triphosphatase (Na<sup>+</sup>/K<sup>+</sup> ATPase or the sodium-potassium pump). Akizawa et al. (1994) were the first to show that bufadienolides in the eggs of *Bufo marinus* inhibit sodium-potassium pump activity typical of cardiac glycosides. Both bufadienolides and cardenolides are termed cardiac glycosides due to their cardiac-active properties (they increase the contractile strength of the heart



and lower heart rate), but chemically the bufadienolides produced by toads do not conjugate with sugars to form true glycosides as they do in plants (Klyne 1957). Rather, toad bufadienolides may conjugate with either suberyl-arginine, suberyl-histidine, or suberyl-glutamine (at C-14) forming bufotoxins (Klyne 1957; Erspamer 1994). Bufotoxins have lower potency than the corresponding un-conjugated bufadienolide and thus produce a weaker effect on the heart (Chen and Kovaříková 1967).

It is the potent inhibition of the sodium-potassium pump and associated cardiac effects that make bufadienolides especially important in the anti-predator chemical defenses of toads. Moreover, bufadienolides can be lethal to vertebrates such as mammals and even to other frogs (Fieser and Fieser 1959; Crossland and Alford 1998). Amphibian chemical defenses are also irritating to buccal tissue (Daly 1995) and have adhesive properties that deter predation (Evans and Brodie 1994).

Chemical defense in toads, as in other amphibians, is a cumulative effect of a diversity of chemicals, in addition to bufadienolides, that collectively makes amphibian skin biochemically and physiologically complex. This complexity is probably necessary to serve the multiple and simultaneous functions of amphibian integument associated with survival (e.g., water balance, respiration, excretion, temperature control, anti-

microbial and anti-fungal properties, as well as defense against predators) (Clarke 1997). Other substances found in toad skin secretions include biogenic amines (basic substances) such as indole alkylamines (e.g., bufotenines are typically present in high levels in *Bufo*) (Daly 1995), catecholamines (e.g., epinephrine and norepinephrine), other non-cardiac active sterols (e.g., cholesterol) (Chen and Kovaříková 1967; Clarke 1997), and peptides and proteins (Mahan and Biggers 1977). Also, some bufonid toads contain alkaloids (Porter 1964; Daly *et al.* 1987; Daly 1998).

Bufadienolides and other chemical defenses are produced in the cutaneous granular glands (poison or serous glands) found throughout the skin as well as in the clusters of granular glands that form the enlarged parotoid glands. Granular glands can be either evenly distributed or concentrated in certain parts of the body (Duellman and Trueb 1986). The concentration of granular glands that form the parotoid glands of toads is an example of such clustering. The term parotid gland (as in salivary gland) should not be confused with the chemical-producing parotoid gland of amphibians. Erroneously, these two terms are often used synonymously in the literature. Another term, paratoid (referring to glands in salamanders) has also been used in place of toad parotoid glands (Cannon and Palkuti 1976; Tyler *et al.* 2001).

The parotoid glands of toads are located on both sides of the head in post-orbital position and may extend over the shoulders (Figure 16a). The parotoids may differ in both morphology and in placement on the body (Lutz 1971). Figure 16b shows differences in the parotoids of *Bufo americanus* and *Bufo fowleri*. When the parotoid glands are stimulated, the chemical secretion exudes onto the skin's surface through multiple pores where it may come into contact with potential predators. Although a defensive function for bufadienolides in toads is the most parsimonious explanation for their presence, very little is known about the ecology of chemical defenses in toads and whether bufadienolides are in fact effective against their natural enemies.

The ecology of bufadienolide defenses in the North American toads, *Bufo americanus* (Holbrook) and *Bufo fowleri* (Hinkley), was the focus of this study. At the outset, I wanted to determine if the bufadienolide composition of *B. americanus* differs from that of *B. fowleri*. I expected that both species should possess similar bufadienolides since they are closely related species and because the production of bufadienolides is presumably under genetic control (Porter 1962; Wittliff 1962), yet differences in bufadienolides could exist between the species due to environmental variation and variable predatory pressures.

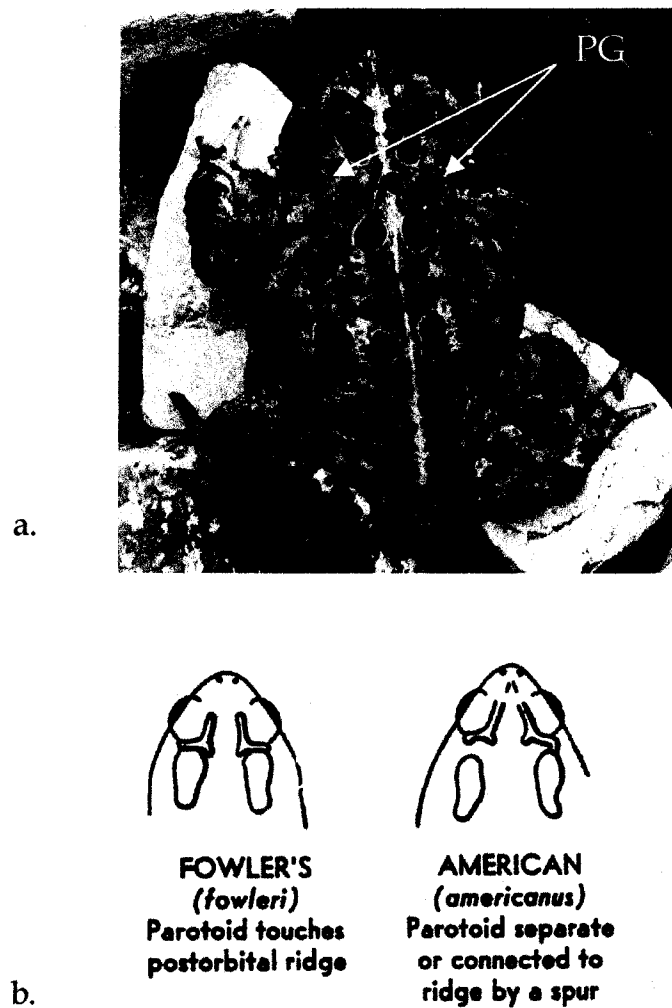


Figure 16. (a) Bufonid toads possess large aggregations of granular glands that form two protuberances, the parotoid glands, on both sides of the body. Shown here is *B. americanus* with enlarged kidney-shaped parotoid glands (PG) and (b) the morphology and placement of the parotoids differs slightly between *B. americanus* and *B. fowleri* (Conant 1975).

Source (Fig. 16b): Conant, R. 1975. A Field Guide to Reptiles and Amphibians. Houghton Mifflin Company. Boston, Massachusetts. Used with permission of Ronald Hussey, Permissions Manager/Serial Rights Manager, Houghton Mifflin Company, 9-22-2004.

*Bufo americanus* and *Bufo fowleri* have largely overlapping ranges but in Michigan the range of *B. fowleri* is more restricted than that of *B. americanus*. *Bufo fowleri* typically occurs along the eastern shore of Lake Michigan, especially in sandy areas, and *B. americanus* occurs ubiquitously throughout the state and beyond (Harding 1997). Thus, I hypothesized that distributional differences among toad populations would influence the frequencies of their chemical phenotypes so that these two bufonids should possess different bufadienolide profiles. For example, if *B. americanus* is prey to a larger diversity of predators, because of their widespread distribution, they should possess either more bufadienolides, different types of bufadienolides, or perhaps different concentrations of bufadienolides in their secretions. Thus, a difference in bufadienolide composition could be attributed to different predatory-pressures experienced by each species or by different populations of the same species. Intra-specific variation in bufadienolides is possible, especially if toad populations are adapted to local conditions. I was also interested to determine whether individual variation in bufadienolide composition was apparent in both species.

Another goal of this study was to investigate the potential induction of bufadienolides in toads that have been subjected to repeated but simulated encounters with predators. When the parotoid glands are stimulated,

thereby releasing the noxious chemical secretions onto the skin's surface (as when a toad is seized by a predator), most if not all of the secretion may be used in the toad's defense. Assuming that the chemical defenses deter predation and the toad survives to encounter another predator, I wanted to determine whether the newly synthesized bufadienolide profile would be altered.

Lastly, I examined the internal structure of the parotoid gland to determine if the stimulation technique ("milking" the gland by gentle compression) was effective in removing most of the chemical secretion. Although bufadienolides can be obtained from other tissues in toads, I focused on the parotoid gland bufadienolides because this represents a morphological and physiological concentration that is thought to target defense against natural enemies.

## Methods

### *Parotoid gland secretions*

I collected toads during their active seasons (spring-fall) of 2000 and 2001 from several locations in southwest Michigan (Kalamazoo, Kent, and Van Buren counties). Snout-vent-length (SVL) and mass were recorded for

each toad and parotoid secretions were obtained in the laboratory. To collect the parotoid secretion, each toad was held in one hand while the parotoid glands were gently compressed between thumb and index finger until no further secretion was observed. The yellowish-white, viscous secretion from each toad was collected onto pre-weighed filter paper, weighed again while wet, dried completely in a drying oven, and reweighed. Filter papers containing secretion were stored separately in plastic bags and kept refrigerated until they were prepared for chemical analysis by high performance liquid chromatography (HPLC).

#### *HPLC of toad bufadienolides*

Filter papers containing parotoid secretions were placed into glass test tubes containing 5 – 7 ml methanol and sonicated for 30 minutes. The filter papers were removed and the methanol was evaporated under nitrogen in a water bath at 60 °C. The remaining residue in each tube was re-suspended in 1 ml of methanol and passed through a 0.45- $\mu$ m filter into a 1 ml autosampler vial for HPLC. Fifty micro-gram samples of a known bufadienolide standard, bufalin (obtained from Sigma Scientific), were run in separate vials as an external standard.

Chemical analyses of toad secretion samples by HPLC were performed on a Waters gradient HPLC system with WISP autosampler, 600E pump, 996 photodiode array detector (at 298 nm) ranging between 270-330 nm using Millennium 2010™ chromatography manager software. Injection volumes for individual samples were 30 µl. Samples were eluted isocratically with a mixture of water and acetonitrile (60:40) at 1.25 ml/min on a 250-4 LiChroCART RP-18 column packed with 5 µl LiChrospher 100 (E. Merck) and a guard column packed with the same material, following the methods of Gella et al. (1995) and Benard and Fordyce (2003) and each sample had a run time of 20 min.

I identified bufadienolides by comparing the absorption spectra of unknown peaks to that of bufalin. I quantified the bufadienolide content of each sample by using a calibration curve obtained from running a bufalin dilution series (between 10 ng and 100 µg). Peaks identified as bufadienolides were constrained within the purity values (purity angle and purity threshold) quantified by the lowest concentration of bufalin. If the purity angle and purity threshold of an unknown peak (with a characteristic absorption spectra) was equal to or less than 9.017 and 12.750 respectively, then the peak was considered a bufadienolide. If the absorption spectra of an unknown



peak fit the characteristics of a bufadienolide but did not fall within these limits of purity, it was not considered a bufadienolide.

The data were not normally distributed, therefore I used Mann Whitney U tests to determine whether the mean total concentrations of bufadienolides differed between *B. americanus* and *B. fowleri* as a function of both toad mass and dry parotoid secretion. A MANOVA was conducted to test for differences in the concentrations (ng bufadienolide/ $\mu$ g dry weight parotoid secretion) of the twelve most common bufadienolides between toad species. A significant analysis was followed by univariate ANOVAs to test for differences in each bufadienolide concentration between toad species. I also used a multivariate, ordination technique, principal component analysis (PCA), on the concentrations of these same twelve bufadienolides in order to synthesize the data and to determine which bufadienolides were most responsible for explaining the variation between species and between habitats. The data were checked for normality using Shapiro Wilk's W tests and could not be normalized by transformation. The data were skewed, however a square-root transformation reduced this problem (raw data had an average skewness value of 3.99 and square-root transformed data had an average skewness value of 1.66). Given the reduced skewness of the data, PCA and MANOVA were considered valid analyses. Moreover, PCA can be

performed on data that are not normally distributed without necessarily producing biased results when the data are not highly skewed (Legendre and Legendre 1998). MANOVA is also especially resistant to non-normality due to skewness (Zar 1999). Analyses were conducted with JMP statistical software version 5.0.1 and Statistica software version 4.5 and considered statistically significant at  $\alpha = 0.05$ .

#### *Inducibility of bufadienolides*

Five *B. americanus* and five *B. fowleri* were collected in southwest Michigan (Kent County) in June of 2000. Four *B. fowleri* and one *B. americanus* were successfully maintained in the laboratory to investigate the effect of simulated encounters with predators (manual and repeated expression of parotoid glands). The cause of mortality for the other animals is unknown and *B. americanus* was subsequently excluded from the analysis. Each toad was housed separately in a plastic rat cage (42.55 cm x 26.67 cm x 29.21 cm) and fed crickets once or twice weekly. Parotoid secretions were collected repeatedly (once a month) over the course of three months in captivity. The parotoid secretion was collected on a pre-weighed filter paper, dried, and analyzed by HPLC as described above. All toads were released at the site of capture following the final parotoid extraction. I used a sign test

(nonparametric equivalent to a dependent t-test) to assess changes in total bufadienolide concentrations between the first and final collection times.

#### *Histology of toad parotoid glands*

A total of ten toads (five *B. americanus* and five *B. fowleri*) were collected in the summer of 2003 from several locations in southwest Michigan (Kalamazoo and Kent Counties). For each toad collected, measurements were taken of mass, snout-vent-length, left and right parotoid length and width. The right parotoid gland was manually compressed while the left gland was not. The parotoid secretion was collected on a pre-weighed filter paper, dried, and analyzed by HPLC as described above.

All animals were euthanized in a cold water bath with an overdose of MS222 (concentration > 250 mg/L buffered pH 7-7.5) and death was confirmed after 10-15 minutes by cutting the aorta of each animal. Compressed glands were dissected in their entirety and then cut in half. One half was immediately fixed in formalin and the other half was frozen to a piece of cork in isobutane over dry ice. Frozen samples were placed in a -80 °C freezer prior to making sections and used in attempts to stain bufadienolides with 2% dinitrobenzoic acid failed. Formalin-fixed tissues were sectioned along the transverse plane at 6 µm and stained with

hemotoxylin and eosin. Slicing and staining was performed by the colleagues of Dr. Robert Eversole. Sections were examined on a Zeiss Axioskop2 Mot light microscope and digital images were captured with an MRC5 camera using AxioVision 4.1 software. Granular gland areas were measured by digitizing polygons around all non-compressed alveoli. The data were checked for normality using Shapiro Wilk's W statistics and dependent t-tests were used to compare total mean granular gland areas (compressed versus non-compressed parotoids) for *B. americanus* and *B. fowleri*. Analyses were conducted with JMP statistical software version 5.0.1 and Statistica software version 4.5 and considered statistically significant at  $\alpha = 0.05$ .

## Results

### *Parotoid gland secretions – bufadienolide composition*

Table 9 shows the average sizes of animals whose parotoid secretions were analyzed. Toads were equal in mean mass ( $U = 807.5$ ;  $P = 0.174$ ) (Figure 17). The mean total concentrations of bufadienolides as a function of toad of bufadienolides as a function of toad mass (ng bufadienolide/g toad) in *B. americanus* and *B. fowleri* were 8.50 and 4.30 respectively and were not statistically different ( $U = 765$ ;  $P = 0.09$ ) (Figure 18).

Table 9

Mean mass and snout-vent length (SVL) of toads whose parotoid gland secretions were analyzed.

Toad	Mean $\pm$ SE	Range	Mean $\pm$ SE	Range
	Mass (g)	(g)	SVL (cm)	(cm)
<i>B. americanus</i> (n = 63)	11.72 $\pm$ 1.57	1.00 - 54.90	4.03 $\pm$ 0.169	2.05 - 7.34
<i>B. fowleri</i> (n = 31)	8.53 $\pm$ 1.77	1.57 - 36.71	3.57 $\pm$ 0.21	2.20 - 6.60

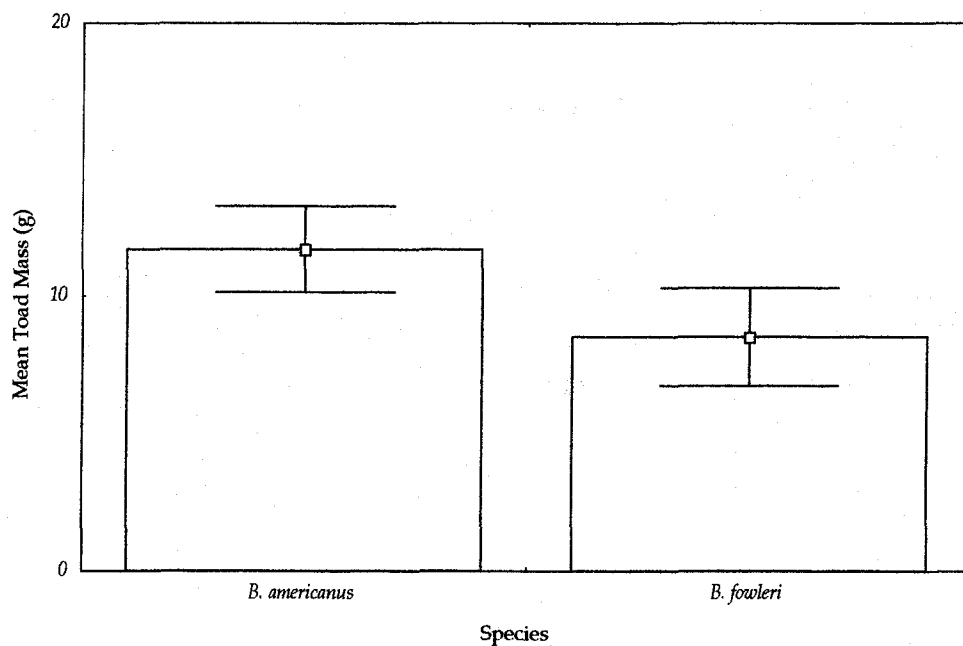


Figure 17. Mean mass ( $\pm$  SE) of adult *B. americanus* and *B. fowleri*.

Cumulatively, 30 bufadienolides were identified in the parotoid gland secretions of *Bufo americanus* and *Bufo fowleri*. Individuals of both species, on average, possessed 6.83 bufadienolides. When each species was considered separately, the average number of bufadienolides in *B. americanus* secretion was 7.66 and in *B. fowleri* secretion was 4.90 (Figure 19a). The difference in the mean number of bufadienolides between species was statistically significant ( $U = 324$ ;  $P < 0.0001$ ). The mean total concentrations of bufadienolides as a function of dry parotoid secretion in *B. americanus* and *B. fowleri* were 46.03 and 34.63 ng bufadienolide/ $\mu$ g dry secretion sample respectively and were not statistically different ( $U = 974$ ;  $P = 0.98$ ) (Figure 19b).

Twelve bufadienolides (B1, B2, B4, B7, B9, B10, B12, B13, B15, B16, B17, and B21) were typically found in both species of toads. These relatively common bufadienolides were present in at least 22.3% (B17) and at most 71.3% (B2) of sampled toads (Table 10). The average concentrations of these bufadienolides in *B. americanus* and *B. fowleri* were between 2.0 – 10.0 and 0.60 – 2.0 ng bufadienolide/ $\mu$ g dry secretion sample respectively (Figures 20 and 21). Of the twelve common bufadienolides in both toad species, five of these (B1, B2, B4, B7, and B10) were present in 53.2% – 71.3% of individuals (Table 10). *Bufo americanus* contributed most to these percentages by accounting for

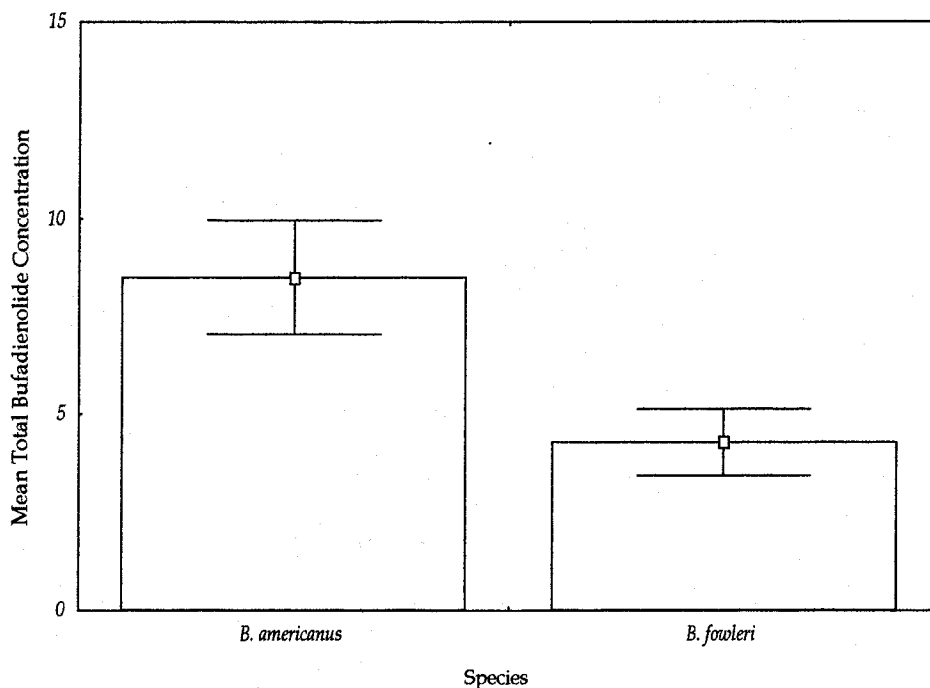
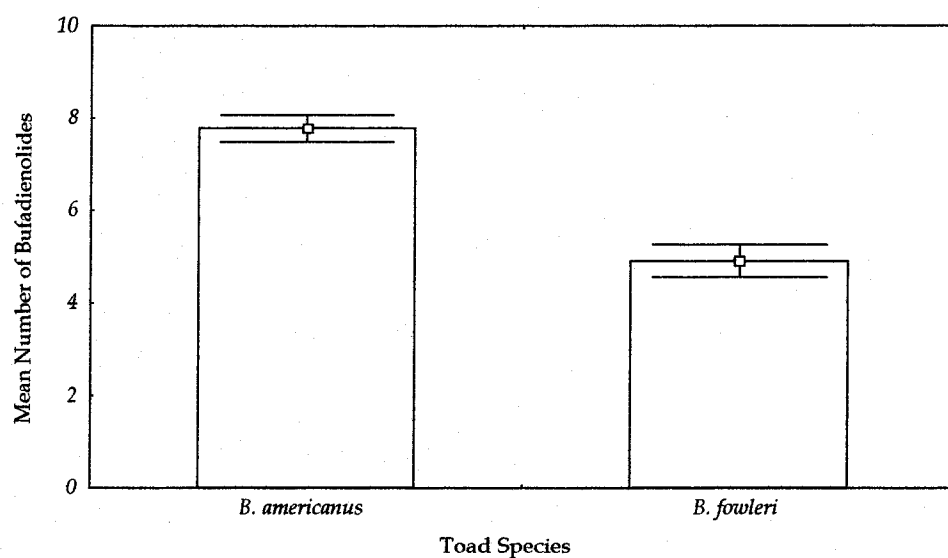
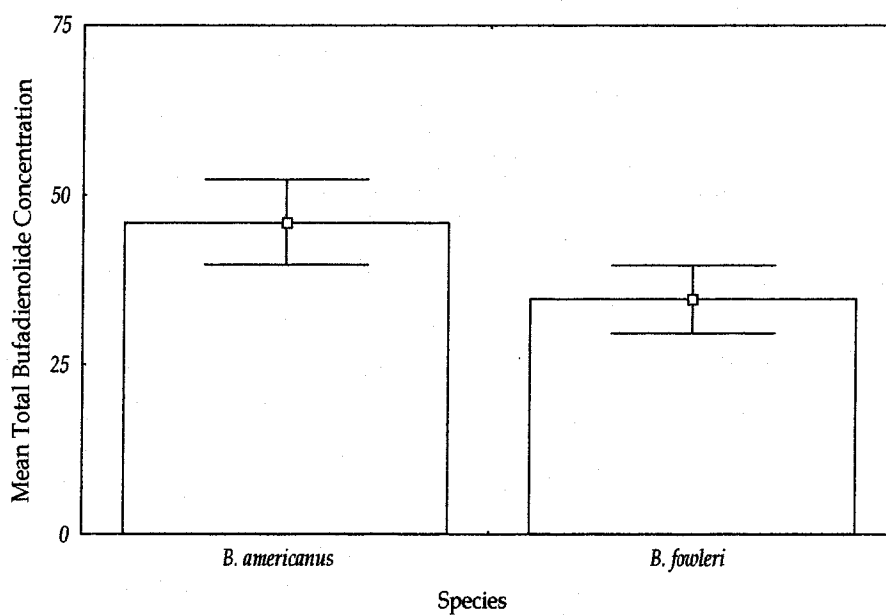


Figure 18. Mean ( $\pm$  SE) total bufadienolide concentration as a function of toad mass. Units are in ng bufadienolide/g toad.

78.4% - 96.9 % of toads that contained each of these five bufadienolides (Table 10). Moreover, these five bufadienolides were the most common bufadienolides among *B. americanus* individuals (present in 74.6% - 100.00% of individuals). One bufadienolide (B7) was also commonly found among *Bufo fowleri* individuals, but four others (B11, B12, B16, and B17), in contrast to those in *B. americanus*, were most common in *B. fowleri* (present in 25.81 % - 51.62 % of individuals) (Table 10). Bufadienolide B11 was the one bufadienolide relatively common to *B. fowleri* (present in 25.8% of *B. fowleri*



a.



b.

Figure 19. (a) The mean number ( $\pm$  SE) of bufadienolides identified in *B. americanus* and *B. fowleri* and (b) the mean ( $\pm$  SE) total concentration of bufadienolides in *B. americanus* and *B. fowleri*. Units are in ng bufadienolide/ $\mu$ g dry parotoid secretion.



Table 10

Number of individuals (*B. americanus* n = 63 and *B. fowleri* n = 31) that possessed each of the identified bufadienolides (B1 – B30). Percent number of individuals with each bufadienolide present in the parotoid secretion is in parentheses.

Bufadienolide	Total	<i>B. americanus</i>	<i>B. fowleri</i>
Average Retention Time (min)			
	# toads	# toads	# toads
	(%)	(%)	(%)
	n = 94	n = 63	n = 31
B1 2.77	65 (69.15)	63 (100.00)	2 (6.45)
B2 3.43	67 (71.28)	59 (93.65)	8 (25.81)
B3 3.82	12 (12.77)	7 (11.11)	5 (16.13)
B4 4.13	53 (56.38)	48 (76.19)	5 (16.13)
B5 4.58	12 (12.77)	5 (7.94)	7 (22.58)
B6 5.04	17 (18.09)	17 (26.98)	0 (0.00)
B7 5.31	51 (54.25)	40 (63.49)	11 (35.48)
B8 5.97	17 (18.09)	10 (15.87)	7 (22.58)
B9 6.55	28 (29.79)	22 (34.92)	6 (19.35)
B10 6.91	50 (53.19)	47 (74.60)	3 (9.68)
B11 7.77	17 (18.09)	9 (14.29)	8 (25.81)
B12 8.27	34 (36.17)	21 (33.33)	13 (41.94)
B13 8.84	23 (24.47)	17 (26.98)	6 (19.35)

Table 10—Continued.

Bufadienolide Average Retention Time (min)	Total	<i>B. americanus</i>	<i>B. fowleri</i>
	# toads	# toads	# toads
	(%)	(%)	(%)
	n = 94	n = 63	n = 31
B14 9.27	2 (2.13)	1 (1.59)	1 (3.23)
B15 9.49	29 (30.85)	22 (34.92)	7 (22.58)
B16 9.80	35 (37.23)	22 (34.92)	13 (41.94)
B17 10.43	21 (22.34)	5 (7.94)	16 (51.62)
B18 11.56	8 (8.51)	5 (7.94)	3 (9.68)
B19 12.21	11 (11.70)	8 (12.70)	3 (9.68)
B20 12.79	14 (14.89)	13 (20.63)	1 (3.23)
B21 13.53	24 (25.53)	18 (28.57)	6 (19.35)
B22 14.11	12 (12.77)	10 (15.87)	2 (6.45)
B23 14.79	9 (9.57)	5 (7.94)	4 (12.90)
B24 15.45	11 (11.70)	4 (6.35)	7 (22.58)
B25 15.98	13 (13.83)	6 (9.52)	7 (22.58)
B26 16.68	3 (3.19)	3 (4.76)	0 (0.00)
B27 17.47	4 (4.26)	4 (6.35)	0 (0.00)
B28 18.13	5 (5.32)	5 (7.94)	0 (0.00)
B29 18.73	4 (4.26)	4 (6.35)	0 (0.00)
B30 19.51	5 (5.32)	5 (7.94)	1 (3.23)

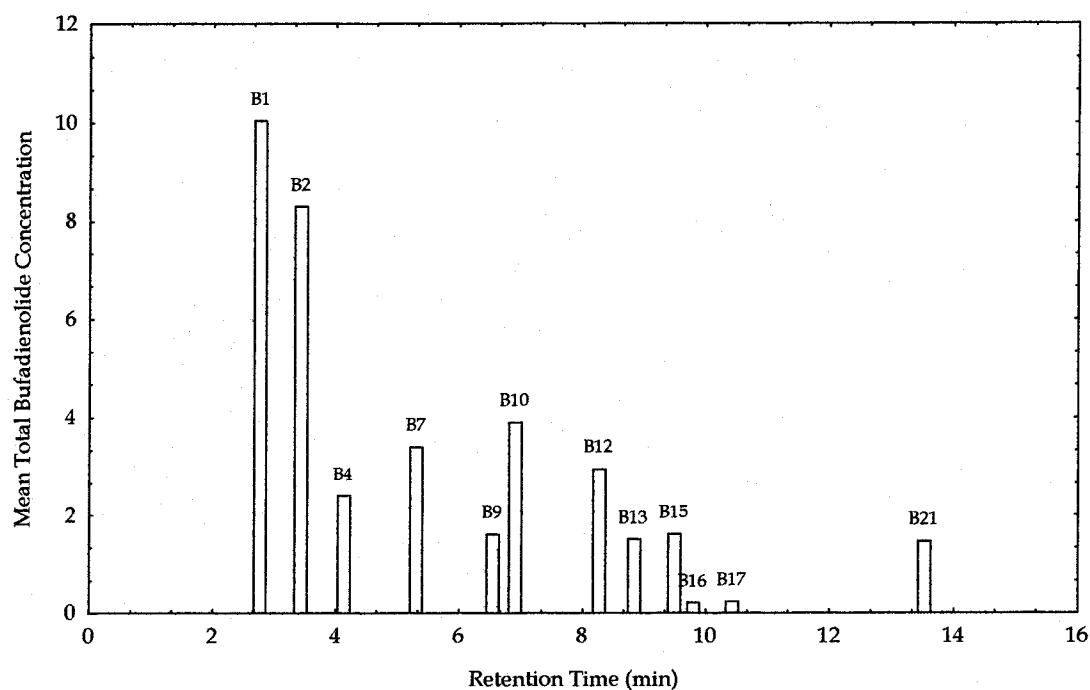


Figure 20. Mean concentrations of each of twelve common bufadienolides in *B. americanus*. Concentrations are in ng bufadienolide/ $\mu$ g dry weight parotoid secretion. Standard errors for each mean: B1 = 2.19, B2 = 1.30, B4 = 0.48, B7 = 0.84, B9 = 0.46, B10 = 0.90, B12 = 0.92, B13 = 0.43, B15 = 0.46, B16 = 0.08, B17 = 0.11, B21 = 0.35.

individuals) but not to *B. americanus* (present in only 14.3 % of *B. americanus* individuals). All 30 bufadienolides were identified in *B. americanus* individuals, although in several cases these numbers were small with only one or two individuals per bufadienolide, and in *B. fowleri* some bufadienolides (B6, B26, B27, B28, B29) were never observed (Table 10).

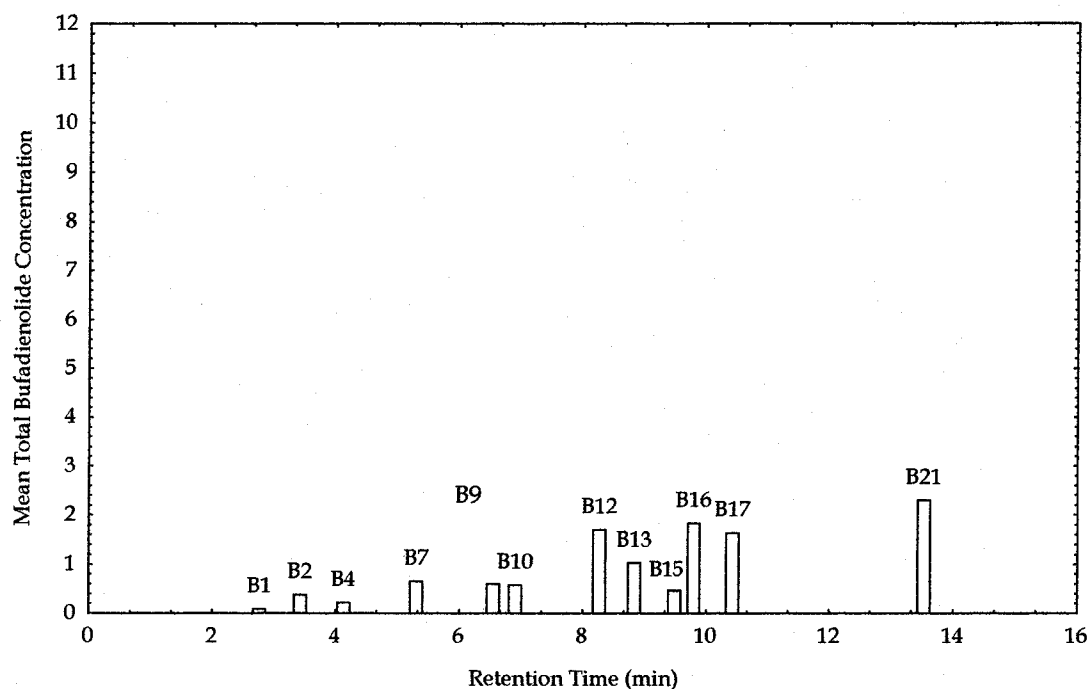
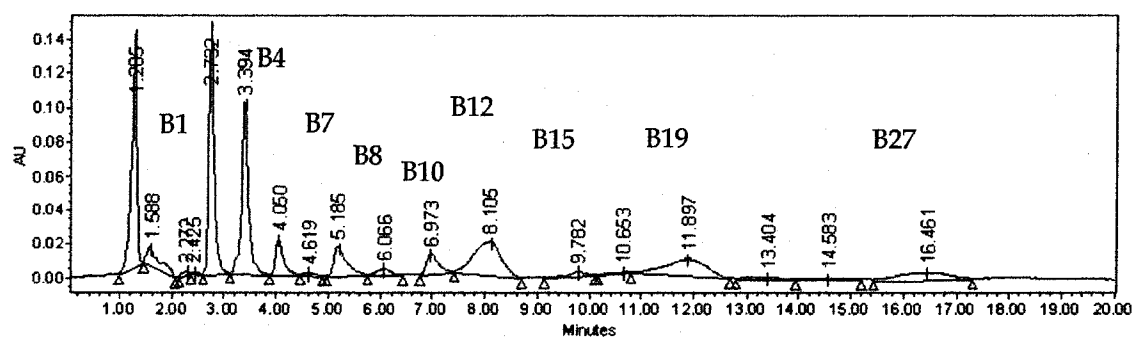
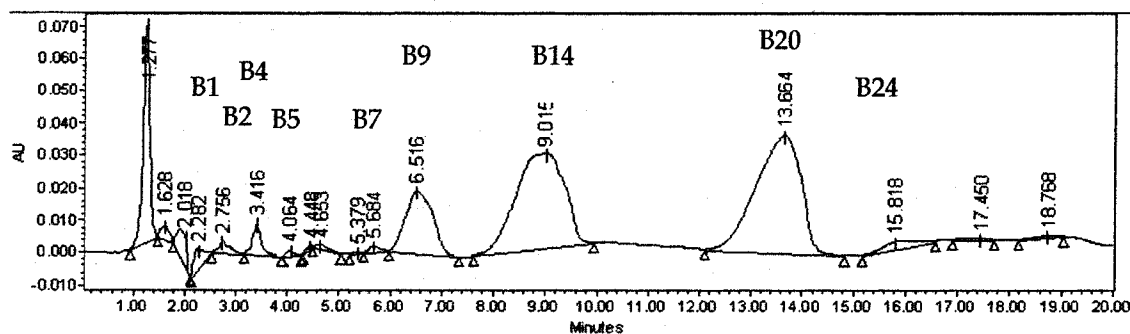


Figure 21. Mean concentrations of each of twelve common bufadienolides in *B. fowleri*. Concentrations are in ng bufadienolide/ $\mu$ g dry weight parotoid secretion. Standard errors for each mean: B1 = 0.06, B2 = 0.13, B4 = 0.09, B7 = 0.18, B9 = 0.27, B10 = 0.35, B12 = 0.45, B13 = 0.43, B15 = 0.16, B16 = 0.62, B17 = 0.33, B21 = 0.95.

Figures 22a and 22b show HPLC chromatograms of example individuals of *B. americanus* and *B. fowleri* with these characteristic bufadienolide profiles.



a.



b.

Figure 22. HPLC chromatograms for (a) one *B. americanus* individual (Ba26FF) collected at Franz Farm (Van Buren County) and (b) one *B. fowleri* individual (Bf5BT) collected along Belmont Trail (Kent County). These bufadienolides were typically found in each species respectively.

#### *Variation in bufadienolides between toad species*

Results of MANOVA on concentrations of the twelve most common bufadienolides (comprising between 22.3 % - 71.3 % of sampled toads) in *B.*

*americanus* and *B. fowleri* parotoid secretion show a highly significant difference between species ( $F_{df = 1,92} = 5.30$ ;  $P < 0.0001$ ). The subsequent univariate ANOVAs showed that the concentrations of seven bufadienolides (B1, B2, B4, B7, B10, B16 and B17) were statistically different between species (Table 11).

A multivariate principal component analysis (PCA) showed that principal component axes one and two (PC1 and PC2) explained 49.9 % of the variability in the concentrations of bufadienolides among toads (Table 12). Principal component axis one alone explained 33.79% of this variance (Figure 23). Eigenvector scores for specific bufadienolides represent the amount of loading or weighting of each variable on each of the principal components. For example, for PC1, the highest weightings (eigenvector scores) are for bufadienolides B1, B2, B4, and B10 and for PC2, the highest weightings are for bufadienolides B13 and B21 (Figure 23). Thus, bufadienolides B1, B2, B4, and B10 are highly positively correlated with PC1 and associated with *B. americanus* and B13 and B21 are highly negatively correlated with PC1 and associated with *B. fowleri*. A scatter plot of the first two principal variable scores (Figure 23) shows the placement of individuals of each species along the gradients created by PC1 and PC2. *Bufo fowleri* are clustered in the negative direction on PC1 in contrast to the more positive clustering of *B.*

Table 11

Univariate ANOVAs on bufadienolide concentrations  
(ng/ $\mu$ g dry weight of parotoid secretions) from  
*B. americanus* and *B. fowleri*.

ANOVA			
Source	df	F	P
B1	1, 92	10.142	< 0.002
B2	1, 92	18.120	< 0.001
B4	1, 92	9.994	< 0.002
B7	1, 92	5.229	< 0.025
B9	1, 92	2.147	0.146
B10	1, 92	6.422	0.013
B12	1, 92	0.843	0.361
B13	1, 92	0.496	0.483
B15	1, 92	0.493	0.485
B16	1, 92	13.173	0.0005
B17	1, 92	24.960	< 0.0001
B21	1, 92	1.001	0.3198

Table 12

Eigen analysis of bufadienolide concentrations  
in adult *B. americanus* and *B. fowleri*  
parotoid gland secretions.

	PC1	PC2	PC3	PC4
Eigenvalue	4.055	1.934	1.456	1.188
Percent	33.793	16.113	12.135	9.896
Cumulative Percent	33.793	49.905	62.040	71.936
Eigenvectors				
B1	0.456	0.078	-0.096	-0.029
B2	0.458	0.060	0.030	-0.087
B4	0.421	0.043	0.012	0.170
B7	0.342	0.159	-0.019	0.442
B9	0.203	-0.001	0.584	-0.107
B10	0.404	0.013	-0.236	0.107
B12	0.027	0.372	0.504	-0.293
B13	0.057	-0.582	0.083	0.201
B15	0.066	0.123	-0.533	-0.419
B16	-0.180	0.212	0.095	0.599
B17	-0.204	0.289	-0.193	0.289
B21	0.049	-0.585	0.044	0.008

*americanus* about PC1. Along PC1 there is less overlap between the species than along PC2.

The PCA conducted on the concentrations of bufadienolides from *B. americanus* collected from six different locations shows that PC1 and PC2 explained 47.22% of the variability in bufadienolide concentrations (Table 13). Principal component one alone explained 31.5% of this variance (Table 13).



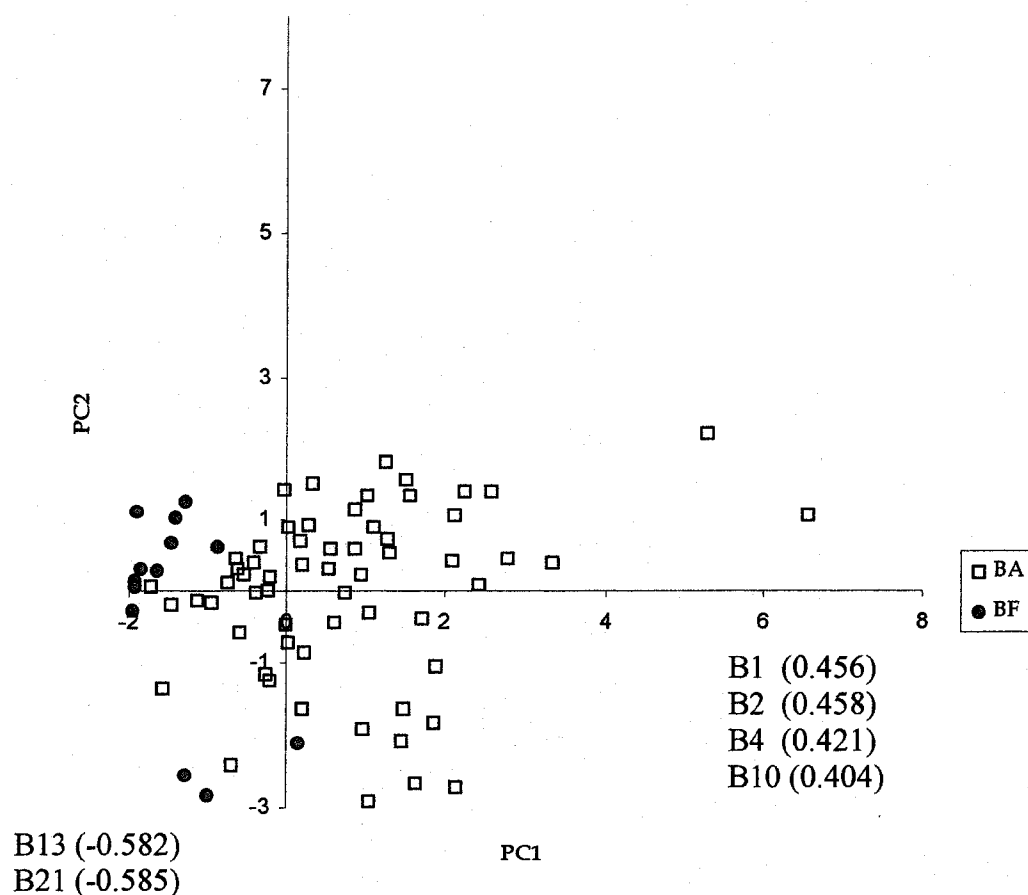


Figure 23. Scatterplot of PCA scores for *B. americanus* (open squares) and *B. fowleri* (filled circles) with respect to concentrations of twelve bufadienolides.

The bufadienolides B1, B2, B4, B9, and B21 were highly positively correlated with PC1 while B12, B17, and B21 were highly positively correlated with PC2 (Figure 24). Bufadienolide B10 was highly negatively correlated with PC2

Table 13

Eigen analysis of bufadienolide concentrations  
in adult *B. americanus* parotoid  
secretions by habitat.

	PC1	PC2	PC3	PC4
Eigenvalue	3.780	1.887	1.570	1.262
Percent	31.496	15.728	13.082	10.519
Cumulative Percent	31.496	47.224	60.307	70.826
	Eigenvectors			
B1	0.465	-0.054	0.026	0.177
B2	0.425	0.166	0.086	0.065
B4	0.401	0.036	0.057	-0.344
B7	0.189	-0.324	0.446	0.218
B9	0.421	0.170	-0.145	-0.080
B10	0.079	-0.481	0.151	0.493
B12	-0.043	0.516	0.420	0.117
B13	0.065	0.026	-0.556	0.218
B15	0.065	-0.179	0.124	-0.633
B16	0.022	0.223	-0.400	0.198
B17	-0.056	0.502	0.279	0.214
B21	0.458	0.050	-0.079	0.046

(Figure 24). Figure 24 shows the scatterplot of the first two principal variable scores for *B. americanus* from each of six collection sites. Therefore, there was overlap in bufadienolide types and concentrations from all habitats.

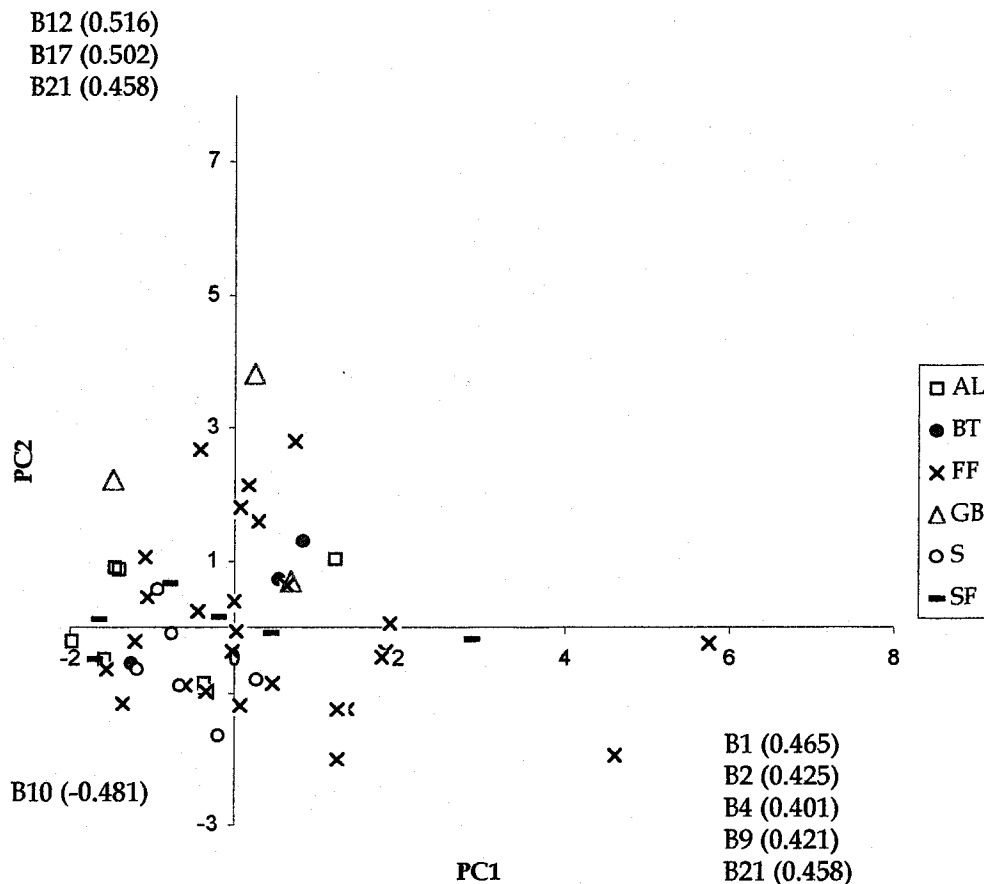


Figure 24. Scatterplot of PCA scores for the concentrations of twelve bufadienolides in *B. americanus* collected from six locations. Kalamazoo County (AL = Asylum Lake, S = Steve's land, and SF = Schwallier Farm); Kent County (BT = Belmont Trail and GB = Guy's Bog); VanBuren County (FF = Franz Farm).

#### *Hisotology of toad parotoid glands*

Adult *B. americanus* and *B. fowleri* have well-developed parotoid glands with visible pores. In *B. fowleri*, the parotoid glands touch the postorbital ridge of the cranial crest and in *B. americanus*, the parotoids either touch the postorbital ridge by a short spur or do not touch the ridge at all

(Figure 16b). The average sizes of the parotoid glands of toads used in this study were 1.11 cm in length by 0.65 cm in width and 1.12 cm in length by 0.59 cm in width for *B. americanus* and *B. fowleri* respectively. The general shape of the parotoids of *B. americanus* and *B. fowleri* is ovoid or sometimes kidney-shaped. The parotoids are located behind the tympanic membranes on both sides of the head and may extend down over the shoulders. The major component of the parotoid glands is the large alveoli (granular glands) that produce and accumulate the granular secretion (white or yellowish in color) (Figures 25 and 26). If stimulated, the parotoid secretion exudes from each alveolus to the surface of the animal through a pore.

Table 14 shows the average size of toads and their parotoid glands used for histological observations. The basic morphology of the internal structure of the parotoid glands of *B. americanus* and *B. fowleri* is similar to that of other *Bufo* species such as *B. arenarum*, *B. crucifer*, *B. granulosus*, *B. ictericus*, *B. jimi*, and *B. paracnemis* (Carlos Jared pers. comm.). In *B. americanus*, there were on average 21.6 granular alveoli and in *B. fowleri* there were on average 31 granular alveoli per parotoid gland section (longitudinal section across the middle of the parotoid). Mucus glands, also found in the parotoid glands of both species, are much smaller and found closer to the epidermis than the granular glands (Figures 25 and 26).

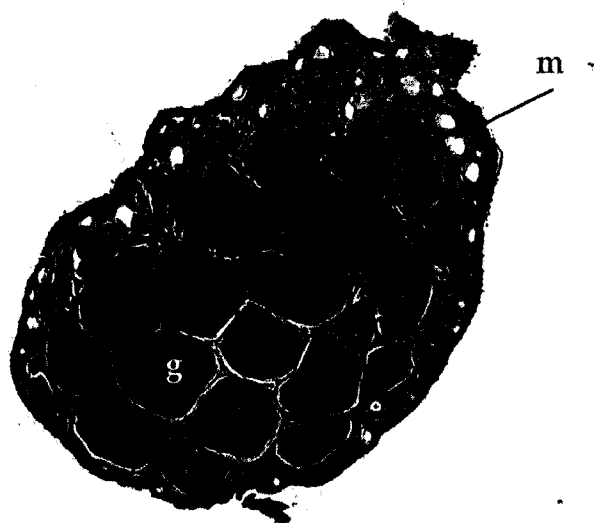


Figure 25. Parotoid section from the left parotoid of *B. americanus* showing non-compressed granular alveoli (g) full of darkly stained secretion and arranged in a honey-comb fashion. Small mucous glands (m) are located in the periphery each with relatively large lumen. Total magnification = 10.25 x.

To determine if the milking technique of secretion extraction was effective in removing the secretion by compressing the parotoids, I observed

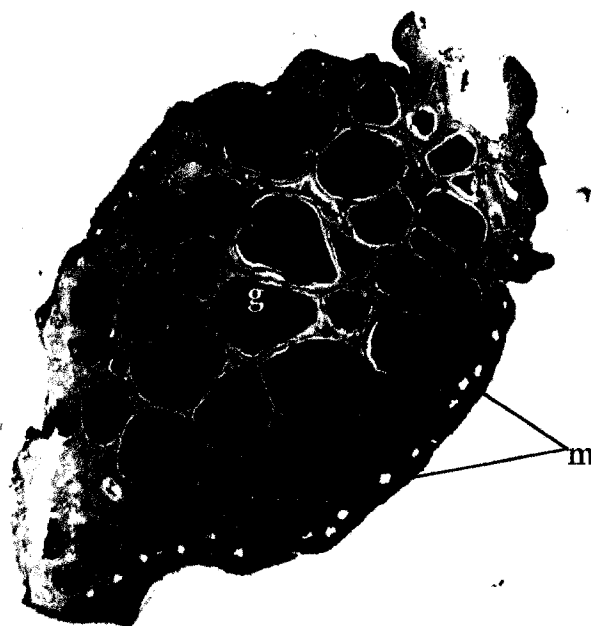


Figure 26. Parotoid section from the left parotoid of *B. fowleri* showing non-compressed granular alveoli (g) full of darkly stained secretion and arranged in a honey-comb fashion. Total magnification = 10.25 x.

sections before and after compression and quantified the area of granular glands containing secretion. Results show that in *B. americanus* and *B. fowleri*, manually compressing the parotoid glands removes most of the secretion in compressed parotoids but not all of it (Figures 27 and 28). The mean area of granular glands containing secretion in the non-compressed parotoids was statistically greater than in the compressed parotoids of *B. americanus* ( $t =$

Table 14

Measurements taken on toads used in the examination of parotoid glands.  
 SVL (snout-vent-length), rPL (right parotoid length), rPW (right parotoid  
 width), lPL (left parotoid length), lPW (left parotoid width),  
 dry (mass of dry secretion)

Toad	Mass (g)	SVL (cm)	rPL (cm)	rPW (cm)	lPL (cm)	lPW (cm)	dry (g)
<i>Bufo americanus</i>							
Ba1	49.75	6.61	1.43	0.80	1.27	1.27	0.00289
Ba2	18.73	4.9	1.03	0.57	1.05	0.61	0.00408
Ba3	12.45	4.65	1.03	0.53	0.96	0.53	0.00086
Ba4	15.45	5.18	1.06	0.51	1.13	0.53	0.00355
Ba5	18.42	4.62	1.09	0.56	1.09	0.57	0.00356
average	22.96	5.192	1.128	0.594	1.10	0.702	0.002988
<i>Bufo fowleri</i>							
Bf1	9.12	4.18	0.84	0.37	0.89	0.39	0.00332
Bf2	31.64	5.87	1.17	0.60	1.16	0.65	0.00745
Bf3	32.59	6.60	1.35	0.64	1.16	0.61	0.00842
Bf4	29.19	5.43	1.09	0.5	1.02	0.63	0.00428
Bf5	36.71	5.95	1.15	0.76	1.35	0.73	0.01158
average	27.85	5.606	1.12	0.574	1.116	0.602	0.00701

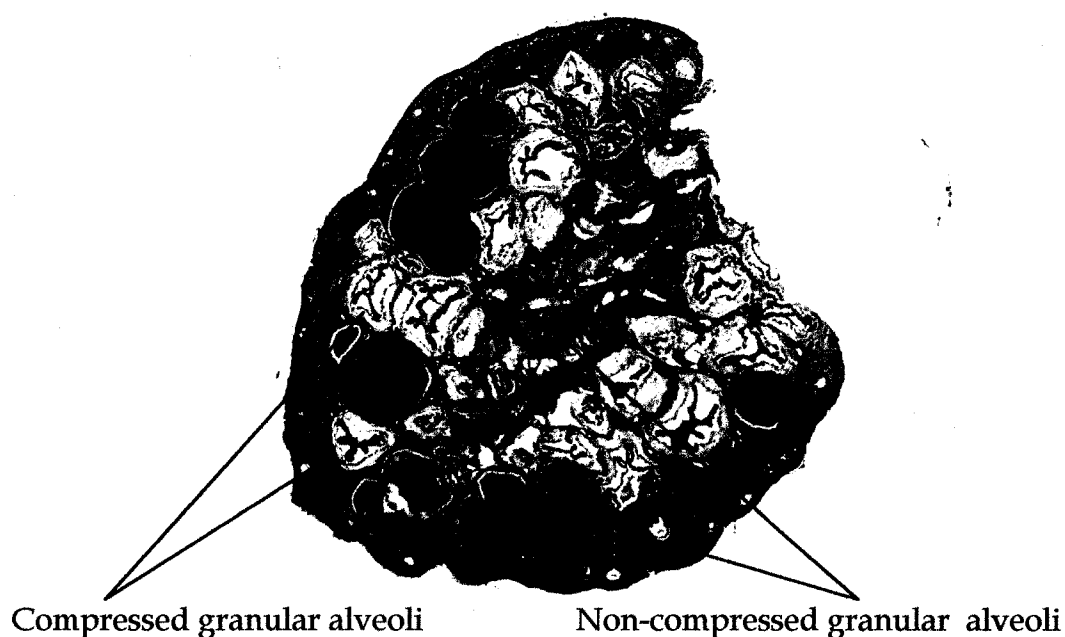


Figure 27. Parotoid section from the right parotoid of *B. americanus* showing compressed granular alveoli including some in the non-compressed state. Note how the syncytia have collapsed into the middle of each alveolus. Total magnification = 10.25 x.

2.81;  $P = 0.05$ ) but not in *B. fowleri* ( $t = 1.14$ ;  $P = 0.34$ ). Interestingly, manual compression of one parotoid gland on a toad does not stimulate the non-compressed parotoid to exude its secretion—at least not between the time of compression and killing of the animal because no collapsed alveoli were observed in non-compressed parotoids (Figures 25 and 26).



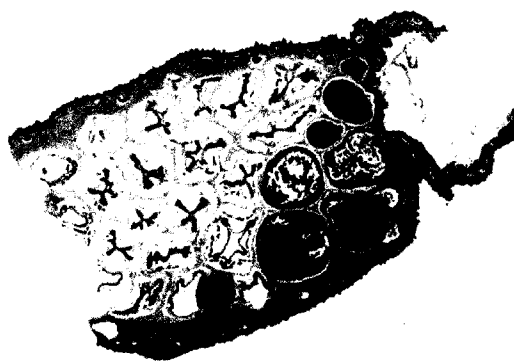


Figure 28. Parotoid section from the right parotoid of *B. fowleri* showing compressed granular alveoli including some in the non-compressed state. Total magnification = 10.25 x.

#### *Bufadienolide induction*

Manual compression of the parotoid glands, to simulate repeated encounters with predators, does change the bufadienolides that are present and the concentrations of those bufadienolides in *B. fowleri*. Table 15 shows the bufadienolides that were detected and their concentrations. The mean

Table 15

Bufadienolides present in *B. fowleri* parotoid secretion and their average concentrations during three sampling periods spaced at one month intervals.

Bufadienolide	(average concentration ng/ $\mu$ g dw secretion)		
	Month One	Month Two	Month Three
B4	6.57	0.73	--
B5	0.36	--	1.54
B6	--	0.59	0.66
B7	0.51	--	--
B8	0.20	3.88	0.034
B9	0.0457	10.12	3.22
B10	--	1.14	--
B13	--	--	0.0295
B14	--	0.31	--
B15	--	--	0.76
B18	--	0.32	--
B21	--	--	0.0126

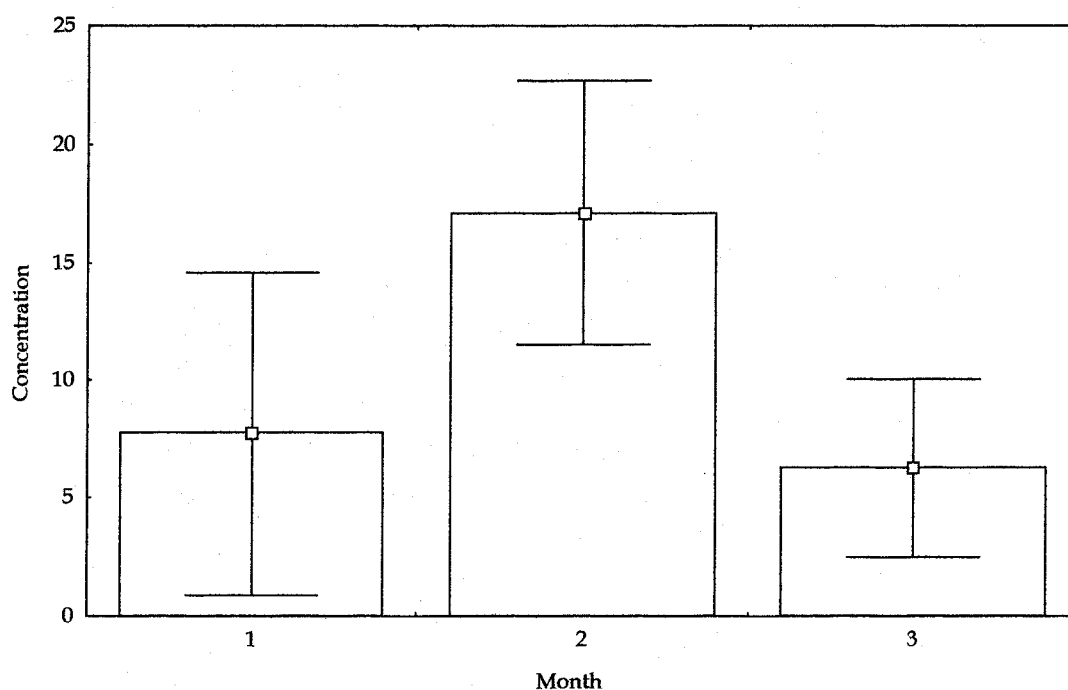


Figure 29. Mean total concentration ( $\pm$  SE) of bufadienolides collected from four *B. fowleri* individuals over a three month period. Concentrations are in ng bufadienolide/ $\mu$ g dry parotoid secretion.

total concentration of bufadienolides between month one and month three were not statistically different ( $\chi^2_{r, df=2} = 3.50$ ;  $P < 0.174$ ) (Figure 29), however, there were large differences among individuals in each sampling period. Individuals varied in the type of bufadienolides that they possessed, the concentrations of bufadienolides, and how the bufadienolides changed over the course of the three months (Figure 30). For example, the number of bufadienolides detected in individual *B. fowleri* ranged from zero to three in

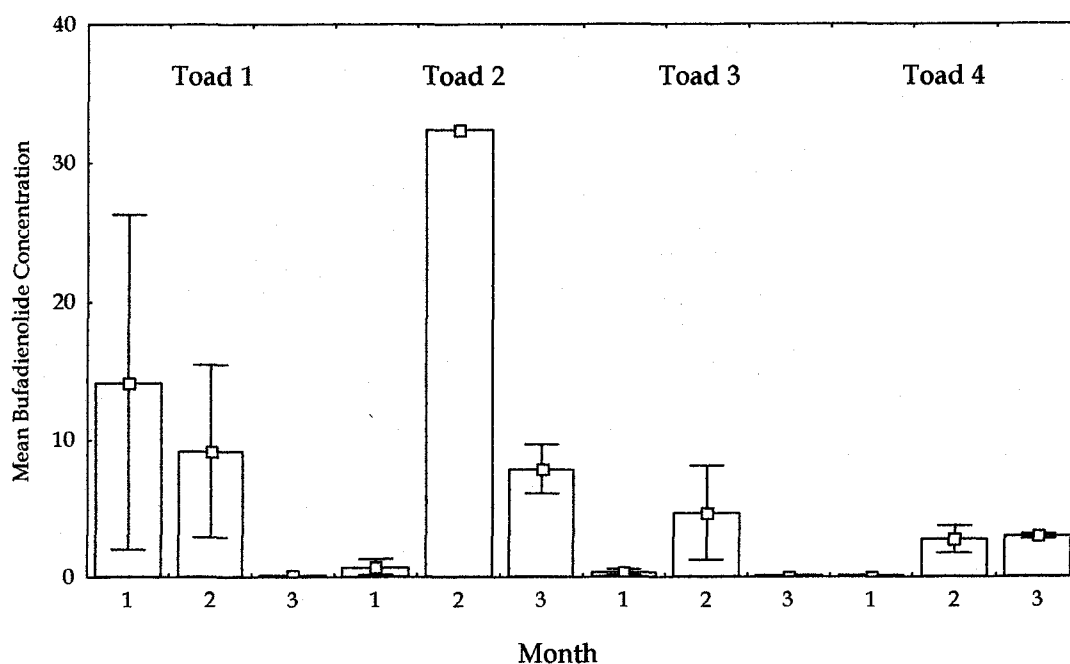


Figure 30. Mean bufadienolide concentrations ( $\pm$  SE) in *B. fowleri* over a three month period. Units are ng bufadienolide/ $\mu$ g dry parotoid secretion.

month one, one to three in month two, and zero to four in month three. Also, the way that these bufadienolides changed from one sample time to the next was different among individuals. For example, in one toad, the final secretion sample showed a loss of bufadienolides and none were detected, while in another toad there was an increase in the number of bufadienolides and four were detected. Further, there was a shift from less polar to more polar bufadienolides with time because bufadienolides with relatively longer

retention times were more frequently observed in months two and three (Table 7).

### Discussion

Steroidal bufadienolides are important components of the chemical secretions of toad integument and parotoid glands. Because the biological activity of bufadienolides involves the potent inhibition of the membrane enzyme,  $\text{Na}^+/\text{K}^+$  ATPase, bufadienolides can provide toads with formidable chemical defenses. However, very little is known about the ecology of these chemical defenses in toads and how the bufadienolides may change or be affected by variable natural conditions. For example, I hypothesized that differences in the distributions of *Bufo americanus* and *Bufo fowleri* would translate into variation in the bufadienolide profiles of both species. Also, regional variation in the chemical defenses within the same toad species (populations may be more or less noxious or toxic) was predicted. Both intra- and inter-specific variability in bufadienolides in toads could be explained by variability in diet, climate, and local adaptation (Palumbo et al. 1975) as well as genetics. Geographical variation in chemical defenses (and predator resistance to prey chemicals) has been well documented in interactions

between rough skinned newts (*Taricha granulosa*) and garter snakes (*Thamnophis sirtalis*) (Brodie et al. 2002; Geffeney et al. 2002; Williams et al. 2003). In toads, regional variation in chemical defenses has also been suggested (Palumbo et al. 1975).

The results of this study indicate that there is individual variation in bufadienolides among toads and that the bufadienolide composition of *B. americanus* and *B. fowleri* differs. Specifically, the mean number of bufadienolides in *B. americanus* was larger than that of *B. fowleri*. *Bufo americanus* has a broader distribution than *B. fowleri* in Michigan and as such may encounter a greater diversity of predatory species which may drive the production of more defensive compounds. However, the mean total concentrations of bufadienolides between toad species were not statistically different. This finding shows that predators are likely to get a similar dose of bufadienolides from the parotoid secretion of *B. americanus* and *B. fowleri* and as a defensive mechanism, this may be more important than the total number of bufadienolides that are present.

Yet, seven bufadienolide concentrations were statistically different between toad species. Bufadienolides with relatively short retention times (B1, B2, B4, B7, and B10) were more concentrated in *B. americanus* while bufadienolides with relatively long retention times (B16 and B17) were more

concentrated in *B. fowleri*. Principal component analysis also revealed the importance of two additional bufadienolides (B13 and B21) for explaining the variation in bufadienolide concentrations between toad species. Based on these data, an examination of bufadienolides in toads could be helpful in distinguishing species. Whether the differences in bufadienolide concentrations are largely attributed to local adaptation and genetic control is unknown. The result that *B. americanus* individuals collectively possessed all 30 bufadienolides and some bufadienolides were never detected in *B. fowleri* is interesting and supports the idea that *B. americanus* is susceptible to more diverse predatory pressures.

Concerning differences in bufadienolide concentrations examined within *B. americanus* collected from several habitats, PCA suggests that there is no association between the collection site and the suite of bufadienolide concentrations that toads possess. Thus, within *B. americanus*, there is no discernable pattern of bufadienolides among habitats despite the fact that toads were sampled from separate locations each greater than one kilometer apart and presumably beyond the range for interbreeding and gene flow.

The internal structure of the parotoid glands of *B. americanus* and *B. fowleri* is similar to that of other toad species (Carlos Jared *pers. comm.*; Jared et al. 2003). Within the parotoids of *B. americanus* and *B. fowleri*, granular

glands (alveoli) were observed full of secretion and arranged in a honeycomb-like fashion. Smaller mucus glands were also observed close to the epidermis each with a relatively large lumen. Pores on the surface of the parotoid glands were also readily visible with the naked eye as they are in other toads (Hostetler and Cannon 1974; Jared et al. 2003; Shipley and Wislocki 1915) and each pore, although not directly observed in this study, is joined to an alveolus via a duct (Wilber and Carroll 1940). Upon stimulation, such as manually compressing the glands ('milking'), the chemical secretion reaches the surface of the toad by exiting the pores. When parotoids of both *B. americanus* and *B. fowleri* are manually compressed, collapsed syncytia are readily observed and their contents are secreted. However, manual compression is not completely effective since some intact granular alveoli are also seen in the compressed parotoid. Interpretation of the granular gland measurement of areas to assess this difference gave conflicting results—in *B. americanus* compression statistically removed more secretion but in *B. fowleri* it did not. This finding is probably a result of the difficulty in obtaining sections cut at the same depth of the parotoids.

Finally, manually and repeatedly compressing the parotoids does appear to cause changes in the types of bufadienolides detected and in their concentrations in *B. fowleri*. However, there was a very large amount of



individual variation in both types of bufadienolides detected and in how the concentrations of these bufadienolides changed during the sampling times.

## CHAPTER IV

### RESPONSE OF A VERTEBRATE PREDATOR TO THE PAROTOID SECRETIONS OF *BUFO AMERICANUS* AND *BUFO FOWLERI*

#### Introduction

Snakes are one of the most important predators of amphibians throughout North America (Conant and Collins 1998; Harding 1997) and here I investigate whether the parotoid secretions of the toads, *Bufo americanus* and *Bufo fowleri*, influences the response of a snake predator, *Thamnophis sirtalis*, and whether there is a difference in response to toad species. Throughout most of the Great Lakes Basin, *Thamnophis sirtalis sirtalis*, is the most commonly encountered subspecies of garter snake (Harding 1997). Survival and reproduction in garter snakes, as in many lizards and virtually all other snakes, depends heavily upon chemoreception (Lanuza and Halpern 1998). In fact, chemoreception is probably more important in squamate reptiles than in any other vertebrate group because it mediates many behaviors such as aspects of reproductive behavior, prey detection and localization, and aggregation of individuals (Lanuza and Halpern 1998). In garter snakes, prey are located by either sight and/or by the tongue and vomeronasal organ. *Thamnophis sirtalis* is considered to have a very broad diet (Seigel 1996) and to

prey heavily upon earthworms (Harding 1997), to prefer fish and small amphibians (Wright and Wright 1967), to be major toad predators (Lagler and Salyer 1945, Licht and Low 1968), and especially to prefer newly metamorphosed frogs and toads (Harding 1997). Occasionally, other types of prey are taken such as small mammals, birds, leeches, slugs, crayfish, insects, and other snakes, as well as carrion (Harding 1997; Conant and Collins 1998).

In Michigan, toads have been found to make up 25% of the diet of garter snakes living near natural waters (Lagler and Salyer 1945). Despite the fact that toads possess a variety of compounds in their cutaneous poison glands such as biogenic amines, peptides, proteins, both water-soluble and lipid soluble alkaloids, steroidal bufadienolides and bufotoxins (Daly 1995); garter snakes still find toads suitable if not preferred prey (Fitch 1941, 1965). Moreover, *Thamnophis sirtalis* seems to be particularly resistant to the poisons of many amphibians (Brodie 1968; Brodie and Brodie 1990a and 1990b; Licht and Low 1968; Macartney and Gregory 1981) and if an individual *T. sirtalis* consumes one to several toads, it is very unlikely to receive a lethal dose of toad chemicals (between 3-10 mg *B. marinus* secretion/g *T. sirtalis*) (Licht and Low 1968). In particular, the bufadienolides can be highly toxic and can give toads formidable chemical defenses by their potent inhibition of the membrane enzyme  $\text{Na}^+/\text{K}^+$  ATPase (Daly 1995) and by adversely affecting heart muscle by increasing the intensity of contraction and decreasing heart

rate (Clarke 1997, Voet and Voet 1990). The bufadienolides appear to be synthesized from cholesterol precursors *de novo* by the animals themselves, in fact, toads appear to produce all of their granular gland compounds with the exception of the alkaloids (Doull et al. 1951; Siperstein, et al. 1957; Daly 1995; Clark 1997). Also, the biological activities induced by bufadienolides are similar to those of plant cardenolides, such as syrioxide, desglucosyrioxide, and calotropin that provide monarch butterflies with their chemical defenses (Malcolm 1991).

Although garter snakes can consume toads with no apparent ill effects, some studies have shown that garter snakes exhibit selective responsiveness to chemical stimuli of preferred prey and that geographic variation in responsiveness is correlated with prey preference (Burghardt 1969, 1970; Arnold 1977, 1981). In a study by Macartney and Gregory (1981), differential susceptibility of garter snake species to toad secretions was considered. They used a tongue-flick assay to determine which of three garter snake species (*Thamnophis sirtalis*, *T. ordinoides*, and *T. elegans*) was most susceptible to toad chemicals. Their results showed that *T. sirtalis* and *T. elegans* had overall higher responsiveness to amphibian prey extracts than *Thamnophis ordinoides*. In fact, *T. ordinoides* did not consume *Bufo boreas* or *Taricha granulosa* as did the other two snake species. This suggested that chemical defenses of prey can act as deterrents to predation in a garter snake species. In addition to

differences in prey preference, garter snakes from different regions also show variation in their abilities to tolerate amphibian poisons (Brodie and Brodie 1991, Williams et. al 2002).

In southwest Michigan, snakes could encounter and prey upon two species of toads, American toad (*Bufo americanus*) and Fowler's toad (*Bufo fowleri*) and in this region *B. fowleri* has a smaller range than *B. americanus* but its populations can be locally large, especially along the southern and eastern shore of Lake Michigan (Harding 1997). Also, hybridization between both toad species is known to occur in some locations (Green 1982, Green and Parent 2003) and they can be heard calling together at the same breeding location in southwest Michigan (personal observation). Thus, snakes may be sympatric with one or both species of toads, or their hybrids, and may exhibit differential preferences for one toad species over the other as prey. Further, differences in prey preference in garter snakes could be attributed to differences in the chemicals (e.g., the bufadienolides) among toad species. I tested the responses of *T. sirtalis* to chemical stimuli from *B. americanus* and *B. fowleri* (in tongue-flick bioassays) to investigate potential differences in prey preference and to determine if toad parotoid secretions would deter predation.

## Methods

### *Collection and maintenance of snakes and toads*

Ten adult eastern garter snakes, *Thamnophis sirtalis sirtalis*, (6 females and 4 males) were collected from three locations in southwest Michigan (Kalamazoo County) in the summer (June-July) of 2001. These adult snakes had an average snout-vent length (SVL) of 47.7 cm and an average mass of 76.2 g. Two females (snakes 1 and 3) gave birth to litters of 15 (Litter A) and 5 (Litter B) young, respectively, while kept in the lab. The average mass of Litter A neonates was 3.1 g and Litter B neonates was 2.8 g. Another adult female was obtained in July of 2002 and gave birth in the lab to 20 young, five of which died prior to the analyses (Litter C). The average mass of Litter C neonates was 2.25 g.

Adult snakes were housed separately in 40 L glass aquaria and fed earthworms, guppies, and frozen mice at least once weekly or as often as they would accept food. Neonate snakes were housed separately in plastic rat cages (42.55 cm x 26.67 cm x 29.21 cm) and fed pieces of earthworms daily. Trials with newborn snakes were conducted 5-10 days after birth. Water was provided to all snakes *ad libitum*.

Snakes and toads were housed at the Animal Facility, Haenicke Hall, Western Michigan University, under the supervision of Lynn Plew. Care of

animals was according to IACUC protocol number 97-07-02. Adult snakes were kept in the Animal Facility for one month prior to the experiment to ensure that proper activity and feeding behavior would occur in captivity. Fluorescent light was provided on a 12-hour light/12-hour dark cycle and incandescent lamps were also used to provide additional heat. Ambient temperatures in the animal facility ranged between 20-22 °C, but temperatures in the aquaria were approximately 10 degrees warmer. Snakes were active and fed consistently under these laboratory conditions.

Adult toads (*Bufo americanus* and *Bufo fowleri*) were collected from several locations in Southwestern Michigan (Kalamazoo, Kent, and VanBuren Counties) in the summers of 2001 and 2002 to provide chemical stimuli for the assays. Toads were housed singly in either 40L glass aquaria or in plastic rat cages with sand substrate, a water dish, and terrarium moss for cover. Crickets and earthworms were provided as food. This research was conducted under the specifications described in IACUC proposal number 97-07-02 and in the Scientific Collector's Permit from the Michigan DNR number CO896.

#### *Tongue-flick bioassays*

A commonly used measure of responsiveness to chemical stimuli by squamate reptiles is the rate of tongue extrusions or tongue flicks that occur

within a set time interval (Halpern and Frumin 1979; Cooper and Vitt 1986; Cooper and Burghardt 1990; Cooper 1992; Halpern et al. 1997). Since the tongues of *T. sirtalis* and many other snakes and lizards are used as chemosensory organs, counting the number of tongue-flicks provides an easily measured, quantitative index of responsiveness to chemical stimuli (Cooper and Vitt 1986). Each tongue-flick bioassay was designed as a randomized block with repeated measures and followed the procedures described by Cooper et al. (1989 and 1990) and Wattiez et al. (1994). The stimuli tested were (1) distilled water (control), (2) *Bufo americanus* skin ("Baskin": dorsal and ventral surfaces), (3) *Bufo americanus* parotoid secretion ("Basec"), (4) *Bufo fowleri* skin ("Bfskin": dorsal and ventral surfaces), and (5) *Bufo fowleri* parotoid secretion ("Bfsec").

The bioassays involved soaking the cotton tip of a 15 cm wooden applicator in distilled water and swabbing both the dorsal and ventral surfaces of *B. americanus* and *B. fowleri* to obtain chemical stimuli. To obtain parotoid secretion, one parotoid gland was gently pressed between thumb and index finger. Expressed secretion was collected onto the entire surface of a cotton applicator.

In each trial, the top of the cage was slowly lifted and after 60 seconds had elapsed, the cotton applicator was slowly moved toward the snake's snout. The trial was started once the applicator was approximately 1 cm from



the snout. I recorded the total number of tongue-flicks directed toward the cotton tip in 60 seconds. Trials were terminated if a snake quickly fled from the applicator or bit the applicator. The elapsed time for both types of terminated trials was recorded as flee latency and bite latency respectively. If a snake moved slowly, as if actively searching, the applicator was slowly moved to stay anterior to the snake's snout. If a snake failed to tongue-flick in two or more trials, data for that individual snake were omitted due to a lack of vomeronasal activity.

I also adjusted tongue-flick data and used tongue-flick attack scores for repeated measures [TFAS(R)s] to weight bitten applicators. Tongue-flick attack scores gave an overall index of response strength where rapid attacks by biting are heavily weighted. If no bite occurred, then  $TFAS(R) = \text{number of tongue-flicks in the trial}$ . If a bite did occur, then  $TFAS(R) = \text{the maximum number of tongue-flicks by that snake in any of its trials} + (60 - \text{latency to bite})$  (Cooper 1998).

Adult snakes were starved for one week prior to the experiment, but neonates were fed during the experiment (post-trials) to prevent mortality. Each snake was randomly exposed to one of five treatments on consecutive days between 1200 and 1600 hrs EST beginning 20 August 2001 (Experiment 1: Adults, Litter A and Litter B neonates] and between the same times beginning 7 September 2002 [Experiment 2: Litter C neonates].

### *Data analyses*

Data (number of tongue-flicks and tongue-flick attack scores for repeated measures [TFAS(R)]) were checked for normality using Shapiro-Wilks W statistics. I used repeated measures analysis of variance to test for differences in the number of tongue-flicks and in tongue-flick attack scores among treatments if the distribution of errors were normality distributed and if variances were homogeneous. If the assumptions of ANOVA were violated, I used square root transformations of the data. If these transformations failed to bring the data into a normal distribution, the nonparametric analog, Friedman's test ( $\chi^2_r$ ) or its adjustment ( $F_F$ ) was used (Zar 1999). All tests were significant at  $\alpha = 0.05$ . In cases of significant tests, multiple comparisons were conducted for parametric tests (Fisher's Least Significant Difference) and for nonparametric tests (Studentized range or q value) according to Zar (1999). Analyses were conducted with JMP statistical software version 5.0.1.

### **Results**

In the first experiment, 30 snakes were tested (9 adults and 21 neonate snakes from Litters A and B). Three snakes failed to tongue-flick in two or more trials (snakes 8, 11, and 23) and were omitted from the analyses. Nine

snakes (snakes 2, 9, 14, 20, 22, 26, 27, 29 and 30) failed to tongue-flick in one trial. All other snakes tongue-flicked in all trials. In the second experiment, 20 snakes were tested (Litter C neonates). Five snakes failed to tongue-flick in two or more trials (snakes 31, 36, 37, 47, and 48) and data for these individuals were omitted from the analysis. Eight snakes failed to tongue-flick in one trial (snakes 33, 35, 39, 44, 45, 46, and 50). The remaining seven snakes tongue-flicked in all trials.

#### *Tongue-flick responses of *Thamnophis sirtalis**

For both adult and neonatal snakes, there was large variation in the total number of tongue-flicks in all experimental conditions, including the control (Tables 16, 17, 18, and 19). The number of tongue-flicks directed toward the applicators by adult snakes was not the same among the treatments ( $F_{df=4} = 3.1835$ ,  $P = 0.0261$ ). The average number of tongue-flicks directed toward secretion samples of *B. americanus* was significantly greater than the number of tongue-flicks directed toward the control (distilled water) ( $t = 22.67$ ,  $P < 0.05$ ) (Figure 31). The average number of tongue-flicks directed toward secretion stimuli of *B. americanus* was also significantly greater than the number of tongue-flicks directed toward skin stimuli of *B. americanus* ( $t = 24.44$ ,  $P < 0.05$ ) and *B. fowleri* ( $t = 20.89$ ,  $P < 0.05$ ) (Figure 31). However, the

Table 16

Adult *Thamnophis sirtalis* (n = 9) responses (number of tongue-flicks directed toward cotton applicators in 60-seconds) to toad skin stimuli, toad secretion stimuli, and distilled water.

Stimulus	Mean	Standard Error	Range
distilled water (control)	25.11 ab	6.46	0-56
<i>Bufo americanus</i> skin	23.33 a	5.09	3-47
<i>Bufo americanus</i> secretion	47.77 ab	7.87	0-80
<i>Bufo fowleri</i> skin	26.88 bc	7.53	1-54
<i>Bufo fowleri</i> secretion	43.00 c	4.70	18-59

Table 17

Litter A neonatal *Thamnophis sirtalis* (n = 13) responses (number of tongue-flicks directed toward cotton applicators in 60-seconds) to toad skin stimuli, toad secretion stimuli, and distilled water.

Stimulus	Mean	Standard Error	Range
distilled water	22.38 ab	6.24	0-77
<i>Bufo americanus</i> skin	17.46 a	4.01	0-42
<i>Bufo americanus</i> secretion	35.08 bc	5.89	5-74
<i>Bufo fowleri</i> skin	32.54 bc	4.96	6-66
<i>Bufo fowleri</i> secretion	37.46 c	5.05	1-63

Table 18

Litter B neonatal *Thamnophis sirtalis* (n = 5) responses (number of tongue-flicks directed toward cotton applicators in 60-seconds) to toad skin stimuli, toad secretion stimuli, and distilled water.

Stimulus	Mean	Standard Error	Range
distilled water	3.60 a	2.46	0-13
<i>Bufo americanus</i> skin	26.60 b	8.85	10-51
<i>Bufo americanus</i> secretion	28.00 b	7.69	0-45
<i>Bufo fowleri</i> skin	31.40 b	10.14	0-56
<i>Bufo fowleri</i> secretion	28.20 b	5.61	16-48

Table 19

Litter C neonatal *Thamnophis sirtalis* (n = 15) responses (number of tongue-flicks directed toward cotton applicators in 60-seconds) to toad skin stimuli, toad secretion stimuli, and distilled water.

Stimulus	Mean	Standard Error	Range
distilled water	7.67 a	2.38	0-30
<i>Bufo americanus</i> skin	18.53 a	3.88	1-49
<i>Bufo americanus</i> secretion	19.00 a	5.07	0-58
<i>Bufo fowleri</i> skin	21.20 a	4.06	0-40
<i>Bufo fowleri</i> secretion	17.80 a	4.20	0-47

secretion and skin stimuli of *B. fowleri* did not elicit more tongue-flicks when compared with the control (Figure 31).

The average number of tongue-flicks directed toward the applicators by neonatal snakes was not the same among the treatments with the exception of responses by Litter C snakes (Litter A:  $F_{df=4} = 2.89$ ,  $P = 0.03$ ; Litter B:  $F_{df=4} = 2.99$ ,  $P < 0.05$ ; Litter C:  $F_F = 1.59$ ,  $P > 0.10$ ) (Tables 17, 18, and 19; Figures 31, 32, and 33). No significant differences were found in the number of tongue-flicks among the treatments for Litter C neonates. When the data for all neonates was combined, combined, the number of tongue-flicks was different among the treatments ( $F_{df=4} = 3.77$ ;  $P = 0.006$ ) (Figure 35).

For the significant analyses, differences in the number of tongue-flicks among treatments were similar to that of adults but not identical. For example, neonates from both litters (A and B) exhibited enhanced responses toward parotoid secretions compared to that of the control (Litter A:  $Bfsec > water$ ,  $t = 15.08$ ,  $P < 0.05$ ; Litter B:  $Basec$  and  $Bfsec > water$ ,  $t = 24.40$ ,  $P < 0.05$ ; and  $t = 24.60$ ,  $P < 0.05$ , respectively) (Figures 32 and 35). Also, neonates from Litter A tongue-flicked more toward parotoid secretion than they did toward skin stimuli ( $Basec > Baskin$ ,  $t = 17.62$ ,  $P < 0.05$ ;  $Bfsec > Baskin$ ,  $t = 20.00$ ,  $P < 0.05$ ) (Figure 31). In the case of Litter B neonates, the average number of tongue-flicks directed toward all of the toad stimuli were greater than that of

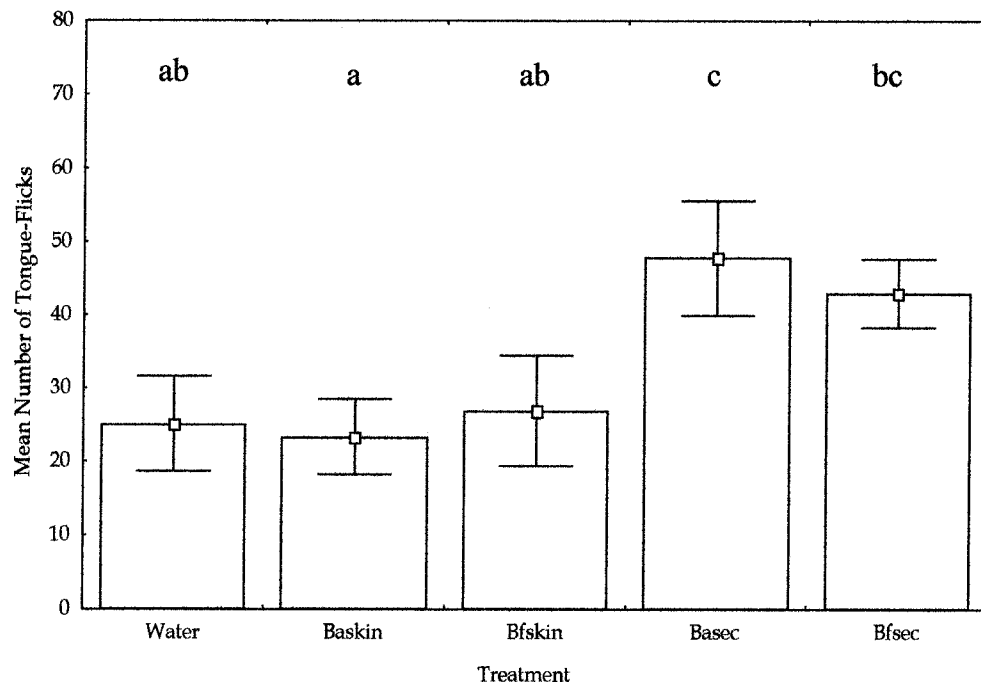


Figure 31. Mean number ( $\pm$  SE) of tongue-flicks directed toward cotton applicators by adult garter snakes from experiment one. Treatments joined by the same letter are not statistically different.

the control (Baskin > water,  $t = 23.00$ ,  $P < 0.05$ ; Basec > water,  $t = 24.40$ ,  $P < 0.05$ ; Bfskin > water,  $t = 27.80$ ,  $P < 0.05$ ; and Bfsec > water,  $t = 24.60$ ,  $P < 0.05$ ) (Figure 33).

*Tongue-flick attack scores for repeated measures [TFAS(R)s]*

In the first experiment, a total of five snakes bit applicators with either toad skin or toad secretion stimuli. Snakes never bit applicators soaked in

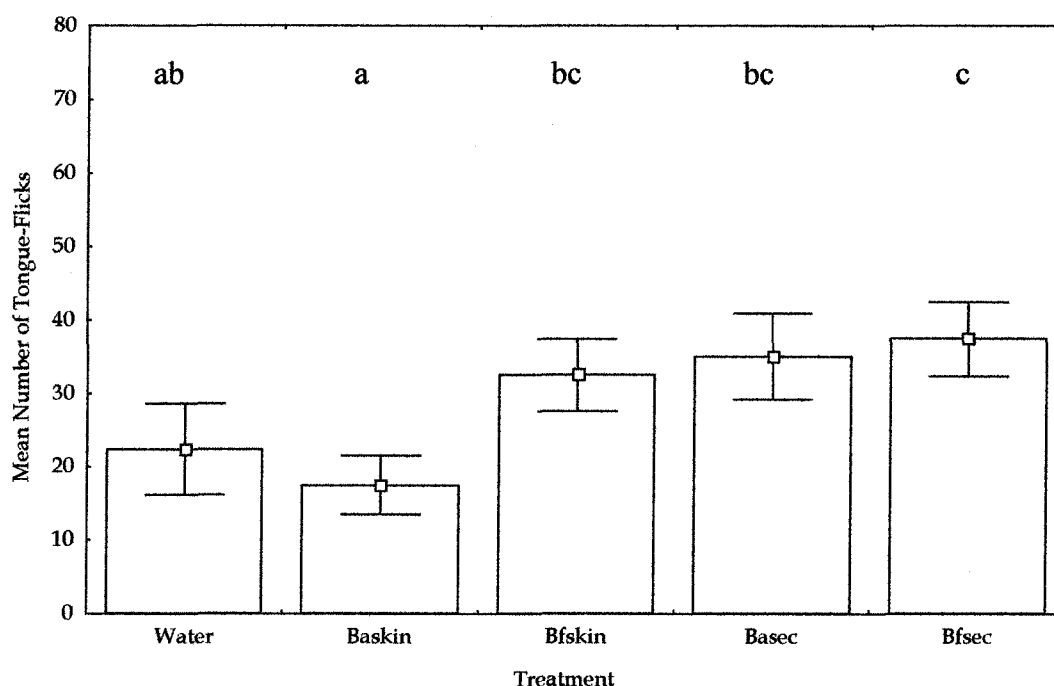


Figure 32. Mean number ( $\pm$  SE) of tongue-flicks directed toward cotton applicators by neonatal snakes (Litter A). Treatments joined by the same letter are not statistically different.

only distilled water. Three adult snakes and one neonate snake bit applicators with toad skin stimuli [Baskin had one bite (snake 1) and Bfskin had three bites (snakes 5, 6, and 13)]. One other adult snake bit an applicator with toad secretion stimuli [Bfsec (snake 4)]. None of the snakes bit applicators in more than one of its trials. Tongue-flick attack scores for repeated measures [TFAS(R)] for each experimental treatment are shown in Tables 20-23. There were no significant differences found in TFAS(R)s for adult and neonate snakes from Litters B and C (adults:  $F_{df=4} = 1.24$ ,  $P = 0.31$ ;



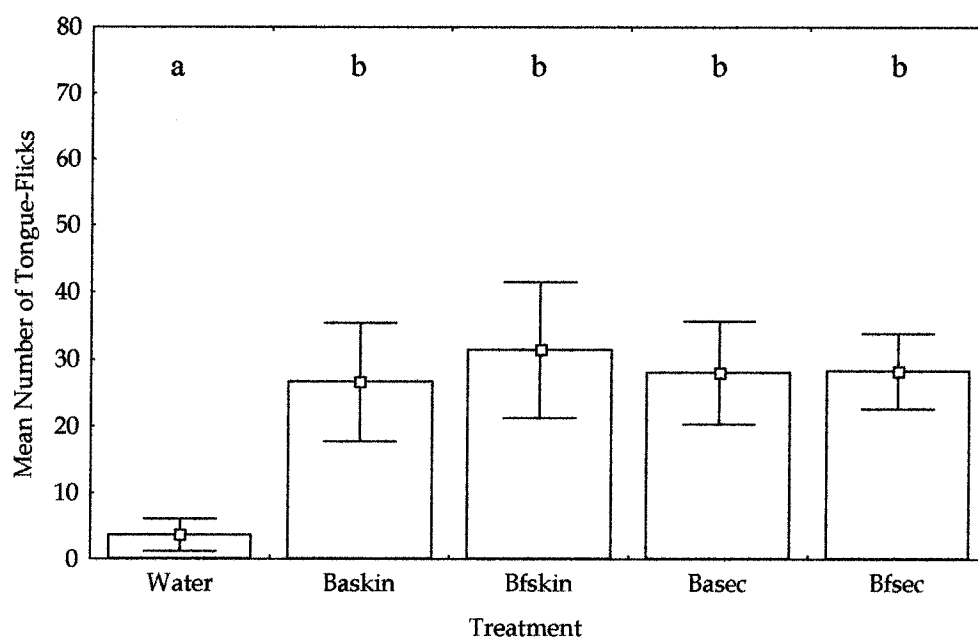


Figure 33. Mean number ( $\pm$  SE) of tongue-flicks directed toward cotton applicators by neonatal snakes (Litter B). Treatments joined by the same letter are not statistically different.

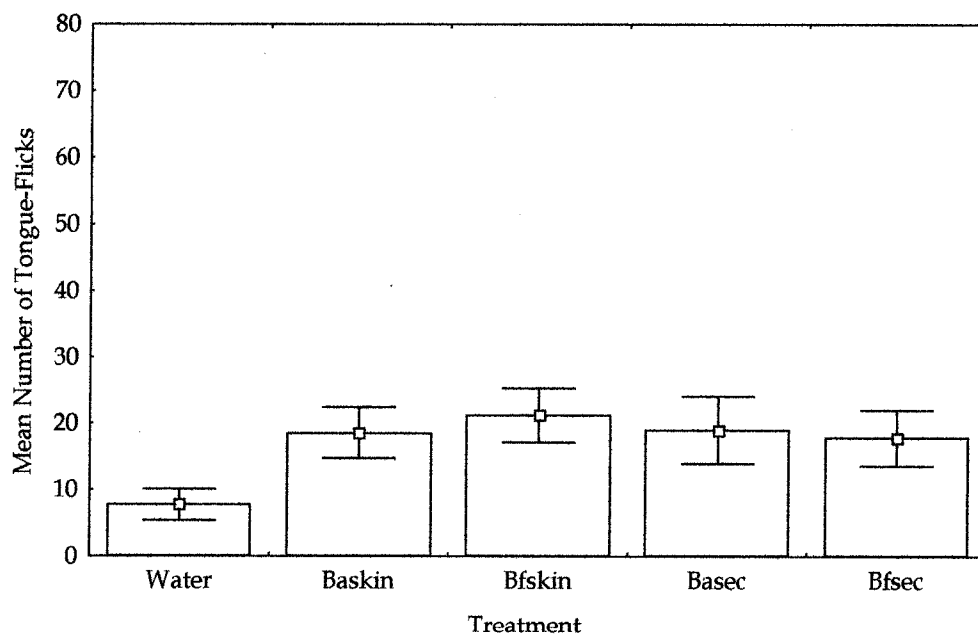


Figure 34. Mean number ( $\pm$  SE) of tongue-flicks directed toward cotton applicators by neonatal snakes (Litter C). Treatments were not statistically different.

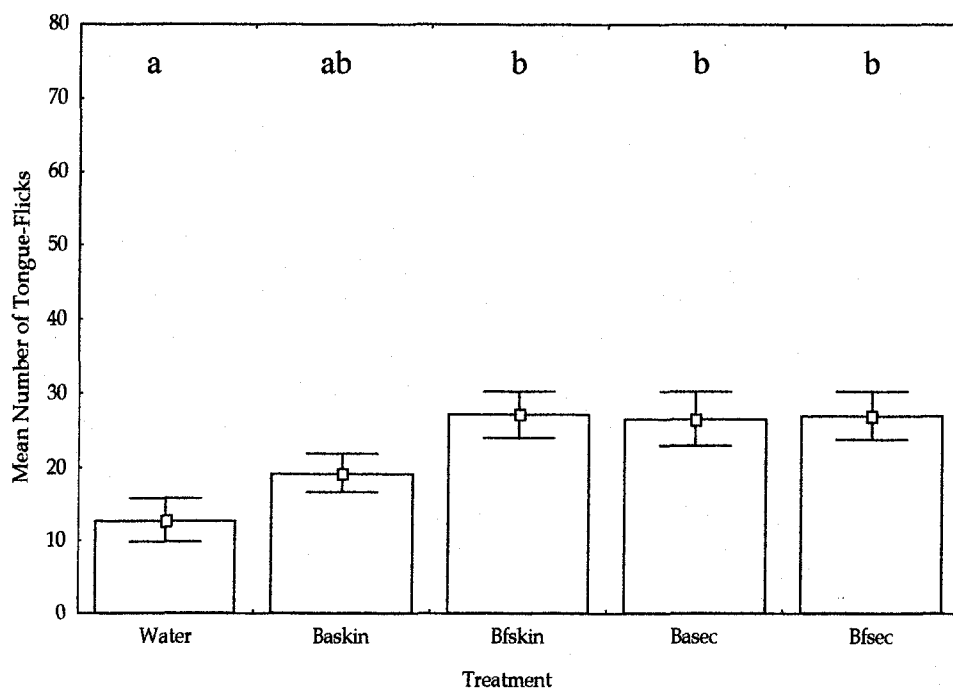


Figure 35. Mean number ( $\pm$  SE) of tongue-flicks directed toward cotton applicators by all neonatal snakes combined (Litters A, B, and C).

Litter B:  $\chi^2 = 5.92$ ,  $P > 0.10$ ; Litter C:  $F_{df=4} = 2.28$ ,  $P = 0.07$ ) (Tables 20, 22, and 23; Figures 36, 38, and 39). Responses of Litter A neonates [TFAS(R)s] were significantly different among treatments ( $F_{df=4} = 3.40$ ,  $P = 0.02$ ) (Table 21 and Figure 37). Specifically, TFAS(R)s were greater in treatments with *B. fowleri* skin and secretion stimuli compared to the control (Bfskin > water,  $t = 1.89$ ,  $P < 0.05$ ; Bfsec > water,  $t = 1.83$ ,  $P < 0.05$ ). Also, *B. americanus* skin stimuli

Table 20

Adult *Thamnophis sirtalis* (n = 9) responses [TFAS(R)s] to toad skin stimuli, toad secretion stimuli, and distilled water.

Stimulus	Mean	Standard Error	Range
Water	25.11 a	6.46	0-56
<i>Bufo americanus</i> skin	34.44 a	10.20	3-107
<i>Bufo americanus</i> secretion	47.78 a	7.87	0-80
<i>Bufo fowleri</i> skin	43.44 a	10.99	1-90
<i>Bufo fowleri</i> secretion	48.11 a	8.28	18-105

Table 21

Litter A neonatal *Thamnophis sirtalis* (n = 13) responses [TFAS(R)s] to toad skin stimuli, toad secretion stimuli, and distilled water.

Stimulus	Mean	Standard Error	Range
Water	28.38 ab	6.24	0-77
<i>Bufo americanus</i> skin	17.46 a	4.01	0-42
<i>Bufo americanus</i> secretion	35.08 bc	5.89	5-74
<i>Bufo fowleri</i> skin	39.08 c	7.62	6-109
<i>Bufo fowleri</i> secretion	37.46 c	5.05	1-63

Table 22

Litter B neonatal *Thamnophis sirtalis* (n = 5) responses [TFAS(R)s] to toad skin stimuli, toad secretion stimuli, and distilled water.

Stimulus	Mean	Standard Error	Range
Water	3.60 a	5.50	0-13
<i>Bufo americanus</i> skin	26.60 a	19.78	10-51
<i>Bufo americanus</i> secretion	28.00 a	17.19	0-45
<i>Bufo fowleri</i> skin	31.40 a	22.67	0-56
<i>Bufo fowleri</i> secretion	28.20 a	12.54	16-48

Table 23

Litter C neonatal *Thamnophis sirtalis* (n = 15) responses [TFAS(R)s] to toad skin stimuli, toad secretion stimuli, and distilled water.

Neonates (n = 15)	Mean	Standard Error	Range
Water	7.67 a	2.38	0-30
<i>Bufo americanus</i> skin	18.53 a	3.88	1-49
<i>Bufo americanus</i> secretion	23.13 a	6.27	0-75
<i>Bufo fowleri</i> skin	28.07 a	6.69	0-105
<i>Bufo fowleri</i> secretion	25.93 a	6.56	0-73

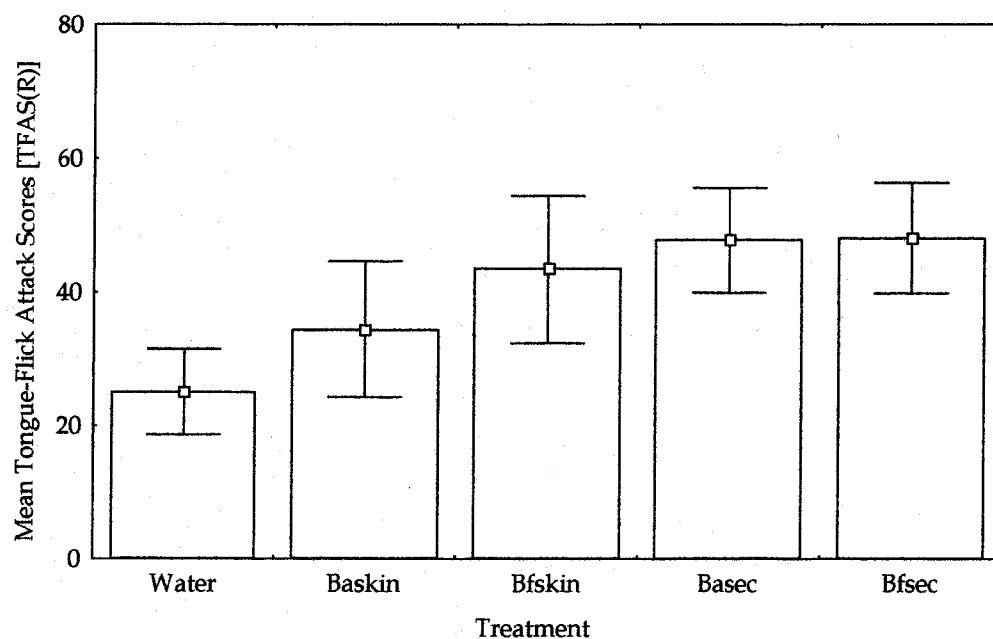


Figure 36. Mean ( $\pm$  SE) tongue-flick attack scores for repeated measures [TFAS(R)s] generated from adult snakes. Treatments were not statistically different.

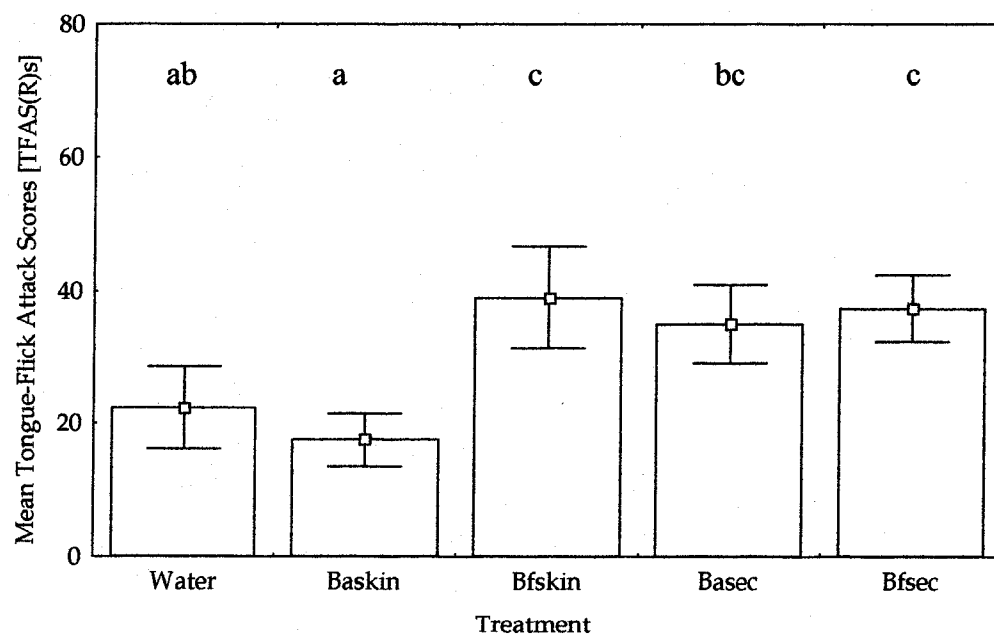


Figure 37. Mean ( $\pm$  SE) tongue-flick attack scores for repeated measures [TFAS(R)s] generated from neonatal snakes from Litter A. Treatments joined by the same letter are not statistically different.

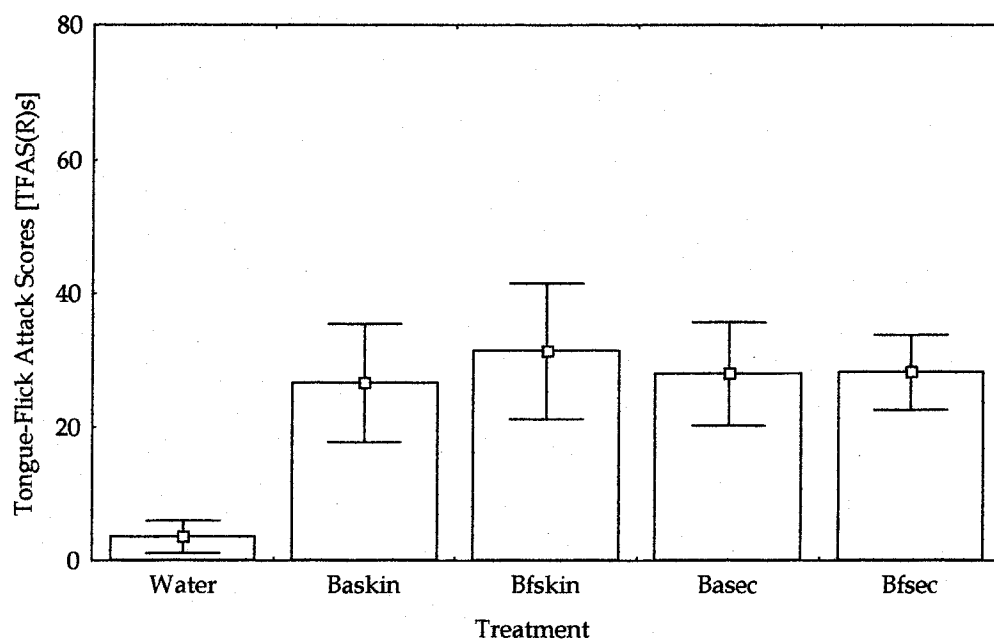


Figure 38. Mean ( $\pm$  SE) tongue-flick attack scores for repeated measures [TFAS(R)s] generated from neonatal snakes from Litter B. Treatments were not statistically different.

received lower responses when compared to *B. americanus* secretion, *B. fowleri* skin and *B. fowleri* secretion stimuli ( $t = 1.91$ ,  $t = 2.21$ , and  $t = 2.14$  respectively). When data for all neonates were combined, [TFAS(R)s] were different among the treatments ( $F_{df = 4} = 4.84$ ;  $P = 0.001$ ) and results were identical to that for the numbers of tongue-flicks with all neonates combined (Figure 40).

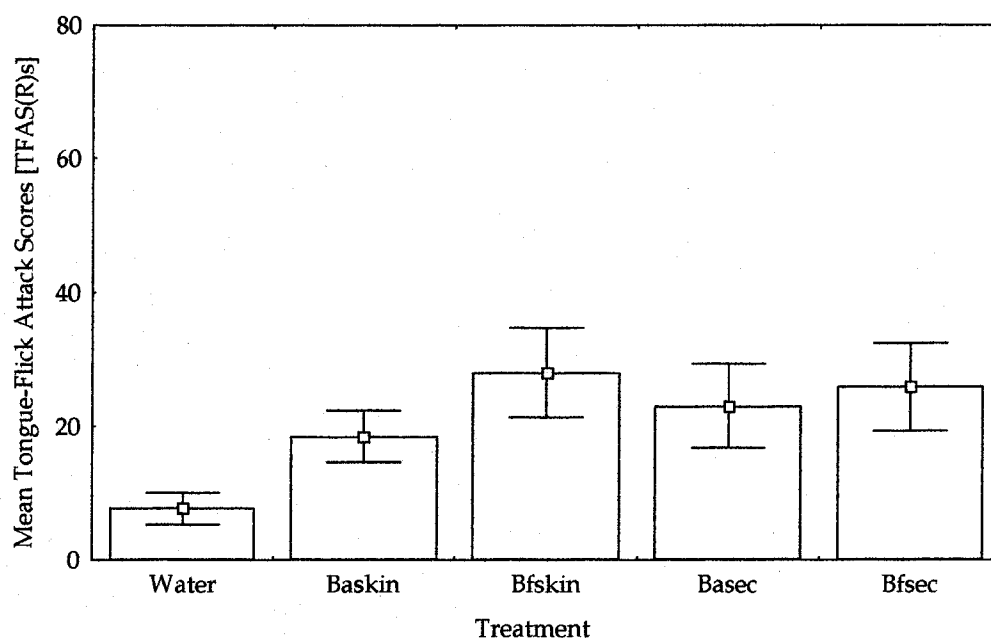


Figure 39. Mean ( $\pm$  SE) tongue-flick attack scores for repeated measures [TFAS(R)s] generated from neonatal snakes (Litter C). Treatments were not statistically different.

## Discussion

Although Bufonid toads possess chemical defenses that include steroidal bufadienolides with potent cardiotonic activity, garter snakes, *Thamnophis sirtalis*, are able to consume them with no apparent ill effects (Brodie 1968; Brodie and Brodie 1990a and 1990b; Licht and Low 1968; Macartney and Gregory 1981). In fact, this snake and other *Thamnophis* species are particularly resistant to a wide variety of amphibian poisons,

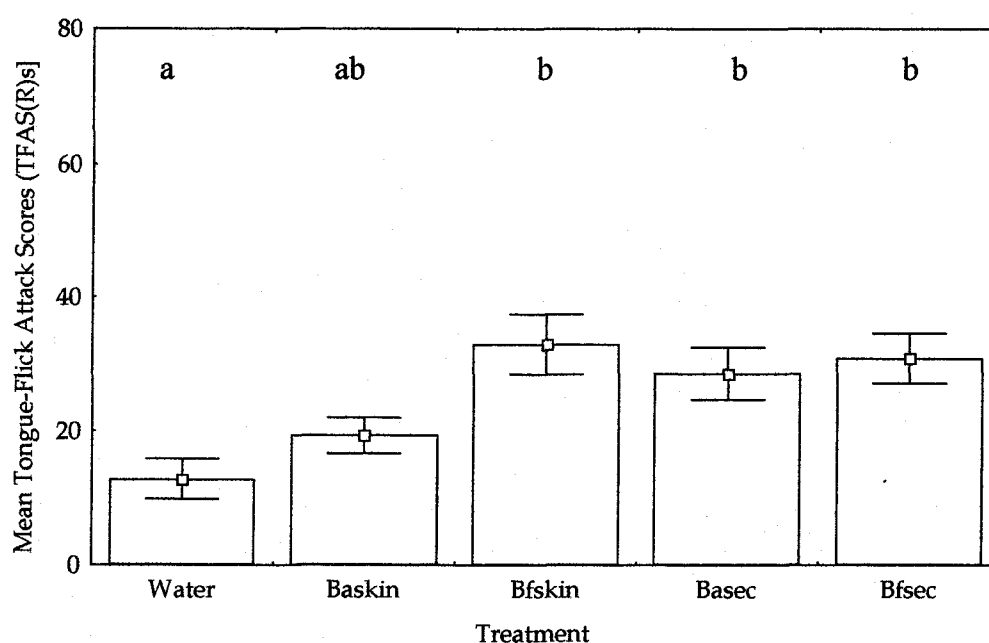


Figure 40. Mean ( $\pm$  SE) tongue-flick attack scores for repeated measures [TFAS(R)s] generated from all neonatal snakes combined (Litters A, B, and C). Treatments joined by the same letter are not statistically different.

including tetrodotoxin from the rough-skinned newt, and this resistance to amphibian chemicals is enhanced in locations where snakes typically encounter noxious or toxic prey (Brodie and Brodie 1990b).

Garter snakes are also known to exhibit selective responsiveness to preferred prey (Burghardt 1969, 1970; Arnold 1977, 1981) and research has shown that *Thamnophis* snakes exhibit some differential susceptibility to toad secretions (Macartney and Gregory 1981). I tested the responsiveness of garter snakes to chemical stimuli from the toads *Bufo americanus* and *Bufo*



*fowleri* to investigate potential differences in prey preference and to determine if toad parotoid secretions would deter predation.

Results of the first experiment indicate that eastern garter snakes (adults and neonates) respond to chemical stimuli from both *B. americanus* and *B. fowleri* with more tongue-flicks and greater tongue-flick attack scores than snakes exposed to distilled water. Snakes in experiment two (Litter C neonates) showed no significant differences in the number of tongue-flicks or in TFAS(R) among the treatments. This suggests that some neonatal garter snakes show no more interest in toad chemicals compared to the control (distilled water). However, data from the neonates in experiment one did show significantly increased responses to toad stimuli. Litter A neonates tongue-flicked more toward parotoid secretion than toward skin stimuli while Litter B neonates responded with more tongue flicks in all toad treatments compared to the control. These differences in responsiveness between groups of siblings could simply reflect genetic differences for prey recognition among litters (Burghardt 1975).

Because snakes in the first experiment tongue-flicked more often and had greater tongue-flick attack scores in treatments with parotoid secretions than in the control (distilled water), toad parotoid secretions elicit investigative and predatory behaviors in these snakes. Results also suggest no preference for one toad species over the other since snakes responded to

both toad species secretion above the control. However, four out of five total bites occurred with *B. fowleri* stimuli (3 bites on skin stimuli and 1 bite on secretion stimuli). Only one bite occurred with *B. americanus* skin stimuli. This might suggest that some snakes are more likely to prefer *B. fowleri* as prey although TFAS(R)s did not specifically show this discrimination. Presumably no differences existed in the amounts of chemical stimuli gathered from the two toad species to affect these results, yet prey odor concentrations are, in fact, important for snakes to locate food by trailing and snakes will also follow intense odor trails more accurately than less intense trails (Halpern 1983; Waters 1993). However, because sibling newborn garter snakes can recognize preferred prey in low concentrations (Burghardt 1975), snakes in this experiment could be exhibiting a true preference for *B. fowleri* as prey. This result is interesting given that the range of *B. fowleri* is smaller than that of *B. americanus* in southwest Michigan. A preference for *B. fowleri* by snake predators may actually account for the smaller abundances and distributions of *B. fowleri* in Michigan. Also, it would be interesting to investigate the actual toad cue molecules that the snakes are sensing with their vomeronasal organs to determine if they can distinguish between species of toads.

Finally, because snakes tongue-flicked more toward parotoid secretions (of both species) than they did toward *B. americanus* skin stimuli, it

may appear as though the snakes are not deterred by toad parotoid chemicals, but rather, are more interested in secretion stimuli. A stronger response toward parotoid secretions could also indicate a strong signal to the snakes, thus stimulating predatory behaviors irrespective of the nature of the cue (indicating either a palatable or an un-palatable prey item). Interpretation of this result should be made cautiously because, as mentioned above, snakes could be responding with more tongue-flicks toward secretion stimuli simply because the secretion presented a more concentrated toad cue as compared to a less intense toad cue as given by the skin stimuli. Parotoid secretion could, however, deter strikes since snakes bit more applicators with toad skin stimuli (4 bites) than they did with secretion stimuli (1 bite). Thus, snakes may recognize a toad as a prey item and be more apt to attack if the cue is toad skin rather than toad secretion, but because only five total bites occurred in the experiments it is not entirely clear whether parotoid secretion actually deters predation by *T. sirtalis*. Rather, parotoid secretion does not appear to deter predation since in the trials where snakes did bite applicators with toad secretions, they did not appear to release the applicators any sooner than they released bitten applicators with toad skin stimuli, a finding which is probably not surprising since *T. sirtalis* is known to have tolerance to the chemical defenses of many amphibian species.

## CHAPTER V

### RESPONSE OF AN INVERTEBRATE PREDATOR TO THE LARVAE OF *BUFO AMERICANUS* AND *BUFO FOWLERI*

#### Introduction

Amphibian larvae are prey for a diverse variety of aquatic and terrestrial natural enemies. With their widespread distributions and large abundances, especially during stages of egg and larval development, amphibians are important parts of many food webs. All amphibian life history stages (eggs, larvae, metamorphs, and adults) are vulnerable to predation (Villa et al. 1982; Arnold and Wassersug 1978) and numerous kinds of vertebrate predators such as birds, turtles, fishes, small mammals, and snakes commonly feed on tadpoles (Duellman and Trueb 1986). In addition, invertebrate predators also prey heavily upon amphibian larvae. Collectively, predation is believed to be the major source of tadpole mortality based on field studies of survival rates (Alford 1999). The focus of this part of my research is on the response of invertebrate predators to larvae of *B. americanus* and *B. fowleri* because these predators can be an important and

abundant component of the freshwater habitats in which toad larvae develop (Brockelman 1969; Brodie et al. 1978).

Aquatic insects such as dragonfly larvae (e.g., *Anax* and *Pantala*), predaceous diving beetle larvae (e.g., *Acilius* and *Dytiscus*), and giant water bugs (e.g., *Belostoma*) are among the most important predators of amphibian larvae (Brockelman 1969; Heyer et al. 1975). Swart and Taylor (2004) observed that belostomatid adults abundantly co-occur with toad tadpoles, *Bufo woodhousii*, and that these bugs readily feed on tadpoles. Larval dytiscid beetles, in particular, are important predators of *Bufo americanus* tadpoles (Brockelman 1969; Brodie et al. 1978) despite the fact that *B. americanus* eggs and tadpoles have defensive characteristics attributed to toxic and noxious chemicals (Brodie et al. 1978; Brodie and Formanowicz 1987; Brodie and Tumbarello 1978).

Even though tadpoles are prey for many organisms, their secretions do repulse some predators. For example, a dytiscid beetle, *Dytiscus verticalis*, avoids newt secretions (Brodie and Formanowicz 1981) and hatchling tadpoles of *B. americanus* are unpalatable to a vertebrate predator, the red-spotted newt (*Notophthalmus viridescens*), to dragonfly naiads (*Anax junius*), and to giant water bugs (*Belostoma*) (Brodie and Formanowicz 1987). Also, *Bufo americanus* in metamorphic climax (Gosner stages 42-46) are unpalatable

to predators (Brodie et al. 1978). Kats et al. (1988) showed that *B. americanus* larvae are unpalatable to two species of sunfish (*Lepomis cyanellus* and *Lepomis macrochirus*) and Lardner and Loman (1995) found crucian carp (*Carassius carassius*) avoid consuming *Bufo bufo* tadpoles. Poisonous characteristics of amphibians are most prominent after transformation when the mature skin secretions contain various compounds that include, depending on the species, such as biogenic amines, peptides, proteins, both water-soluble and lipid soluble alkaloids, and steroids (bufadienolides and bufotoxins) (Daly 1995).

Some studies have suggested that the piercing and sucking technique used by some aquatic insects allows them to avoid consuming too much of the tadpole skin where chemical defenses are most concentrated (Flier et al. 1980; Wassersug 1971; and Wassersug 1973). However, anuran larvae lack the skin glands (granular and mucus glands) that are found in adults (Hayes and Gill 1995). *Rana sylvatica* tadpoles were found to have many small developing granular glands, with little or no secretion, when they were fully palatable to *Dytiscus* (between Gosner stages 38-39), while in later stages (44+) the glands were larger in size and were presumed to be actively producing secretion (Formanowicz and Brodie 1982). Furthermore, the large clusters of granular glands that comprise the parotoid glands are not found in tadpoles.

For example, in three species of North American *Bufo* that Licht (1967) raised in the laboratory, the initial appearance of parotoid glands requires between 18-29 days post metamorphosis. Also, in a salamander, *Ambystoma gracile*, the parotoid glands are only present in metamorphosed individuals (Licht and Sever 1993). These data suggest that larval toads are more vulnerable to predation than metamorphosed toads and that predation is contingent upon toad development. Larval toad defense may also vary in effectiveness according to predator feeding tactics. Thus, "bite-and-chew" predators may be more susceptible to prey chemical defenses than "pierce-and-suck" predators that may circumvent skin defenses.

Dytiscid beetle larvae use a pierce-and-suck strategy for consuming prey. These larvae inject a cocktail of protease enzymes that liquefies body contents and then ingest the resulting fluid (Young 1967). This pierce-and-suck feeding technique is thought to be superior to other techniques (i.e., bite-and-chew) to avoid tadpole chemical defenses (Wassersug 1973). However, Dytiscid larvae are known to consume surface tissues as well as entire tadpoles (*B. marinus*) (Crossland 1998) and are classified as predatory swallowers that consume parts or whole animals (Cummins 1973 as cited by Williams and Feltmate 1992). The pierce-and-suck strategy may not completely enable an insect to avoid tadpole unpalatability because some

predators that suck body fluids are in fact repulsed by the secretions of newly metamorphosed *Bufo americanus* (Brodie et al. 1978).

Palatability of tadpoles to predators has been shown to change with ontogeny in some studies (Brodie et al. 1978; Formanowicz and Brodie 1982; Brodie and Formanowicz 1987; Denton and Beebee 1997; Crossland 1998; Crossland and Alford 1998), but no stage-dependent palatability was found in *Bufo boreas* larvae under *Dytiscus* predation (Peterson and Blaustein 1992). Interestingly, dytiscids are also known for their own defensive steroids and may perhaps be immune to the defenses of toads (Miller and Mumma 1974).

Brodie and Formanowicz (1987) described a graphical model that, in part, illustrates the change in tadpole palatability during ontogeny. This model shows that tadpoles garner protection from predation due to chemical defenses early in development and during metamorphic climax as depicted by lines B<sub>1</sub> and B<sub>2</sub> (Chapter II, Figure 5). Specifically, during the very early developmental stages (hatchlings, Gosner stages 20-25), individuals are unpalatable to predators perhaps as a result of chemical defenses present in the yolk of eggs through parental investment. Protection during these early stages is followed by a drop in stage-specific survival when intermediate-stage tadpoles [within the range of Gosner stages 26-41 but the explicit stages of palatability examined by Brodie and Formanowicz (1987) were between



Gosner stages 29-33] lose their chemical defenses and become palatable to predators. Then unpalatability and survivorship increase again as the tadpoles approach metamorphosis (beginning with the emergence of forelimbs, Gosner stage 42) and reach a peak when metamorphosis is complete (Gosner stage 46) (Chapter II, Figure 5). The prediction of this stage-specific palatability model is that amphibian larvae should possess chemical defenses during their development when they are not palatable to predators. In one bufonid species, *Bufo calamita*, early developmental stages (just after hatching) suffer high mortality due to invertebrate predation and then the relative strength of predation falls as tadpoles become larger (Denton and Beebee 1997). Thus, there are exceptions to this generalized survivorship model.

I tested the responses of predaceous diving beetle larvae (*Acilius sp.* and *Dytiscus sp.*) to the larvae of American toad (*Bufo americanus*) and Fowler's toad (*Bufo fowleri*). My interest was to determine if these aquatic insect larvae would respond differently (by attack, consumption, and survival) when exposed to tadpoles in intermediate stages of development and in metamorphic climax. If predators respond differently to intermediate stages (i.e., with greater attack, consumption, and survival) than they do with tadpoles approaching metamorphosis, this could be related to chemical

defenses that appear when the granular glands mature and become active as individuals approach and complete metamorphosis. With this in mind, I wanted to relate the presence and/or absence of one group of chemical defenses, the steroidal bufadienolides, to this variability in palatability during toad development. My other interest was to determine if dytiscid larvae would discriminate between *B. americanus* and *B. fowleri* as prey. Again, a difference in prey preference could be attributed to variation in chemical defenses between these toad species. In Chapter II, I found that on average bufadienolides were much more concentrated in the early toad developmental stages than in later stages but that the average number of bufadienolides in transformed individuals was not distinctly different from earlier stages like eggs (Chapter II, Figures 10 and 11).

## Methods

### *Animal collection*

Dytiscid larvae (*Acilius sp.* and *Dytiscus sp.*) and toad tadpoles (*Bufo americanus* and *Bufo fowleri*) were collected from several freshwater ponds in southwest Michigan (Kalamazoo and VanBuren Counties) by dip-netting in June 2002. Total lengths of individuals were measured with vernier calipers

to the nearest 0.01 cm and wet masses were measured to the nearest 0.0001 g. Masses were taken prior to the experiment for all predators and prey and at the end of the experiments for surviving individuals. Tadpoles were staged according to Gosner (1960) prior to the experiments. Feeder guppies, *Poecilia reticulata*, of similar lengths and masses to tadpoles were obtained from a local pet shop and served as alternative prey to *Bufo*. Dyticid beetles were starved for 24 hr prior to the start of the experiments.

#### *Experimental design*

I conducted a laboratory experiment designed following the methods of Crossland (1998) and Crossland and Alford (1998) to assess the responses of aquatic insect predators to toad tadpoles. The experiments were conducted in glass finger bowls containing 250 mL aged tap water. The insect larvae were able to breathe at the surface and predators could not escape from the containers. Fresh water was added as needed during the experiments.

All glass bowls were placed on a laboratory bench-top in a 5 x 5 array (5 treatments and 5 replicates) in a completely randomized design. The five treatments were: (1) one predator alone (predator control), (2) one *B. americanus*, one *B. fowleri*, and one *P. reticulata* alone (prey control), (3) one

predator plus one *B. americanus* and one *P. reticulata* (predator treatment), (4) one predator plus one *B. fowleri* and one *P. reticulata* (predator treatment), (5) one predator plus one *B. americanus*, one *B. fowleri*, and one *P. reticulata* (predator treatment). Experiments were monitored at 12 hr intervals for the condition of both predators and prey for a total of 72 hrs. Alternative prey were replaced when consumed. Predators that consumed prey were kept for 24 hr after the experiment to assess their condition.

#### *Data analyses*

I tested the survival of predators exposed to *Bufo* prey using Chi-square contingency analyses. The Chi-square analysis was constructed as a 2x2 table that classified predators as either exposed or not exposed to *Bufo* and predator response (survived or did not survive). If a statistical difference was found using the 2x2 Chi-square contingency analysis, I subdivided the exposure to *Bufo* by species to represent the five treatments as described above and conducted a 5x2 Chi-square contingency analysis.

I used t-tests to examine if (1) tadpoles of both toad species were similar in both length and mass, (2) predators were similar in length and mass throughout the experiment, and (3) alternative prey were similar in length and mass to *Bufo* prey. I also used a one-way analysis of variance to

determine if the sizes of predators were similar among the experimental treatments. Analyses were conducted with JMP statistical software version 5.0.1 and considered statistically significant at  $\alpha = 0.05$ .

## Results

Table 24 shows the average lengths and masses of animals used in the experiment. Although *Acilius* were smaller than *Dytiscus* in both length and mass, there were no differences in their sizes (in length or mass) among treatments ( $F = 0.44$ ,  $P = 0.52$ ;  $F = 0.01$ ,  $P = 0.94$  respectively). *Bufo americanus* and *B. fowleri* tadpoles were similar in both length ( $t = 1.58$ ,  $P = 0.12$ ) and in mass ( $t = 1.69$ ,  $P = 0.102$ ) (Table 1). Also, *P. reticulata* were similar in mass to both *B. americanus* and *B. fowleri* ( $t = 0.51$ ,  $P = 0.66$  and  $t = 0.75$ ,  $P = 0.53$  respectively) (Table 24).

### *Predator response – attack and consumption*

Seven predators attacked *Bufo* tadpoles and three predators attacked both *B. americanus* and *B. fowleri* for a total of ten attacks on separate individuals (5 on *B. americanus* and 5 on *B. fowleri*). Thus, predator response by attack was equal for the two toad species. All attacks occurred within the

Table 24

Mean lengths and masses ( $\pm$  SE) for predators and prey used in the experiment. Starting masses were measured prior to the experiment and the ending masses were measured at 72 hrs.

	Mean total length (cm) $\pm$ SE	Mean start mass (g) $\pm$ SE	Mean end mass (g) $\pm$ SE
<u>Predators</u>			
Dytiscidae			
<i>Acilius sp.</i>	1.65 $\pm$ 0.05	0.17 $\pm$ 0.016	0.32 $\pm$ 0.13
<i>Dytiscus sp.</i>	4.17 $\pm$ 0.31	1.17 $\pm$ 0.22	1.21 $\pm$ 0.20
<u>Prey</u>			
Bufonidae			
<i>Bufo americanus</i>	2.38 $\pm$ 0.08	0.26 $\pm$ 0.03	0.15 $\pm$ 0.04
<i>Bufo fowleri</i>	2.55 $\pm$ 0.07	0.21 $\pm$ 0.02	0.13 $\pm$ 0.03
Poeciliidae			
<i>Poecilia reticulata</i>	1.77 $\pm$ 0.41	0.16 $\pm$ 0.07	

first 12 hours of observation. In fact, within the first 15 minutes of adding prey, both *Bufo* species were attacked and eaten by five different predators. These five predators and two others preyed on *Bufo* before *Poecilia*. Three predators attacked and consumed part or all of both *Bufo* prey before attacking or ignoring *Poecilia*. In only one instance did a predator attack

*Poecilia* prior to *Bufo*. More *Poecilia* were consumed in the predator control compared to the *Bufo* treatments ( $F = 13.09$ ,  $P = 0.002$ ).

All ten attacks on *Bufo* resulted in tadpole mortality with eight of the attacks resulting in partial or total consumption of the tadpole prey. Two attacks involved refusal of *B. fowleri* tadpoles with no obvious signs that any consumption had occurred. All *B. americanus* tadpoles were partially or totally consumed by either *Acilius* or *Dytiscus*. Six tadpoles between Gosner stages 40-42 were attacked and four between Gosner stages 34-38 were attacked. Eight predators did not attack *Bufo* tadpoles in the experiment.

#### *Predator response—survival*

Out of 15 predators that were exposed to *Bufo*, a total of nine predators died within 72 hours of exposure and a statistical difference between predator survival and *Bufo* exposure was found ( $\chi^2 = 7.335$ ,  $P = 0.0068$ ). However, there was no difference in predator survival when each toad species was considered separately (5 x 2 Chi-square contingency analysis with  $\chi^2 = 7.335$ ,  $P = 0.0619$ ). Of the nine predators that died under *Bufo* exposure, only three of these died after consuming tadpoles. Interestingly, these same three predators were the only ones that consumed both *B.*

*americanus* and *B. fowleri* tadpoles. The other predators that consumed only one tadpole (of either species) survived 24 hrs past the end of the experiment.

## Discussion

The anti-predator effectiveness of chemical defenses in larval amphibians has not been as extensively studied as it has been for adult amphibians. Palatability of tadpoles has been studied for many *Bufo* species to a diversity of predators (Brodie et al. 1978 and references therein), yet these studies did not determine if defensive chemicals were present. The main purpose of these earlier studies was to address whether *Bufo* larvae are distasteful to predators. In this experiment, I investigated the responses of larval dytiscid beetles to *Bufo americanus* and *Bufo fowleri* tadpoles during both intermediate stages of larval development and during stages approaching metamorphosis. I hypothesized that beetle larvae would respond differently to tadpoles based on their developmental stage by the predictions of the Brodie and Formanowicz (1987) palatability model.

Dytiscid beetles attacked equal numbers of *B. americanus* and *B. fowleri* tadpoles and nearly equal numbers of tadpoles in both developmental groups (intermediate stages and stages approaching metamorphosis). These findings



also show that dytiscid larvae readily attack and consume tadpoles approaching metamorphosis (Gosner stage 40-42). With respect to chemical defenses, neither *B. americanus* nor *B. fowleri* have detectable bufadienolides during a large stretch of tadpole development, including stages approaching metamorphosis (as described in Chapter II) and this fact could explain tadpole palatability to dytiscid larvae. The finding that some *Bufo* tadpoles were not entirely consumed is probably not indicative of their unpalatability since this behavior could be attributed to optimal foraging by the beetle larvae or by a gut-limiting model (Kruse 1983). Because the assumptions of the Chi-square analysis were not met for this direct test (predator survival by toad developmental stage), significant results are suspect.

In this experiment, dytiscid predators attacked *Bufo* within the first 12 hours of observation and this finding is typical (Young 1967). In total, five *Bufo* were rapidly attacked (within the first 15 minutes) and eaten by separate predators. Seven predators selected *Bufo* as the first prey item when alternative prey (*Poecilia*) were available. When *Bufo* were not available as prey, beetle larvae readily captured and consumed *Poecilia*. This suggests that bufonid tadpoles may be preferred prey or were easier to capture.

The significant Chi-square analysis on *Bufo* exposure and predator survival showed that more beetle predators died under *Bufo* exposure than in

the control. This result is likely due to variability in predatory behavior between the dytiscid species (*Acilius* and *Dytiscus*). Although both dytiscid species were observed capturing and consuming *Bufo*, more *Dytiscus* preyed on *Bufo* than did *Acilius*. Further, the mortality experienced by the beetle larvae within the *Bufo* exposure treatments was the result of starving *Acilius* that failed to consume prey.

An interesting finding of this study is that three predators died after consuming larvae of both toad species. Although both *Bufo* were devoid of detectable bufadienolide defenses during the larval stages used in this study, this does not negate the possibility that other classes of defensive compounds could be involved in tadpole noxiousness or toxicity. Moreover, there may be synergistic effects between these compounds when both species are combined in the diet of dytiscid beetle larvae. This deserves further attention especially because the sample size for predators consuming both toad species was small.

## BIBLIOGRAPHY

- Abel, J. J. and D. I. Macht. 1912. Two crystalline pharmacological agents obtained from the tropical toad *Bufo agua*. *Journal of Pharmacology and Experimental Therapeutics* 3: 319-377.
- Akizawa, T., T. Mukai, M. Matsukawa, M. Yoshioka, J. F. Morris, and V. P. Butler, Jr. 1994. Structures of novel bufadienolides in the eggs of a toad, *Bufo marinus*. *Chemical and Pharmaceutical Bulletin* 42: 754-756.
- Alford, R. A. 1999. Ecology: resource use, competition, and predation. In: R. W. McDiarmid and R. Altig (Eds.) *Tadpoles the Biology of Anuran Larvae*. pp. 240-278. The University of Chicago Press, Chicago and London.
- Argay, G., and A. Kálmán. 1992. Structures of two bufadienolides: telocinobufagin ( $3\beta$ ,  $5\beta$ -dihydroxy- $5\beta$ ,  $14\beta$ -bufa-20,22-dienolide) monohydrate and  $14\alpha$ -artebufogenin ( $3\beta$ -hydroxy-15-oxo- $5\beta$ ,  $14\alpha$ -bufa-20,22-dienolide). *Acta Crystallographica* C48: 1823-1827.
- Arnold, S. J. 1977. Polymorphism and geographic variation in the feeding behavior of the garter snake *Thamnophis elegans*. *Science* 197: 676-678.
- Arnold, S. J. 1981. Behavioral variation in natural populations. II. The inheritance of feeding response in crosses between geographic races of the garter snake, *Thamnophis elegans*. *Evolution* 35: 510-515.
- Arnold, S. J. and R. J. Wassersug. 1978. Differential predation on metamorphic anurans by garter snakes (*Thamnophis*): Social behavior as a possible defense. *Ecology* 59: 1014-1022.
- Barry, T. L., G. Petzinger, and S. W. Zito. 1996. GC/MS comparison of the West Indian aphrodisiac "Love Stone" to the Chinese medication

- "Chan Su": Bufotenine and related bufadienolides. *Journal of Forensic Science* 41(6): 1068-1073.
- Benard, M. F. and J. A. Fordyce. 2003. Are induced defenses costly? Consequences of predator-induced defenses in western toads (*Bufo boreas*). *Ecology* 84 (1): 68-78.
- Blaustein, M. P., M. Juhaszova, and V. A. Golovina. 1998. The cellular mechanism of cardiotonic steroids: A new hypothesis. *Clinical and Experimental Hypertension* 20 (5 & 6): 691-703.
- Bovbjerg, A. M. 1963. Development of the glands of the dermal plicae in *Rana pipiens*. *Journal of Morphology* 113: 231-243.
- Bradford, A. M., J. H. Bowie, M. J. Tyler, and J. C. Wallace. 1996. New antibiotic uperin peptides from the dorsal glands of the Australian toadlet *Uperoleia mjobergii*. *Australian Journal of Chemistry* 49: 1325-1331.
- Brockelman, W. Y. 1969. An analysis of density effects and predation in *Bufo americanus* tadpoles. *Ecology* 50 (4): 632-644.
- Brodie, E. D., Jr. 1977. Hedgehogs use toad venom in their own defence. *Nature* 268: 627-628.
- Brodie, E. D., Jr. 1968. Investigations on the skin toxin of the adult rough-skinned newt, *Taricha granulosa*. *Copeia* 1968: 307-313.
- Brodie, E. D., and E. D. Brodie, Jr. 1990a. Genetics of the garter's getaway. *Natural History* July 1990: 45-51.
- Brodie, E. D., and E. D. Brodie, Jr. 1990b. Tetrodotoxin resistance in garter snakes: an evolutionary response of predators to dangerous prey. *Evolution* 44(3): 651-659.
- Brodie, E. D., III., and E. D. Brodie, Jr. 1991. Evolutionary response of predators to dangerous prey: reduction of toxicity of newts and resistance of garter snakes on island populations. *Evolution* 45: 221-224.

- Brodie, E. D. Jr. and D. R. Formanowicz. 1981. Larvae of the predaceous diving beetle *Dytiscus verticalis* acquire an avoidance response to skin secretions of the newt *Notophthalmus viridescens*. *Herpetologica* 37 (3): 172-176.
- Brodie, E. D., Jr., and D. R. Formanowicz, Jr. 1987. Antipredator mechanisms of larval anurans: protection of palatable individuals. *Herpetologica* 43: 369-373.
- Brodie, E. D., Jr., D. R. Formanowicz, Jr., and E. D. Brodie, III. 1978. The development of noxiousness of *Bufo americanus* tadpoles to aquatic insect predators. *Herpetologica* 34: 302-306.
- Brodie, E. D., Jr., B. J. Redenhour, E. D. Brodie, III. 2002. The evolutionary response of predators to dangerous prey: hotspots and coldspots in the geographic mosaic of coevolution between garter snakes and newts. *Evolution* 56 (10): 2067-2082.
- Brodie, E. D., Jr. and N. J. Smatresk. 1990. The antipredator arsenal of fire salamanders: spraying of secretions from highly pressurized dorsal skin glands. *Herpetologica* 46(1): 1-7.
- Brodie, E. D., Jr., and M. S. Tumbarello. 1978. The antipredator functions of *Dendrobates auratus* (Amphibia, Anura, Dendrobatidae) skin secretion in regard to a snake predator (*Thamnophis*). *Journal of Herpetology* 12: 264-265.
- Brubacher, J. R., D. Lachmanen, P. R. Ravikumar, and R. S. Hoffman. 1999. Efficacy of digoxin specific Fab fragments (Digibind®) in the treatment of toad venom poisoning. *Toxicon* 37: 931-942.
- Burghardt, G. M. 1969. Comparative prey-attack studies in newborn snakes of the genus *Thamnophis*. *Behaviour* 33: 77-114.
- Burghardt, G. M. 1970. Chemical perception of reptiles. In: J. W. Johnston, Jr., D. G. Moulton, and A. Turk (Eds.) *Communication by chemical signals*. pp. 241-308. Appleton-Century-Crofts, New York.
- Burghardt, G. M. 1975. Chemical prey preference polymorphism in newborn garter snakes *Thamnophis sirtalis*. *Behavior* 52: 202-225.

- Butler, V. P., Jr., J. F. Morris, T. Akizawa, M. Matsukawa, P. Keating, A. Hardart, and I. Furman. 1996. Heterogeneity and lability of endogenous digitalis-like substances in the plasma of the toad, *Bufo marinus*. *American Journal of Physiology* 271: 325-332.
- Caldwell, J. P. 1989. Structure and behavior of *Hyla geographica* tadpole schools with comments on classification of group behavior in tadpoles. *Copeia* 1989: 938-948.
- Cannon, M. S. and G. A. Palkuti. 1976. The 'parotoid' gland of bufonidae. *Toxicon* 14: 149-151.
- Cannon, M. S. and J. R. Hostetler. 1976. The anatomy of the parotoid gland in Bufonidae with some histochemical findings II. *Bufo alvarius*. *Journal of Morphology* 148: 137-160.
- Cei, J. M., V. Erspamer, and M. Roseghini. 1972. Biogenic amines. In: W. F. Blair (Ed.). *Evolution in the genus Bufo*. University of Texas Press, Austin, Texas.
- Chen, K. K. and A. Kovaříková. 1967. Pharmacology and toxicology of toad venom. *Journal of Pharmaceutical Sciences* 56 (12): 1535-1541.
- Chen, C. and M. V. Osuch. 1969. Biosynthesis of bufadienolides— $3\beta$ -hydroxycholanates as precursors in *Bufo marinus* bufadienolide synthesis. *Biochemical Pharmacology* 18: 1797-1802.
- Clarke, B. T. 1997. The natural history of amphibian skin secretions, their normal functioning and potential medical applications. *Biological Reviews* 72: 365-379.
- Conant, R. 1975. *A Field Guide to Reptiles and Amphibians*. Houghton Mifflin Company. Boston, Massachusetts.
- Conant, R. and J. T. Collins. 1998. *A Field Guide to Reptiles and Amphibians of Eastern and Central North America*. Third Edition, Expanded. Houghton Mifflin Company, New York.
- Cooper, W. E. 1992. Post-bite elevation in tongue-flick rate by neonatal garter snakes (*Thamnophis radix*). *Ethology* 91: 339-345.

- Cooper, W. E. 1998. Prey chemical discrimination indicated by tongue-flicking in the Eublepharid gecko *Coleonyx variegatus*. *The Journal of Experimental Zoology* 281: 21-25.
- Cooper, W. E., Jr., and G. M. Burghardt. 1990. A comparative analysis of scoring methods for chemical discrimination of prey by squamate reptiles. *Journal of Chemical Ecology* 16(1): 45-65.
- Cooper, W. E., Jr., D. G. Buth and L. J. Vitt. 1990. Prey odor discrimination by ingestively naïve coachwhip snakes (*Masticophis flagellum*). *Chemoecology* 1: 86-91.
- Cooper, W. E., Jr., S. G. McDowell and J. Ruffer. 1989. Strike-induced chemosensory searching in the colubrid snakes *Elaphe g. guttata* and *Thamnophis sirtalis*. *Ethology* 81: 19-28.
- Cooper, W. E. and L. J. Vitt. 1986. Thermal dependence of tongue-flicking and comments on use of tongue-flicking as an index of squamate behavior. *Ethology* 71: 177-186.
- Cott, H. B. 1941. Adaptive coloration in animals. Oxford University Press, New York.
- Crossland, M. R. 1998. Ontogenetic variation in toxicity of tadpoles of the introduced toad, *Bufo marinus*, to native Australian aquatic invertebrate predators. *Herpetologica* 54: 364-369.
- Crossland, M. R. and R. A. Alford. 1998. Evaluation of the toxicity of eggs, hatchlings and tadpoles of the introduced toad *Bufo marinus* (Anura: Bufonidae) to native Australian aquatic predators. *Australian Journal of Ecology* 23: 129-137.
- Cummins, K. W. 1973. Trophic relations of aquatic insects. *Annual Review of Entomology* 18: 183-206.
- Daly, J. W. 1995. The chemistry of poisons in amphibian skin. In: T. Eisner and J. Meinwald (Eds.) *Chemical Ecology: The Chemistry of Biotic Interaction*. pp. 17-28. National Academy Press, Washington, D. C.
- Daly, J. W. 1998. Thirty years of discovering arthropod alkaloids in amphibian skin. *Journal of Natural Products* 61: 162-172.

- Daly, J. W., C. W. Myers, and N. Whittaker. 1987. Further classification of skin alkaloids from neotropical poison frogs (Dendrobatidae), with a general survey of toxic/noxious substances in the Amphibia. *Toxicon* 25: 1023-1095.
- Daly, J. W., W. L. Padgett, R. L. Saunders, and J. F. Cover, Jr. 1997. Absence of tetrodotoxin in a captive-raised riparian frog, *Atelopus varius*. *Toxicon* 35(5): 705-709.
- Daly, J. W. and B. Witkop. 1971. Chemistry and pharmacology of frog venoms. In: *Venomous Animals and Their Venoms*, volume 2, W. Bücherl and E. E. Buckley (Eds). Academic Press, New York.
- Delfino, G., R. Brizzi, and G. Borrelli. 1988. Cutaneous glands in anurans: differentiation of the secretory syncytium in serous anlagen. *Zoologische Jahrbücher* 117: 255-275.
- Delfino, G., R. Brizzi, S. Jantra and L. Feri. 1995. Post-golgian maturative processes during the biosynthesis of poison secretion in cutaneous glands of the European common toad *Bufo bufo*. *Journal of Natural Toxins* 4 (2): 97-113.
- Denton and Beebee. 1997. Effects of predator interactions, prey palatability and habitat structure on survival of natterjack toad *Bufo calamita* larvae in replicated semi-natural ponds. *Ecography* 20: 166-174.
- Deulofeu, V. and E. A. Rúveda. 1971. The basic constituents of toad venoms. In: W. Bücherl and E. E. Buckley (Eds.) *Venomous Animals and Their Venoms, VII Venomous Vertebrates*. pp. 475-495. Academic Press, New York.
- DeVito, J. 2003. Metamorphic synchrony and aggregation as antipredator responses in American toads. *Oikos* 103(1): 75-80.
- Doris, P. A. and A. Y. Bagrov. 1998. Endogenous sodium pump inhibitors and blood pressure regulation: an update on recent progress. *Proceedings of the Society for Experimental Biology and Medicine* 218: 156-167.



- Doull, J., K. P. Dubois and E. M. K. Geiling. 1951. The biosynthesis of radioactive bufagin. *Archives internationales de pharmacodynamie et de therapie* 86: 454-464.
- Duellman, W. E., and L. Trueb. 1986. *Biology of Amphibians*. The Johns Hopkins University Press, Baltimore and London.
- Ducey, P. K., and E. D. Brodie, Jr. 1991. Evolution of antipredator behavior: Individual and populational variation in a neotropical salamander. *Herpetologica* 47(1): 89-95.
- Edmunds, M. 1974. *Defence in animals. A survey of anti-predator defences*. Longman, Harlow United Kingdom.
- Edwards, M. W., J. W. Daly, and C. W. Myers. 1988. Alkaloids from a Panamanian poison frog, *Dendrobates speciosus*: Identification of pumiliotoxin-a and allopumiliotoxin class alkaloids, 3,5-disubstituted indolizidines, 5-substituted 8-methylindolizidines, and a 2-methyl-6-nonyl-4-hydroxypiperidine. *Journal of Natural Products* 51(6): 1188-1197.
- Eklöv, P. 2000. Chemical cues from multiple predator-prey interactions induce changes in behavior and growth of anuran larvae. *Oecologia* 123: 192-199.
- Erspamer, V. 1994. Bioactive secretions of the amphibian integument. *In*: H. Heatwole (Ed.) *Amphibian Biology, Vol. I: The Integument*. pp. 178-350. Surrey Beatty and Sons, Chipping Norton, NSW.
- Farquhar, M. G. and G. E. Palade. 1964. Functional organization of amphibian skin. *Proceedings of the National Academy of Sciences* 51 (4): 569-577.
- Fieser, L. F. and M. Fieser. 1959. *Steroids*. Reinhold Publishing Company, New York.
- Fitch, H. S. 1941. The feeding habits of California garter snakes. *California Fish and Game* 27: 1-32.

- Fitch, H. S. 1965. An ecological study of the garter snake, *Thamnophis sirtalis*. University of Kansas Publications of the Museum of Natural History. 15: 493-564.
- Flier, J., M. W. Edwards, J. W. Daly, and C. W. Myers. 1980. Widespread occurrence in frogs and toads of skin compounds interacting with the ouabain site of Na<sup>+</sup>,K<sup>+</sup>-ATPase. *Science* 208: 503-505.
- Formanowicz, D. R., Jr., and E. D. Brodie, Jr. 1982. Relative palatabilities of members of a larval amphibian community. *Copeia* 1982(1): 91-97.
- Garraffo, H. M. and E. G. Gros. 1986. Biosynthesis of bufadienolides in toads. IV. Experiments with [1,2-<sup>3</sup>H]cholesterol, [21-<sup>14</sup>C]coprostanol, and 5β-[21-<sup>14</sup>C]pregnanolone in the toad *Bufo arenarum*. *Steroids* 48 (3-4): 251-257.
- Geffeney, S., E. D. Brodie, Jr., P. C. Ruben, and E. D. Brodie, III. 2002. Mechanisms of adaptation in a predator-prey arms race: TTX resistant sodium channels. *Science* 297: 1336-1339.
- Gosner, K. L. 1960. A simplified table for staging anuran embryos and larvae with notes on identification. *Herpetologica* 16: 183-190.
- Green, D. M. 1982. Mating call characteristics of hybrid toads (*Bufo americanus* x *B. fowleri*) at Long Point, Ontario. *Canadian Journal of Zoology* 60: 3293-3297.
- Green, D. M. 1988. Antipredator behaviour and skin glands in the New Zealand native frogs, genus *Leiopelma*. *New Zealand Journal of Zoology* 15: 39-45.
- Green, D. M. and C. Parent. 2003. Variable and asymmetric introgression in a hybrid zone in the toads, *Bufo americanus* and *Bufo fowleri*. *Copeia* 2003(1): 34-43.
- Halpern, M. 1983. Nasal chemical cues in snakes. In: J-P. Ewert, R. R. Capranica, and D. J. Ingle (Eds.) *Advances in Vertebrate Neuroethology*. Plenum Press, New York and London.

- Halpern, M. 1992. Nasal chemical senses in reptiles. *In*: C. Gans and D. Crews (Eds.) *Biology of the Reptilia*, Vol. 18: Hormones, Brain and Behavior. University of Chicago Press, Chicago.
- Halpern, M. and N. Frumin. 1979. Roles of the vomeronasal and olfactory systems in prey attack and feeding in adult garter snakes. *Physiology and Behavior* 22: 1183-1189.
- Halpern, M., J. Halpern, E. Erichsen, and S. Borghjid. 1997. The role of nasal and chemical senses in garter snake response to airborne odor cues from prey. *Journal of Comparative Psychology* 111(3): 251-260.
- Harding, J. H. 1997. *Amphibians and Reptiles of the Great Lakes Region*. The University of Michigan Press, Ann Arbor, Michigan.
- Hayes, T. B. and T. N. Gill. 1995. Hormonal regulation of skin gland development in the toad (*Bufo boreas*): The role of thyroid hormones and cortisosterone. *General and Comparative Endocrinology* 99: 161-168.
- Heyer, R. W., R. W. McDiarmid, and D. L. Weigmann. 1975. Tadpoles, predation and pond habits in the tropics. *Biotropica* 7: 100-111.
- Hong, Z., K. Chan, and H. W. Yeung. 1992. Simultaneous determination of bufadienolides in the traditional Chinese medicine preparation, Liu-Shen-Wan, by liquid chromatography. *Journal of Pharmacy and Pharmacology* 44: 1023-1026.
- Hostetler, J. R. and M. S. Cannon. 1974. The anatomy of the parotoid gland in Bufonidae with some histochemical findings I. *Bufo marinus*. *Journal of Morphology* 142: 225-240.
- Jared, C. 2004. Laboratório de Biologia Celular, Instituto Butantan—[carlosjared@butantan.gov.br](mailto:carlosjared@butantan.gov.br)
- Jared, C., M. M. Antoniazzi, P. R. Neves, J. R. M. C. Silva, and S. M. Carneiro. 2003. Cutaneous glands in anuran amphibians: Comparative morphology in *Bufo paracnemis* (Bufonidae) and *Phyllomedusa distincta* (Hylidae) parotoid glands. *Acta Microscopica* 12: 561-562.

- Kamano, Y., A. Kotake, H. Hashima, M. Inoue, H. Morita, K. Takeya, H. Itokawa, N. Nandachi, T. Segawa, A. Yukita, K. Saitou, M. Katsuyama, and G. R. Pettit. 1998. Structure-cytotoxic activity relationship for the toad poison bufadienolides. *Bioorganic and Medicinal Chemistry* 6: 1103-1115.
- Kats, L. B., J. W. Petranka, and A. Sih. 1988. Antipredator defenses and the persistence of amphibian larvae with fishes. *Ecology* 69 (6): 1865-1870.
- Kiesecker, J. M., D. P. Chivers, and A. R. Blaustein. 1996. The use of chemical cues in predator recognition by western toad tadpoles. *Animal Behavior* 52: 1237-1245.
- Klyne, W. 1957. *The Chemistry of the Steroids*. John Wiley and Sons, New York.
- Kopp, B., L. Krenn, M. Draxler, A. Hoyer, R. Terkola, T. Vallaster, and W. Robien. 1996. Bufadienolides from *Urginea maritima* from Egypt. *Phytochemistry* 42(2): 513-522.
- Kren, L., M. Jelovina, and B. Kopp. 2000. New bufadienolides from *Urginea maritima sensu strictu*. *Fitoterapia* 71: 126-129.
- Kruse, K. C. 1983. Optimal foraging by predaceous diving beetle larvae on toad tadpoles. *Oecologia* 58: 383-388.
- Lagler, K. F. and J. G. Salyer. 1945. Influence of availability on the feeding habits of the common garter snake. *Copeia* 1945: 159-162.
- Lanuza, E. and M. Halpern. 1998. Efferents and centrifugal afferents of the main and accessory olfactory bulbs in the snake *Thamnophis sirtalis*. *Brain, Behavior and Evolution* 51: 1-22.
- Laurila, A., S. Pakkasmaa, P. A. Crochet, and J. Merilä. 2002. Predator-induced plasticity in early life history and morphology in two anuran amphibians. *Oecologia*

- Laurila, A., J. Kujasalo, E. Ranta. 1997. Different antipredator behavior in two anuran tadpoles: effects of predator diet. *Behavioral Ecology and Sociobiology* 40: 329-336.
- Laurila, A., S. Pakkasmaa, P. A. Crochet, J. Merilä. 2002. Predator-induced plasticity in early life history and morphology in two anuran amphibians. *Oecologia* 132(4): 524-530.
- Lawler, K. L. and J. M. Hero. 1997. Palatability of *Bufo marinus* tadpoles to a predatory fish decreases with development. *Wildlife Research* 24: 327-334.
- Legendre, P. and L. Legendre. 1998. *Numerical Ecology*. Second English Edition. Elsevier, New York.
- Licht, L. E. 1967. Initial appearance of the parotoid gland in three species of toads (Genus *Bufo*). *Herpetologica* 23(2): 115-118.
- Licht, L. E. and B. Low. 1968. Cardiac response of snakes after ingestion of toad parotoid venom. *Copeia* 1968: 547-551.
- Licht, L. E. and D. M. Server. 1993. Structure and development of the parotoid gland in metamorphosed and neotenic *Ambystoma gracile*. *Copeia* 1993 (1): 116-123.
- Lichtstein, D., S. Kachalsky, and J. Deutsch. 1986. Identification of a ouabain-like compound in toad skin and plasma as a bufadienolide derivative. *Life Sciences* 38: 1261-1270.
- Low, B. S. 1972. Evidence from parotoid gland secretions. In: W. F. Blair (Ed.) *Evolution in the Genus Bufo*. pp. 244-264. University of Texas Press, Austin.
- Lutz, B. 1971. Venomous toads and frogs. In: W. Bücherl and E. E. Buckley (Eds.) *Venomous Animals and Their Venoms, Volume II: Venomous Vertebrates*. pp. 423-473. Academic Press, New York.
- Macartney, J. M., and P. T. Gregory. 1981. Differential susceptibility of sympatric garter snake species to amphibian skin secretions. *The American Midland Naturalist* 106: 271-281.

- Mahan, J. T. and C. J. Biggers. 1977. Electrophoretic investigation of blood and parotoid venom proteins in *Bufo americanus* and *Bufo woodhousei fowleri*. *Comparative Biochemistry and Physiology* 57 (2): 121-126.
- Majinda, R. T., R. D. Waigh, and P. G. Waterman. 1997. Bufadienolides and other constituents of *Urinea sanguinea*. *Planta Medica* 63(2): 188-190.
- Malcolm, S. B. 1991. Cardenolide-mediated interactions between plants and herbivores. In: G. A. Rosenthal and M. R. Berenbaum (Eds.) *Herbivores: Their Interactions with Secondary Plant Metabolites*, Second Edition. Volume I: The Chemical Participants. pp. 251-296. Academic Press, Inc., San Diego.
- Matsukawa, M., T. Akizawa, J. F. Morris, V. P. Butler, Jr., and M. Yoshioka. 1996. Marionic acid, a novel bufadienolide-related substance in the skin of the giant toad, *Bufo marinus*. *Chemical and Pharmaceutical Bulletin* 44 (1): 255-257.
- Matsukawa, M., T. Akizawa, M. Ohigashi, J. F. Morris, V. P. Butler, Jr. and M. Yoshioka. 1997. A novel bufadienolide, marinosin, in the skin of the giant toad, *Bufo marinus*. *Chemical and Pharmaceutical Bulletin* 45 (2): 249-254.
- Mattos, R. L., M. M. Antoniazzi, C. Haddad, and C. Jared. 2003. Structure of venom glands in the frog *Physalaemus nattereri* (Anura, Leptodactylidae). *Acta Microscopica* 12: 563-564.
- 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.
- Meyer, K. 1966. Cardiotoxic steroids from toads. *Memórias do Instituto de Butantan* 33: 433-440.
- Meyer, K. and H. Linde. 1971. Collection of toad venoms and chemistry of the toad venom steroids. In: W. Bücherl and E. E. Buckley (Eds.) *Venomous Animals and Their Venoms, VII Venomous Vertebrates*. pp. 521-556. Academic Press, New York.

- Miller, J. R. and R. O. Mumma. 1974. Seasonal quantification of the defensive steroid titre of *Agabus seriatus* (Coleoptera: Dytiscidae). *Annals of the Entomological Society of America* 67(6): 850-852.
- Moore, R. D., R. A. Griffiths, C. M. O'Brien, A. Murphy, and D. Jay. 2004. Induced defenses in an endangered amphibian in response to an introduced snake predator. *Oecologia* 141: 139-147.
- Muhse, E. F. 1909. The cutaneous glands of the common toads. *American Journal of Anatomy* 9: 321-361.
- Neuwirth, M., J. W. Daly, C. W. Meyers, and L. W. Tice. 1979. Morphology of the granular secretory glands in skin of poison-dart frogs (Dendrobatidae). *Tissue & Cell* 11 (4): 755-771.
- Okada, M. 1966. Pharmacology of the components of toad venom and allied substances. *Memórias do Instituto de Butantan* 33: 589-602.
- Palumbo, N. E., S. F. Perry, and G. Read. 1975. Experimental induction and treatment of toad poisoning in the dogs. *Journal of the American Veterinary Medical Association* 167: 1000-1005.
- Peterson, J. A. and A. R. Blaustein. 1992. Relative palatabilities of anuran larvae to natural aquatic insect predators. *Copeia* 1992 (2): 577-584.
- Phisalix, C. and G. Bertrand. 1902. Sur les principes actifs du venin de Crapaud commun (*Bufo vulgaris* L.). *Bulletin des Sciences Pharmacologiques* 5: 211-214.
- Porter, K. R. 1964. Chromatographic comparisons of the parotoid gland secretions of six species in the *Bufo valliceps* group. In: C. A. Leone (Ed.) *Taxonomic Biochemistry and Serology*. Chapter 27. pp. 451-456. The Ronald Press Company, New York.
- Porter, K. R., and W. F. Porter. 1967. Venom comparisons and relationships of twenty species of New World toads (genus *Bufo*). *Copeia* 1967: 298-307.
- Relyea, R. and K. L. Yurewicz. 2002. Predicting community outcomes from pairwise interactions: integrating density- and trait-mediated effects. *Oecologia* 131(4): 569-579.

- Santa Coloma, T. A., H. M. Garraffo, O. P. Pignataro, E. H. Charreau, and E. G. Gros. 1984. Biosynthesis of bufadienolides in toads. V. The origin of the cholesterol used by toad parotoid glands for biosynthesis of bufadienolides. *Steroids* 44 (1): 11-22.
- Schoner, W. 2002. Endogenous cardiac glycosides, a new class of steroid hormones. *European Journal of Biochemistry* 269: 2440-2448.
- Seigel, R. A. 1996. Ecology and conservation of garter snakes: masters of plasticity. In: D. A. Rossman, N. B. Ford, and R. A. Seigel (Eds.) *The Garter Snakes*, pp. 55-89, University of Oklahoma Press, Norman and London.
- Semlitsch, R. D. and H. Reyer. 1992. Modification of anti-predator behavior in tadpoles by environmental conditioning. *Journal of Animal Ecology* 61: 353-360.
- Shimada, K., and T. Nambara. 1980. Isolation and characterization of a new type of bufotoxin from the skin of *Bufo americanus*. *Chemical and Pharmaceutical Bulletin* 28: 1559-1562.
- Shipley, P. G. and G. B. Wislocki. 1915. The histology of the poison glands of *Bufo agua* and its bearing upon the formation of epinephrine within the glands. *Carnegie Institution, Washington* 3: 71-90.
- Siperstein, M. D., A. W. Murray, and E. Titus. 1957. Biosynthesis of cardiotonic sterols from cholesterol in the toad, *Bufo marinus*. *Archives of Biochemistry and Biophysics* 67: 154-160.
- Smith, M. A. and D. M. Green. 2002. *Bufo fowleri* (Fowler's toad) predation. *Hepetological Review* 33 (2): 125.
- Smith, H. M. and F. N. White. 1955. Adrenal enlargement and its significance in the hognose snakes (*Heterodon*). *Herpetologica* 11: 137-144.
- Smith, B. P. C., M. J. Tyler, B. D. Williams, and Y. Hayasaka. 2003. Chemical and olfactory characterization of odorous compounds and their precursors in the parotoid gland secretion of the green tree frog, *Litoria caerulea*. *Journal of Chemical Ecology* 29 (9): 2085-2100.



- Steyn, P. S. and F. R. van Heerden. 1998. Bufadienolides of plant and animal origin. *Natural Product Reports: A Journal of Current Developments in Bioorganic Chemistry* 15: 397-413.
- Swart, C. C. and R. C. Taylor. 2004. Behavioral interactions between the giant water bug (*Belostoma lutarium*) and tadpoles of *Bufo woodhousii*. *Southeastern Naturalist* 3 (1): 13-24.
- Toledo, R. C. and C. Jared. 1995. Cutaneous granular glands and amphibian venoms. *Comparative Biochemistry and Physiology* 111A (1): 1-29.
- Toledo, R. C., C. Jared, A. Brunner, Jr. 1992. Morphology of the large granular alveoli of the parotoid glands in the toad (*Bufo ictericus*) before and after compression. *Toxicon* 30: 745-753.
- Trong, Y. L. 1973. Structure et développement de la peau et des glandes cutanées de *Bufo regularis* Reuss. *Bulletin de la Société zoologique de France* 98: 449-485.
- Tschesche, R. 1972. Biosynthesis of cardenolides, bufadienolides and steroid sapogenins. *Proceedings of the Royal Society of London. Series B, Biological Sciences* 180 (1059): 187-202.
- Tyler, M. J., T. Burton, and A. M. Bauer. 2001. Parotid or parotoid: On the nomenclature of an amphibian skin gland. *Hepetological Review* 32 (2): 79-81.
- Van Bushkirk, J. 2002. Phenotypic lability and the evolution of predator-induced plasticity in tadpoles. *Evolution* 56(2): 361-370.
- Van Bushkirk, J. and G. Saxer. 2001. Delayed costs of an induced defense in tadpoles? Morphology, hopping, and development rate at metamorphosis. *Evolution* 55(4): 821-829.
- Villa, J., R. W. McDiarmid, and J. M. Gallardo. 1982. Arthropod predators of leptodactylid frog foam nests. *Brenesia* 19/20: 577-589.
- Voet, D. and J. G. Voet. 1990. *Biochemistry*. John Wiley and Sons, New York.
- Wassersug, R. J. 1971. On the comparative palatability of some dry-season tadpoles from Costa Rica. *American Midland Naturalist* 86: 101-109.

- Wassersug, R. J. 1973. Aspects of social behavior in anuran larvae. In: J. L. Vial (Ed.) *Evolutionary biology of the anurans*. University of Missouri Press, Columbia.
- Waters, R. M. 1993. Odorized air current trailing by garter snakes, *Thamnophis sirtalis*. *Brain Behavior and Evolution* 41: 219-223.
- Wattix, R., C. Remy, P. Falmagne, and G. Toubeau. 1994. Purification and preliminary characterization of a frog-derived proteinaceous chemoattractant eliciting prey attack by checkered garter snakes (*Thamnophis marcianus*). *Journal of Chemical Ecology* 20(5): 1143-1160.
- Werner, E. E. 1986. Amphibian metamorphosis: growth rate, predation risk, and the optimal size at transformation. *The American Naturalist* 128 (3): 319-341.
- Wieland, H. and R. Alles. 1922. Über den Giftstoff der Kröte. *Ber. Deut. Chem. Ges.* 55: 1789-1797.
- Wilber, C. G. and P. L. Carroll, S. J. 1940. Studies on the histology of the glands in the skin of Anura I. The parotoid gland of *Bufo americanus* Holbrook. *Transactions of the American Microscopical Society* 49: 123-128.
- Wilbur, H. M., P. J. Morin, and R. N. Harris. 1983. Salamander predation and the structure of experimental communities: anuran responses. *Ecology* 64(6): 1423-1429.
- Williams, B. L., E. D. Brodie Jr., and E. D. Brodie III. 2002. Comparisons between toxic effects of tetrodotoxin administered orally and by intraperitoneal injection to the garter snake *Thamnophis sirtalis*. *Journal of Herpetology* 36(1): 112-115.
- Williams, B. L., E. D. Brodie, Jr., and E. D. Brodie, III. 2003. Coevolution of deadly toxins and predator resistance: self-assessment of resistance by garter snakes leads to behavioral rejection of toxic newt prey. *Herpetologica* 59 (2): 155-163.
- Williams, D. D. and B. W. Feltmate. 1992. *Aquatic Insects*. Redwood Press Ltd., Melksham, UK.

- Wittliff, J. L. 1962. Parotoid gland secretions in two species groups of toads (genus *Bufo*). *Evolution* 16: 143-153.
- Wittliff, J. L. 1964. Venom constituents of *Bufo fowleri*, *Bufo valliceps*, and their natural hybrids analyzed by electrophoresis and chromatography. In: C. A. Leone (Ed.) *Taxonomic Biochemistry and Serology*. Chapter 28. pp. 457-464. The Ronald Press Company, New York.
- Wright, A. H., and A. A. Wright. 1967. *Handbook of Frogs and Toads*. Comstock Publishing Company, Ithaca, New York.
- Young, A. M. 1967. Predation in the larvae of *Dytiscus marginalis* Linneaus (Coleoptera: Dytiscidae). *The Pan-Pacific Entomologist* 43: 113-117.
- Zar, H. 1999. *Biostatistical Analysis*. 4th Edition. Prentice-Hall, Inc., Upper Saddle River, New Jersey.

## **Appendix A**

### **IACUC Protocol Clearance For the Use of Vertebrate Animals in Research**

IACUC Number 97-07-02  
Date of Receipt  
Date of Approval 9/17/97

158  
**RECEIVED**  
JUL 24 1997  
I.A.C.U.C!

**WESTERN MICHIGAN UNIVERSITY  
INSTITUTIONAL ANIMAL CARE  
AND USE COMMITTEE (IACUC)**

**Application to use Vertebrate Animals for Research or Testing**

The use of any vertebrate animals in research and/or teaching without prior approval of the Institute Animal Care and Use Committee (IACUC) is a violation of Western Michigan University policies and procedures. This Committee is charged with the institutional responsibility for assuring the appropriate care and treatment of vertebrate animals.

Mail the signed original and five (5) copies of the typed application and any supplements to Research and Sponsored Programs, 301 Walwood Hall, (616) 387-8270.

Any application that includes use of hazardous materials, chemicals, radioisotopes or biohazard must be accompanied with SUPPLEMENT A.

Any application the includes survival surgery must be accompanied with SUPPLEMENT B.

Catherine E. Link Biological Sciences 387-5600  
Principal Investigator/Instructor Department Campus Phone  
Catherine E. Link 7-23-1997  
Signature Date

Dr. Stephen B. Malcolm Biological Sciences 387-5604  
Responsible Faculty Member Department Campus Phone  
(if PI not Faculty member)  
Stephen B. Malcolm 23 July 1997  
Signature Date

Title of Project/Course The effectiveness of bufadienolides in the chemical defenses of toads.

Check One: Teaching ☐ Research ☒ Other Ph. D. Dissertation

**I. ANIMAL USE CATEGORIES (check ONLY one category)**

- A. ☒ Projects that involves little or no discomfort (including injections).  
B. ☐ Projects that may result in some discomfort or pain, but short in duration.  
Anesthetics, analgesics or tranquilizers will be used.  
C. ☐ Project that may result in significant discomfort or pain, Anesthetics,  
analgesics, or tranquilizers will not be used

## II. ANIMAL USE FACILITIES

The animal(s) will be housed and maintained in accordance with the WMU Humane Care and Use of Animals Policies and Procedures.

Yes   X   No       

If no, give explanation.

Please indicate the building and room(s) where the animals(s) will be housed and cared for as well as the location of the experiments and procedures if different from where housed.

The laboratory of Dr. Stephen Malcolm  
5022 McCracken Hall

## III. ANIMAL USE SUMMARY

In language understandable to a layperson, summarize your primary aims and describe the proposed use of animals as concisely as possible. Bear in mind the IACUC is primarily interested in the responsible, necessary, humane use of animals. Included a description of procedures designed to assure that discomfort and pain to animals will be minimized. It should include method of restraint; methods of dosing with test compound; and methods of euthanasia or deposition of the animal after the experiment.

My research focuses on the chemical compounds that occur naturally in the parotoid glands of toads and their effectiveness against potential predators. I am interested in sampling from both species that occur in Michigan --Fowler's and American toads (*Bufo americanus* and *Bufo woodhousii fowleri*). Chemical secretions found in these large warts (parotoid glands) can easily be removed using a hypodermic syringe. The principle aim of my research is to understand how these parotoid chemicals are used to protect toads from predators. Specifically, I wish to focus on one main group of chemicals called bufadienolides. Adult toads would be collected in the field, measured and extracts from the parotoid gland would be removed with a sterile 1ml hypodermic syringe for chemical analysis. Toads would be released in the field within 30 minutes of capture at the site of capture.

During the breeding season immature toads (larvae (tadpoles) and juveniles) will also be collected for analysis during May and June. At two-weekly intervals during this period local ponds will be sampled for larval toads and 10 individuals will be sacrificed. The ten larvae of each toad species at each sample period (duration of larval sampling will depend on the season) will be placed on ice and then frozen on return to the laboratory. Juveniles with legs will be sampled as above for adults with a microsyringe extraction of the parotoid gland and then measured and returned to the collection site.

In addition to sampling for annual variation and life cycle variation in skin secretion chemistry, we wish to test the effectiveness of toad skin secretions against a potential predator. For this we propose to use the common garter snake (*Thamnophis sirtalis sirtalis*) to assay parotoid secretion extracts. Twelve individual snakes will be housed in 6, 40L glass aquaria separated into two equal compartments. Parotoid skin secretions will be offered at known concentrations on either an inert test substrate (a movable 1cm diameter polystyrene ball), or on the back of a domestic cricket (*Acheta domestica*) to 8 of the snakes. Snake response will be recorded by timed behaviors such as tongue flick rate, orientation, taste, strike, rejection, consumption, or regurgitation. Snake responses will be voluntary and it is not anticipated that the experiments will cause distress to the 8 replicate snakes. Snakes will be tested three times each (at 24 h intervals) with a random sequence of 3 concentrations of toad parotoid secretion (one normal parotoid concentration and the other two at one natural logarithm below normal and 1ln above normal), and a single

control. The remaining four snakes will be tested with commercially available bufadienolides at similar concentrations using the same protocol.

#### IV. JUSTIFICATION FOR ALL ANIMAL EXPERIMENTS

Please provide a narrative with reference sources which addresses each of the following:

##### A. What assurance can be provided to indicate that the procedure is not duplicative?

Studies have addressed the chemistry of toad skin and parotoid secretions (Chen, K. K., and A. Kovariková. 1967. *Pharmacology and toxicology of toad venom*. *Journal of Pharmaceutical Sciences* 56: 1535-1541; Bücherl, and E. E. Buckley (eds.) 1971, *Venomous Animals and Their Venom*. Academic Press, New York and London; Daly, J. W. 1995. *The chemistry of poisons in amphibian skin*. In, T. Eisner and J. Meinwald (eds.), *Chemical Ecology: The Chemistry of Biotic Interaction*. National Academy Press, Washington D.C.) but little is known about their antipredator properties or those of bufadienolides (Lichtstein et al. 1986. *Identification of a ouabain-like compound in toad skin and plasma as a bufodienolide derivative*. *Life Sciences* 38: 1261-1270).

Literature searches of databases (BIOSIS, AGRICOLA) have not revealed publications that consider the ecology of toad defensive secretions. At a recent meeting of the American Society of Ichthyologists and Herpetologists (June 1997, Seattle, Washington), Cathy Link discussed the proposed research with experts in the field and it was clear that the research proposed here is the first attempt to understand natural variation and effectiveness of toad chemical defenses.

##### B. Have non-live animal techniques (e.g. *in vitro* biological systems, computer simulation, audiovisual demonstration) been considered? Explain why they have not been utilized.

It is not known how toads synthesize their defensive chemicals or how they vary naturally. Thus because the study compares two toad species and seeks to understand the natural eceology of chemical defense in these species it is not possible to use non-live animal techniques.

##### C. Why has this species been selected for this procedure?

*B. americanus* and *B. woodhousii fowleri* were chosen because they are two common and widely distributed species in Michigan. Also, these species are presumably affected by the same natural predators in the wild. According to the Michigan Department of Natural Resources in Plainwell, Michigan, neither these two toad species, nor the common garter snake, *Thamnophis sirtalis*, are subject to special protection beyond the general protection afforded all reptiles and amphibians in Michigan.

##### D. How many animals will be used in this project? How often will its procedure be done and over what duration?

If possible parotoid samples will be collected from 10 individual toads of each species for each month for one year (10 individuals x 2 species x 12 months = 240 individual toads). No toads or snakes will be collected between November 15 and the last Saturday in May, although we will attempt to sample parotoid gland secretions between these dates with the permission of the DNR. In addition, it may prove necessary to keep a small colony of 12 toads housed in 3, 40L aquaria as a source of parotoid gland secretion for the predator experiments.

For predator experiments we will use 12 snakes as indicated above. As soon as the repeated-measures experimnts have been completed we will release the snakes back into the wild.

These numbers are just above the threshold for legal holdings of native amphibians and reptiles in the state of Michigan (for species like the toads and snake we propose to use that are not subject to specific protection) and for this we will apply for the required "Scientific Collectors Permit" from the Michigan Department of Natural Resources.

E. In light of concern to minimize the number of animal used in the experimentation, how will you determine the number of animal to be used?

Most animals will be released within 30 minutes of capture. The number of animals used in laboratory experiments have been determined by the anticipated variation among individuals and to ensure statistical rigor for analysis of the results.

**NOTE : ITEMS F, G, H and I require the approval of the Consulting Veterinarian**

F. What is the anticipated pain or distress response of the animal; and what is the duration of discomfort? (Injection not included.)

Aspiration of parotoid secretion will involve inserting a fine needle in the upper most layer of skin above the parotoid glands on either side of the neck immediately behind the head. Pain and distress is assumed to be minimal. Removal of the poison will not harm the toad because they are able to regenerate the poison. Prior work with toad skin secretions has involved sacrifice of toads. The only published alternative we have found has been to press a glass sheet against the parotoid glands which burst and smear the glass with secretion (Meyer, K. and H. Linde. 1971. *Collection of toad venoms and chemistry of the toad venom steroids*. In W. Bücherl, and E. E. Buckley (eds.), *Venomous Animals and Their Venom*. Academic Press, New York and London). This method is difficult to quantify and is likely to lead to contamination of the parotoid gland secretion with skin debris and blood. We propose to aspirate the secretion with a hypodermic syringe as a more accurate and clean method that is less likely to cause distress to the toads.

Larval and juvenile toads that are collected for sacrifice will be chilled on ice as soon as they are collected. Once returned to the laboratory these chilled individuals will be frozen rapidly to  $-70^{\circ}\text{C}$  and kept at this temperature for at least one week before being freeze dried and extracted for analysis by High Performance Liquid Chromatography. No vertebrate species is known that can revive after having been frozen at this temperature for this period of time.

G. How will the pain in the animal be monitored?

Toads typically show signs of distress by hissing, or making other release calls. Presumably if inserting a needle causes distress it could be ameliorated by using a local anesthetic.

H. Sedative, analgesic, anesthetics will be used, if any? Include dose, route and frequency of administration.

If toads show signs of distress anesthetics will be used. Sacrificed larval and juvenile individuals will be chilled on ice before being frozen at  $-70^{\circ}\text{C}$ .

I. What is the justification if pain relieving drugs are not used?

Their efficacy is not known in toads and the procedure may not cause any pain.

NA  
Signature: Consulting Veterinarian

NA  
Date



# WESTERN MICHIGAN UNIVERSITY

## INVESTIGATOR IACUC CERTIFICATE

Title of Project: *The effectiveness of bufadienolides in the chemical defenses of toads.*

The information included in this IACUC application is accurate to the best of my knowledge. All personnel listed recognize their responsibility in complying with university policies governing the care and use of animals.

I declare that all experiments involving live animals will be performed under my supervision or that of another qualified scientist. Technicians or students involved have been trained in proper procedures in animal handling, administration of anesthetics, analgesics, and euthanasia to be used in this project.

If this project is funded by an extramural source, I certified that this application accurately reflects all procedures involving laboratory animal subjects described in the proposal to the funding agency noted above.

Any proposed revision to or variation from the animal care and use data will be promptly forwarded to the IACUC for approval.

☐ Disapproved ☐ Approved ☒ Approved with the provisions listed below

**Provisions or Explanations**

- ① State anesthetic route & dosing dose be ~~subcutaneous~~ indicated
- ② State animals to be swabbed with alcohol before aspiration of parotid gland
- ③ Are gland secretions toxic to snakes? Give reference if possible

IACUC Chairperson

8/5/97  
Date

**Acceptance of Provisions**

Signature: Principal Investigator/Instructor

19 August 1997  
Date

IACUC Chairperson Final Approval

9/17/97  
Date

Approved IACUC Number 97-07-02

# WESTERN MICHIGAN UNIVERSITY

Department of Biological Sciences,  
Kalamazoo, Michigan, 49008,  
U.S.A.

(Tel: 616-387-5604; Fax: 616-387-2649; E-mail: [steve.malcolm@wmich.edu](mailto:steve.malcolm@wmich.edu))  
(<http://www.wmich.edu/bios/staff/malcolm/>)

Dr Bruce Bejcek,  
Department of Biological Sciences,  
Western Michigan University,  
Kalamazoo,  
Michigan 49008.

17 September 1997

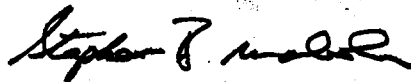
Re: IACUC protocol to investigate *"The effectiveness of bufadienolides in the chemical defenses of toads"*

Dear Dr Bejcek,

Thank you for your comments on our application for an IACUC protocol to investigate, *"The effectiveness of bufadienolides in the chemical defenses of toads"*. Cathy Link and I have considered your comments and provisions in depth and attach with this letter our response.

Thank you again for your comments and help and I look forward to hearing again from the committee.

Yours sincerely,



Stephen B. Malcolm, D. Phil.  
Associate Professor

Provisions to IACUC protocol. 17 September 1997

***The effectiveness of bufadienolides in the chemical defenses of toads***

Catherine E. Link under the advisement of Dr. Stephen B. Malcolm

**1. State anesthetic, route of dosing, and dose to be indicated**

We have been in touch with Dr Cindy Hoorn DVM to discuss toad anesthesia and the use of MS-222 and benzocaine as general anesthetics, or lidocaine (xylacaine) and benzocaine (cetacaine) as local anesthetics. Current Vet Therapy XI says that "Benzocaine (Sigma Chemical) is also an effective anesthetic and is added to the water at concentrations of 300 mg/L for frogs." Xylacaine (lidocaine) was used by Hoogstraten-Miller and Dunham (1997) to anesthetize the skin of African clawed frogs in order to place sutures. However, in this article the authors comment that amphibian skin is very permeable to chemical compounds and anesthetics may result in toxic effects from systemic absorption.

Given the likelihood that chemical anesthetics will influence the validity of the parotoid samples necessary for the proposed research, we would prefer to use a non-chemical method of anesthetic. Thus we propose to cool toads on ice in the field to about 4°C. To do this we would like to place field-collected toads for 30 minutes on moist paper towels in a cooler containing ice. We would then either take a parotoid sample in the field by syringe aspiration and warm the toad again to ambient over a further 30 minutes, or we would return the cooled toad to the laboratory and sample it then keep it in laboratory aquaria for 24 hours (offered live crickets as food) before release back to the capture site.

Cannon and Hostetler (1976) studied the parotoid gland in the Colorado River toad, *Bufo alvarius*, and did not use any chemical anesthetic because its application tended to cause skin gland secretion. Instead they cooled the animals at 4°C until inactive. We also feel that the use of chemical anesthetic on *B. americanus* and *B. fowleri* will likely cause premature secretion of the parotoid chemicals we are trying to obtain.

**2. State animals to be swabbed with alcohol before aspiration of parotoid gland**

Because the skin of toads is highly permeable to organic solvents and these are likely to influence parotoid gland secretions, we would prefer not to swab the gland with alcohol before aspiration. Bufadienolides are highly soluble in ethanol (or any alcohol) and swabbing would influence the validity of our results. Instead we propose to swab the parotoid gland with distilled water (using cotton wool) before and after aspiration.

**3. Are parotoid secretions toxic to snakes? Give reference if possible.**

The eastern garter snake, *Thamnophis sirtalis*, is a major toad predator (Lagler and Salyer, 1945; Licht and Low, 1968) that can only eat toads whole and consequently consumes all of the compounds within the toad parotoid glands. In fact, this snake species appears to be particularly resistant to toxins contained in the secretions of many amphibians (Brodie 1968; Macartney and Gregory 1981) including the rough-skinned newt, *Taricha granulosa*, which is extremely toxic and has few natural predators.

Macartney and Gregory (1981) tested the debilitating and/or lethal effects of amphibian defensive chemicals by force-feeding several amphibians species to snakes in the laboratory. They

showed that of three *Thamnophis* species examined, *T. sirtalis* ate amphibians most readily and did not suffer any apparent ill effects from doing so. Rough-skinned newts were not lethal to snakes but they showed signs of tarichatoxin poisoning and needed approximately 20 h to recover. Licht and Low (1968) showed that *T. sirtalis* can survive oral administration of small doses (3 mg/g body weight) of *Bufo marinus* parotoid venom while non toad-eating snakes suffer lethal cardiac and muscular tetany.

Our chemical assay with toad parotoid venom does not necessitate the feeding of chemicals to snakes. We are simply interested in measuring a voluntary tongue-flick response/strike rate to components of the secretions. Incidental consumption of toad parotoid chemicals by *T. sirtalis* or other *Thamnophis* snakes is very unlikely to cause toxicity. Snakes apparently have digestive physiological mechanisms for dealing with parotoid venom. Moreover, the assay has been designed specifically to minimize distress or toxicity to snakes and all assays will investigate voluntary rather than forced behaviors.

### Literature Cited

- Brodie, E.D., Jr. 1968. Investigations on the skin toxins of the adult rough-skinned newt, *Taricha granulosa*. *Copeia* 1968: 307-313.
- Cannon, M.S., and J.R. Hostetler. 1976. The anatomy of the toad parotoid gland in Bufonidae with some histochemical findings. II. *Bufo alvarius*. *Journal of Morphology* 148: 137-160.
- Hoogstraten-Miller, S., and D. Dunham. 1997. Practical identification methods for African clawed frogs (*Xenopus laevis*). *Lab Animal* 26(7): 36-38.
- Lagler, K.F., and J.G. Salyer. 1945. Influence of availability on the feeding habits of the common garter snake. *Copeia* 1945: 159-162.
- Licht, E.E., and B.S. Low. 1968. Cardiac response of snakes after ingestion of toad parotoid venom. *Copeia* 1968: 547-551.
- Macartney, J.M., and P.T. Gregory. 1981. Differential susceptibility of sympatric garter snake species to amphibian skin secretions. *The American Midland Naturalist* 106: 271-281.

# ADJUSTMENTS IACUC PROTOCOL NUMBER 97-07-02

Application to use Vertebrate Animals for Research or Testing

RECEIVED  
APR 28 1999  
I.A.C.U.C.

Catherine E. (Link) Merovich  
Principal Investigator

Biological Sciences  
Department

387-5600  
Campus Phone

Catherine E. Merovich  
Signature

April 14, 1999  
Date

Dr. Stephen B. Malcolm  
Responsible Faculty Member

Biological Sciences  
Department

387-5604  
Campus Phone

Stephen B. Malcolm (Chair)  
Signature

4-21-99  
Date

The following adjustments must be made under sections II. Animal Use Facilities.

1. The building and room(s) where the animals will be housed and cared for as well as the location of the experiments and procedures:

The laboratory of Dr. Stephen Malcolm  
Room 5022 McCracken Hall and

Room 5010 McCracken Hall

Animals will need to be moved to Wood Hall room 1119.

Animals should only be housed in Wood only until ~~either~~ the Animal Facilities in Haencke Hall are operational or it is requested by the animal facilities manager to move them into the facility. ~~At that time~~ ~~these~~ ~~time~~ animals will be moved into the facility.

4/28/99  
[Signature]  
Chairman IACUC

---

**WESTERN MICHIGAN UNIVERSITY**

---

Date: 5 May 1999

To: Stephen Malcolm, Principal Investigator  
Catherine Merovich, Student Investigator

From: Bruce Bejcek, IACUC Chair *b/f r B. Bejcek*

Re: Changes to IACUC Protocol No. 97-07-02

This letter will serve as confirmation that the changes to your research project "The Effectiveness of Bufadienolides in the Chemical Defenses of Toads" requested in your memo received 28 April 1999 have been approved by the Institutional Animal Care and Use Committee.

Please note, as indicated by the comments on the attached copy of your request, animals may be temporarily housed in Wood Hall. Animals are to be moved into the new facility in Haenicke Hall when it becomes operational or when otherwise requested by the animal facilities manager. If you have any questions, you may contact the research compliance coordinator by phone (7-8293), FAX (7-8276), or email (loreene.broker@wmich.edu).

MAILED  
A  
APR 29 1999  
40176

10/91

**RECEIVED**  
JUL 23 1999

**I.A.C.U.C.**

**WESTERN MICHIGAN UNIVERSITY  
YEARLY RENEWAL FORM APPLICATION TO USE  
VERTEBRATE ANIMALS FOR RESEARCH OR TEACHING**

Date Received \_\_\_\_\_ 168 \_\_\_\_\_  
IACUC Number 97-07-02  
First Renewal Request 07/20/98  
Second Renewal Request 07/15/99

GENERAL INFORMATION: Fill in all appropriate information

<u>Stephen Malcolm</u>	<u>BIOS</u>	<u>7-5604</u>
Principal Investigator/Instructor	Department	Campus Phone
<u>Catherine (Link) MEROVICH</u>	<u>BIOS</u>	<u>7-5600</u>
Co-Principal/Student Investigator	Department	Campus Phone

Title of Project/Course The Effectiveness of Bufadienolides in the Chemical Defense of Toads

**PRINCIPAL INVESTIGATOR/INSTRUCTOR DECLARATION**

I assure that I have obtained IACUC approval prior to implementing this project and that there are no changes in the protocol submitted in the original application to use vertebrate animals for research or teaching. I understand that if at any time changes are made in the use of animals as described in the original application, a letter or amended protocol must be filed for review. I assure that the activities do not unnecessarily duplicate previous experiments.

Signatures:

S.B. Malcolm  
Principal Investigator/Instructor  
Catherine E. Merovich  
Co-Principal/Student Investigator  
(If PI not a faculty member)

20 July 1999  
Date  
21 July 1999  
Date

**INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE APPROVAL**

[Signature]  
IACUC Chairperson

7/28/99  
Date

PLEASE MAIL COMPLETED APPLICATION TO:

Research Compliance Coordinator  
Western Michigan University  
327E Walwood Hall  
Kalamazoo, MI 49008  
(616) 387-8293

**NOTE:** It is the responsibility of the Principal Investigator to obtain the signature of any Co-Principal/Student Investigators.

IAC-E


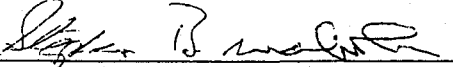
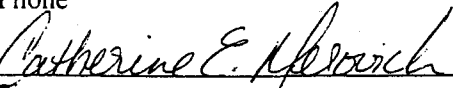
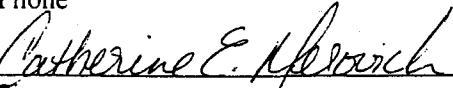
IACUC Number 97-07-02 00-07-02  
 Date of Receipt: 5/17/01  
 Date of Approval 5/19/01

**WESTERN MICHIGAN UNIVERSITY  
 INSTITUTIONAL ANIMAL CARE  
 AND USE COMMITTEE (IACUC)**

Application to use Vertebrate Animals for Research or Teaching

The use of any vertebrate animals in research and/or teaching without prior approval of the Institutional Animal Care and Use Committee (IACUC) is a violation of Western Michigan University policies and procedures. This Committee is charged with the institutional responsibility for assuring the appropriate care and treatment of vertebrate animals.

**Adjustment:** Tadpoles of *B. americanus* and *B. fowleri* will be raised in room 2404 Wood Hall for the Spring and Summer Sessions of 2001. Key WC1 will be available for access to this room.

		
<b>Dr. Stephen B. Malcolm</b>	<b>Biological Sciences</b>	<b>387-5604</b>
Principal Investigator	Department	Campus
Phone		
		
Signature	Date	<u>15 May 2001</u>
		
<b>Catherine E. Merovich</b>	<b>Biological Sciences</b>	<b>387-5687</b>
Student or Co-Principal Investigator	Department	Campus
Phone		
		
Signature	Date	<u>14 May 2001</u>

Title of Project/Course The Role of Bufadienolides in the Chemical Defenses of *Bufo americanus* and *Bufo fowleri* (Anura: Bufonidae).

Approved  
 5/23/01  
 [Signature]



# WESTERN MICHIGAN UNIVERSITY

## Institutional Animal Care and Use Committee

### ANNUAL REVIEW OF VERTEBRATE ANIMAL USE

**PROJECT OR COURSE TITLE:** Role Of Bufadienolides In The Chemical Defenses Of Bufo Americanus And Bufo

Fowleri (Anura: Bufonidae)

IACUC Protocol Number: 00-07-02

Date of Review Request: 09/04/02

Date of Last Approval: 10/02/01

Purpose of project (select one): ☐ Teaching ☒ Research ☐ Other (specify):

**PRINCIPAL INVESTIGATOR OR ADVISOR**

Name: Stephen Malcolm

Title: Assoc./Assist. Professor

Department: BIOS Electronic Mail Address: steve.malcolm@wmich.edu

**CO-PRINCIPAL OR STUDENT INVESTIGATOR**

Name: Catherine Merovich

Title: Student

Department: BIOS Electronic Mail Address: catherine.merovich@wmich.edu

**RECEIVED**

SEP 05 2002

**I.A.C.U.C.**

1. The research, as approved by the IACUC, is completed:

☐ Yes (Continue with items 4-5 below.) ☒ No (Continue with items 2-5 below.)

If the answer to any of the following questions (items 2-4) is "Yes," please provide a detailed explanation on an attached sheet of paper. Include details of any modifications made to the protocol based on new findings or publications, adverse events or mortalities.

2. Have there been any changes in Principal or Co-Principal Investigators? ☐ Yes ☒ No

3. Have there been any new findings or publications relative to this research? ☐ Yes ☒ No

Describe the sources used to determine the availability of new findings or publications:

☐ No search conducted (Please provide a justification on an attached sheet.)

☐ Animal Welfare Information Center (AWIC)

☒ Search of literature databases (select all applicable)

☒ AGRICOLA

☐ Current Research Information Service (CRIS)

☒ Biological Abstracts

☐ Medline

☐ Other (please specify):

Date of search: multip

Years covered by the search: all

Key words:

☐ Additional search strategy narrative:

4. Are there any adverse events, in terms of animal well being, or mortalities to report as a result of this research? ☐ Yes ☒ No

Cumulative number of mortalities:

5. Animal usage: Number of animals used during this quarter (3 months): 38

Cumulative number of animals used to date: 500+

24 released

☒ Stephen B. Malcolm 9/3/02  
Principal Investigator/Faculty Advisor Signature Date

☒ Catherine E. Merovich 3 Sep 2002  
Co-Principal or Student Investigator Signature Date

\* many released -  
I can provide details  
of the exact  
numbers if  
needed.

#### IACUC REVIEW AND APPROVAL

Upon review of the relevant information regarding this protocol, the IACUC approval for this project has been extended for one year from the date of this signature.

☒ [Signature] 8/6/02  
IACUC Chair Signature Date

10/91

Date Received 10/5/01<sup>171</sup>  
IACUC Number 00-07-02  
First Renewal Request X  
Second Renewal Request \_\_\_\_\_

**WESTERN MICHIGAN UNIVERSITY**  
**YEARLY RENEWAL FORM APPLICATION TO USE**  
**VERTEBRATE ANIMALS FOR RESEARCH OR TEACHING**

GENERAL INFORMATION: Fill in all appropriate information

Stephen Malcolm Biological Sciences 387-5604  
Principal Investigator/Instructor Department Campus Phone

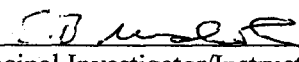
Catherine Merovich Biological Sciences  
Co-Principal/Student Investigator Department Campus Phone

Title of Project/Course Role of Bufadienolides in the Chemical Defenses of Bufo americanus and Bufo fowleri  
(Anura: Bufonidae)

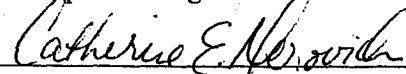
**PRINCIPAL INVESTIGATOR/INSTRUCTOR DECLARATION**

I assure that I have obtained IACUC approval prior to implementing this project and that there are no changes in the protocol submitted in the original application to use vertebrate animals for research or teaching. I understand that if at any time changes are made in the use of animals as described in the original application, a letter or amended protocol must be filed for review. I assure that the activities do not unnecessarily duplicate previous experiments.

Signatures:

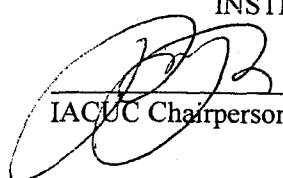
  
Principal Investigator/Instructor

9/24/2001  
Date

  
Co-Principal/Student Investigator  
(If PI not a faculty member)

9/29/2001  
Date

**INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE APPROVAL**

  
IACUC Chairperson

10/2/01  
Date

PLEASE MAIL COMPLETED APPLICATION TO:  
Research Compliance Coordinator  
Western Michigan University  
251 W Walwood Hall  
Kalamazoo, MI 49008  
(616) 387-8293

**NOTE:** It is the responsibility of the Principal Investigator to obtain the signature of any Co-Principal/Student Investigators.

IAC-E

1

## **Appendix B**

### **Permission Letters To Use Published Materials**

18 July 2004

Dr. Edmund D. Brodie, Jr.  
Department of Biology  
BNR 149  
Utah State University  
5305 Old Main Hill  
Logan, Utah 84322

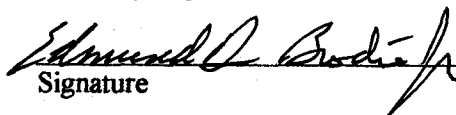
Dear Dr. Brodie,

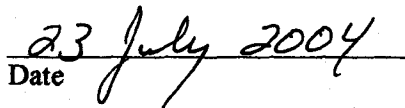
I would like to request your permission to include an excerpt from the following item in my doctoral dissertation and/or in a publication that results from my dissertation research:

Brodie, E. D. Jr. and D. R. Formanowicz, Jr. 1987. Antipredator mechanisms of larval anurans: protection of palatable individuals. *Herpetologica* 43(3): 369-373.

Specifically, I would like permission to use your Figure 1. from this paper showing a model of palatability and ability to escape as they affect stage-specific survivorship of anuran larvae. In my dissertation, I have applied the hypothesis that *Bufo* tadpoles will have bufadienolide defenses when they are unpalatable to predators and will have less or no bufadienolide defenses when they are palatable. I will gratefully acknowledge the source with full credit in the manuscript of the dissertation and/or in a publication.

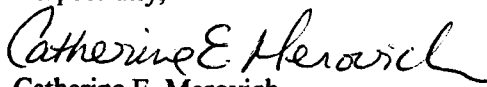
For your convenience, I am including a space for your signature to indicate your permission for my use of this material. By signing below, you give ProQuest Information and Learning (formerly University Microfilms) the right to supply copies of this material on demand as part of my doctoral dissertation. Please attach any other terms or conditions for the proposed use of this item below. If you no longer hold the copyright to this work, please indicate whom I should direct my request on the bottom of this page and return it to me.

  
Signature

  
Date

Please return this letter in the self-addressed, stamped envelop provided. Thank you for your time and attention to this matter.

Respectfully,

  
Catherine E. Merovich  
Department of Biology  
West Virginia University  
Morgantown, WV 26505  
(304) 293-5201 ext. 31543

**Ronald Hussey**

Permissions Manager/  
Serial Rights Manager

215 Park Avenue South  
New York, NY 10003  
phone 212-420-5802  
fax 212-420-5899  
ron\_hussey@hmco.com  
www.hmco.com

September 22, 2004

Catherine E. Merovich  
Department of Biology  
West Virginia University  
P.O. Box 6057  
Morgantown, WV 26506

Dear Ms. Merovich:

This is in reply to your request of September 8, 2004.

We are pleased to grant you permission for use of material, as cited in your request, from the book, **A FIELD GUIDE TO REPTILES AND AMPHIBIANS OF EASTERN AND CENTRAL NORTH AMERICA**, Third Edition for use in your dissertation. Our requirement is that you cite the source as a footnote or in your bibliography.

The permission applies to all copies of your dissertation made to meet degree requirements of Western Virginia University, and to ProQuest Information and Learning, formerly known as University Microfilms editions, which produces copies on demand.

Please re-apply to this department if your dissertation is later accepted for publication and you wish to retain our material.

Best wishes for the successful completion of your work.

Sincerely,

  
Ronald Hussey



**Ronald Hussey**

Permissions Manager/  
Serial Rights Manager

215 Park Avenue South  
New York, NY 10003  
phone 212-420-5802  
fax 212-420-5899  
ron\_hussey@hmco.com  
www.hmco.com

December 21, 2004

Catherine Merovich  
Department of Biology  
West Virginia University  
P.O. Box 6057  
Morgantown, WV 26506

Dear Ms. Merovich

This is in reply to your request of November 20, 2004.

We are pleased to grant you permission for use of material, as cited in your request of November 20, 2004 from the book, *A FIELD GUIDE TO REPTILES AND AMPHIBIANS OF EASTERN AND CENTRAL NORTH AMERICA*, Third Edition for use in your dissertation. Our requirement is that you cite the source as a footnote or in your bibliography.

The permission applies to all copies of your dissertation made to meet degree requirements of Western Michigan University, and to Pro Quest Information and Learning editions, which produces copies on demand.

Please re-apply to this department if your dissertation is later accepted for publication and you wish to retain our material.

Best wishes for the successful completion of your work.

Sincerely,

Ronald Hussey



**Ronald Hussey**

Permissions Manager/  
Serial Rights Manager

215 Park Avenue South  
New York, NY 10003  
phone 212-420-5802  
fax 212-420-5899  
ron\_hussey@hmco.com  
www.hmco.com

December 21, 2004

Catherine Merovich  
Department of Biology  
West Virginia University  
P.O. Box 6057  
Morgantown, WV 26506

Dear Ms. Merovich:

This is in reply to your request of November 20, 2004.

We are pleased to grant you permission for use of material, as cited in your request of November 20, 2004 from the book, *A FIELD GUIDE TO REPTILES AND AMPHIBIANS OF EASTERN AND CENTRAL NORTH AMERICA*, Third Edition for use in your dissertation. Our requirement is that you cite the source as a footnote or in your bibliography.

The permission applies to all copies of your dissertation made to meet degree requirements of Western Michigan University, and to Pro Quest Information and Learning editions, which produces copies on demand.

Please re-apply to this department if your dissertation is later accepted for publication and you wish to retain our material.

Best wishes for the successful completion of your work.

Sincerely,

Ronald Hussey