

Utah State University

DigitalCommons@USU

All Graduate Theses and Dissertations

Graduate Studies

8-2013

Presence and Function of Tetrodotoxin in Terrestrial Vertebrates and Invertebrates

Amber N. Stokes
Utah State University

Follow this and additional works at: <https://digitalcommons.usu.edu/etd>



Part of the [Biology Commons](#), and the [Ecology and Evolutionary Biology Commons](#)

Recommended Citation

Stokes, Amber N., "Presence and Function of Tetrodotoxin in Terrestrial Vertebrates and Invertebrates" (2013). *All Graduate Theses and Dissertations*. 1751.
<https://digitalcommons.usu.edu/etd/1751>

This Dissertation is brought to you for free and open access by the Graduate Studies at DigitalCommons@USU. It has been accepted for inclusion in All Graduate Theses and Dissertations by an authorized administrator of DigitalCommons@USU. For more information, please contact digitalcommons@usu.edu.



PRESENCE AND FUNCTION OF TETRODOTOXIN IN TERRESTRIAL
VERTEBRATES AND INVERTEBRATES

by

Amber N. Stokes

A dissertation submitted in partial fulfillment
of the requirements for the degree

of

DOCTOR OF PHILOSOPHY

in

Biology

Approved:

Edmund D. Brodie, Jr.
Major Professor

Susannah S. French
Committee Member

Michael E. Pfrender
Committee Member

Peter K. Ducey
Committee Member

Lee Rickords
Committee Member

Mark McLellan
Vice President for Research and
Dean of the School of Graduate
Studies

UTAH STATE UNIVERSITY
Logan, Utah

2013

Copyright © Amber N. Stokes 2013

All Rights Reserved

ABSTRACT

Presence and Function of Tetrodotoxin in Terrestrial Vertebrates and Invertebrates

by

Amber N. Stokes, Doctor of Philosophy

Utah State University, 2013

Major Professor: Dr. Edmund D. Brodie, Jr.
Department: Biology

Tetrodotoxin (TTX) is a potent neurotoxin that acts by blocking the pore region of voltage-gated sodium channels in nerve and muscle tissue. This causes paralysis, and often death due to asphyxiation. Interestingly, TTX is found in an array of organisms ranging from bacterial species to vertebrates. Further, TTX is found in both aquatic and terrestrial environments. This range of taxa and environments has led to three common lines of study for ecological research on this toxin: production, predation, and identification of novel TTX bearing taxa.

I began my research by also refining a Competitive Inhibition Enzymatic Immunoassay technique for fast, easy, and inexpensive quantification of TTX. I then focused on the three previously mentioned areas of research. Female newts (*Taricha granulosa*) are known to endow their eggs with TTX in order to protect them from predation. I looked at whether females allocated TTX to their eggs evenly over three years in captivity and compared those levels to TTX levels in eggs directly after capture. I found that eggs had lower levels of TTX following initial capture, but those levels did

not change over the next three years. This provides evidence that TTX is endogenously produced in this species.

Because of the high levels of TTX in newts, there are few known predators. I observed river otters feeding on newts in a high elevation lake in Oregon. I found that these newts have very low levels of TTX, and that in general high elevation populations in Oregon have low levels of TTX relative to low elevation populations. Finally, I documented TTX in two species of terrestrial flatworm (*Bipalium adventitium* and *Bipalium kewense*). Tetrodotoxin has never before been identified in a terrestrial invertebrate species. Further, I found evidence that suggests that TTX is used for both defense and prey capture in these worms. These studies add to our understanding of the evolution of TTX and how it influences interactions between organisms and their biotic and abiotic environments.

(120 pages)

PUBLIC ABSTRACT

Presence and Function of Tetrodotoxin in Terrestrial Vertebrates and Invertebrates

by

Amber N. Stokes, Doctor of Philosophy

Utah State University, 2013

Tetrodotoxin (TTX) is a potent neurotoxin found in a variety of species. This toxin has long been of concern to human health as it is found in puffer fish, which are a delicacy in Japan. Since the distribution of this toxin is so great, there are many questions regarding the evolution and ecology of organisms that have TTX. My research has focused on further investigating three topics with this research: production, predation, and identification of novel TTX bearing taxa. In order to perform this research I first refined a Competitive Inhibition Enzymatic Immunoassay methodology to quantify levels of TTX in tissue.

Production: There is not a consensus among the scientific community as to how TTX is produced. Given that it is found in such a wide variety of species, it has been thought that perhaps bacteria and then bioaccumulated through the food chain to larger organisms. However, there is little support for this in newts (*Taricha granulosa*). I investigated this question by looking at TTX levels in newt eggs over time in the lab. It is thought that if they acquire TTX from somewhere else, levels will drop in the lab because they have non-TTX bearing diets. However, if TTX is produced by the newts TTX levels should remain relatively constant. We found that after the initial capture,

TTX levels declined. However, they remained constant in the three following years. I believe that the initial decline was due to the shortened breeding period in the lab, and that this study is further evidence for TTX production in these newts.

Predation: The high levels of TTX found in newts has resulted in few predators. I observed river otters feeding on these newts in a high elevation lake in Oregon. I found that this population has low levels of TTX, which enabled otters to eat them. Further, I found that high elevation populations in Oregon tend to have lower TTX levels than do low elevation populations in general.

Novel species: Tetrodotoxin has never been identified in a terrestrial invertebrate. I identified TTX in two species of terrestrial flatworms (*Bipalium adventitium* and *B. kewense*). Further, I found that TTX likely is utilized by these species in order to protect them from predation and to subdue larger prey items.

These studies have provided further evidence for the production of TTX as well as the biotic and abiotic interactions surrounding organisms that have TTX. This will help us understand the evolution of this toxin.

ACKNOWLEDGMENTS

As an undergrad I was pre-med up until the very end. Then, with the guidance of some professors at CSU, Bakersfield I decided to try graduate school out instead. I contacted many professors, and didn't get much response back. Until, I contacted "Doc" (Edmund D. Brodie, Jr.). Despite my lack of research experience, especially with organismal/field biology, he took me on as a master's student. I had no idea what I was doing! But, he took a chance on me and helped me become a biologist. He has shown me that honesty, hard work, and love for the research will carry me through my career. He also has taught me that collaboration and discussion are critical to the scientific process. He has created a lab environment that allows all students to be mentors and mentees. I have learned so much from this and hope to carry that forward into my own lab. And, I'll try to be less critical of my own writing so that manuscripts won't take so long. I'll try. I cannot thank him enough for all that he has done for me, and am happy to have had him as my friend as well as my mentor.

Our collaborator and co-author, Edmund (Butch) D. Brodie III, has been a huge help with my work. He has great suggestions with both writing and statistical analyses. He also has taken an interest in Doc's students, helping to mentor our development. I am grateful for his input and look forward to our future collaborations.

I was lucky to have the best PhD committee, ever! I have been fortunate to work with five great minds that have helped shape me. Susannah French has been amazing, and more of a co-advisor than simply a committee member. She helped me get the assay working that was instrumental to my research. I don't think I could have done it without

you! She has always been willing to discuss my data, read drafts of manuscripts for me, and has written many letters of recommendation for me. She has become not only a great colleague, but also a good friend. Luckily for me, we still have a lot of work to do together! Mike Pfrender has been an amazing mentor for me. He stuck it out with me for my master's as well as my PhD. He has always been supportive of my work and willing to help in any way that he can. Even now that he is all the way in Indiana. He has spent time speaking with me about my work and wrote many letters of recommendation for me. I'm looking forward to continuing to work out sodium channels with him. Peter Ducey has been instrumental to my work. He is my *Bipalium* expert, and I could not be more grateful for his expertise, kindness, and mentorship. He has helped me refine my writing, which has been crucial to my success. Finally, I thank Lee Rickords for his help in this process. He has always been willing to listen and help in any way that I've needed. I appreciate his kindness and interest in my work.

I am thankful for the Utah State University Herp group (all iterations since I've been here). It has been crucial for my development to read and critique the papers of others, as well as to have my own papers critiqued. It has helped me better my writing skills, and our discussions have taught me so much about science and the scientific process. I'm not sure that I could have learned these things outside of that setting.

I have been fortunate to have a whole group of amazing students to collaborate with, mentor, and be mentored by. I would especially like to thank Kristin Bakkegard, Leigh Latta, Chris Feldman, and Megan Lahti for being such great mentors to me when I first arrived. They were all amazing at guiding me through grad school, and always willing to give me advice at any turn. I especially am grateful to Megan and Kristin.

They are both great biologists in their own right, and have been wonderful friends as well as colleagues. I am also grateful to Becky Williams. We actually never were enrolled at the same time, but we had an opportunity to collaborate together. She is incredibly organized, thoughtful, and knowledgeable, and I have attempted to implement some of these traits in my own work. It has been a pleasure working with her and being her friend. Charles Hanifin has also been a huge help. He stepped in to help with HPLC for both my master's and my PhD. He's also always been willing to discuss science and give advice at any time. We've got some collaborations going now, and I look forward to more! Also, the faculty and staff in the Biology Department at USU have always been really supportive.

My most recent lab mates Brian Gall and Gareth Hopkins have been amazing. Brian and I have worked on many projects together. We have had the opportunity to do both field and lab work together. We started our PhD programs at the same time and have suffered and celebrated together over the years. I'm grateful for his friendship and collaboration. I know we have only just begun!! Gareth has always been a kind person who is willing to help as much as he can. He has definitely kept us all entertained. He's Canadian (garborator!), which is fun to tease him about even though we really like Canadians. Lori Neuman-Lee, Geoff Smith, Andrew Durso, and Shab Mohammadi have all been great friends and collaborators. I have learned much from you all. I especially want to thank Lori Neuman-Lee and Nick Kiriazis for always being there for me. They have been both intellectual and emotional supports for me. They have often fed and entertained me when my husband was away working, and I couldn't be more grateful for their kindness.

I could not have done any of this without the support of my family. My parents, Maurice and Jackie Brouillette, have always encouraged me to follow my dreams. I got my love of science and school from both of them. They've always supported me, and didn't freak out when I decided not to go to medical school so that I could play with newts instead. My sister Lauren Hammack has been my built-in best friend from her birth. She's willing to fight my battles when I can't, and is always willing to talk and give me advice. Thanks, Sparkles. My grandparents Byron and Mable Zeek have always made me feel that they would travel to the ends of the universe for me. They've supported every decision I've made, even ones they didn't quite agree with. My grandparents Eugene and Jeannie Brouillette have also been great supporters of me and have always encouraged my love of science. My in-laws Berry and Diane Stokes and Wanda Reynolds have been so supportive of my education and have helped at every turn. I also have a whole slew of aunts, uncles (in-law, too!), brothers- and sisters-in-law, and nieces and nephews whom I love very much. You have all been an inspiration to me.

I cannot thank my husband, Tyson Stokes, enough. He didn't think twice when I said that I wanted to quit my relatively well-paying job and move to Utah for grad school. He has always been my biggest supporter, and best friend. He has worked hard to make it possible for me to return to school and follow my dream. Tyson has been willing to help in the field, and always listens to me when I talk about work. He knows almost as much about my system as I do because he listens and cares. Without him I don't know that I would have had the courage to follow this dream, and likely would not have survived all of this. Thank you for your love.

Chapter Acknowledgements

Chapter 2. I would like to thank the Center for Integrated BioSystems (CIB) at Utah State University (USU) for the use of equipment. Dr. Kum Park and Dr. Mark Signs of CIB, Dr. Edmund D. Brodie, Jr. and Dr. Joseph Li from the Department of Biology at USU, Dr. Bignami from Hawaii Biotech, and Dr. Elizabeth Lehman for their helpful suggestions vetting this technique. I thank the USU Herpetology Group for their help with this manuscript.

Chapter 3. I thank Joe Beatty and Oregon State University for access to their research ponds. Thanks go to Leticia Hoffmann for help with animal care. Newts were collected under Oregon Department of Fish and Wildlife permit 045-09 and 004-10. This research was approved under Utah State University's IACUC protocol #1008. Financial support was provided by the Utah State University Biology Department.

Chapter 4. The U.S. Geological Survey and Crater Lake National Park provided support for this work. I would especially like to thank D. Hering, S. Girdner, M. Parker, A. Denlinger, and H. Griffin for help in the field. Any use of trade, product, or firm names is for descriptive purposes only and does not imply endorsement by the U.S. Government.

Chapter 5. I would like to thank Brian Rivest, Michelle Hamerslough, and Dan Hodgson for help collecting these flatworms in the field. We also would like to thank SUNY Cortland, Utah State University, for funding for this project. Susan Durham at Utah State University provided helpful insight with statistical analyses.

CONTENTS

	Page
ABSTRACT.....	iii
PUBLIC ABSTRACT.....	v
ACKNOWLEDGMENTS.....	vii
LIST OF TABLES.....	xiii
LIST OF FIGURES.....	xiv
CHAPTER	
1. INTRODUCTION.....	1
2. AN IMPROVED COMPETITIVE INHIBITION ENZYMATIC IMMUNOASSAY METHOD FOR TETRODOTOXIN QUANTIFICATION.....	13
3. FEMALE NEWTS (<i>TARICHA GRANULOSA</i>) PRODUCE TETRODOTOXIN LADEN EGGS AFTER LONG TERM CAPTIVITY.....	24
4. OTTER PREDATION ON <i>TARICHA GRANULOSA</i> AND VARIATION IN TETRODOTOXIN LEVELS WITH ELEVATION.....	44
5. THE FIRST KNOWN CASE OF TETRODOTOXIN IN A TERRESTRIAL INVERTEBRATE: TERRESTRIAL FLATWORMS <i>BIPALIUM ADVENTITIUM</i> AND <i>BIPALIUM KEWENSE</i>	63
6. SUMMARY.....	85
APPENDIX.....	96
CURRICULUM VITAE.....	102

LIST OF TABLES

Table		Page
2.1	Comparisons of the calculated concentrations from two different plates run the exact same way.....	15
4.1	Results from Wilcoxon pairwise comparisons for high elevation populations.....	53
5.1	The amount of TTX adjusted for weight of body segment for <i>Bipalium adventitium</i> and <i>Bipalium kewense</i>	73

LIST OF FIGURES

Figure	Page
2.1	Tetrodotoxin standard curves.....15
2.2	A typical template used for plates.....20
3.1	Mean total TTX and TTX concentration (ng TTX/mg egg mass) present in eggs from eight different female <i>Taricha granulosa</i>32
3.2	Mean mass (mg) of eggs from eight female newts (<i>Taricha granulosa</i>) from initial collection in the wild through three additional years in captivity.....33
3.3	Comparison of the mean amount of TTX regenerated in the skin of adult newts (<i>Taricha granulosa</i>) over 9 months (skin) versus the estimated mean amount of TTX provisioned by eight female newts in an entire clutch of 525 eggs.....36
4.1	<i>Lontra canadensis</i> feeding on <i>Taricha granulosa</i> and <i>Ambystoma gracile</i> at Lake in the Woods, Oregon.....47
4.2	Map of the four high elevation locations in Oregon where <i>Taricha granulosa</i> were collected.....50
4.3	Total whole body TTX for each individual newt (<i>Taricha granulosa</i>) at each of the five populations in western Oregon.....52
4.4	Relationship between elevation and TTX levels across populations from Hanifin et al. (2008), Ridenhour (2004), and the present study.....54
5.1	Concentration values for each segment for <i>Bipalium adventitium</i> and <i>Bipalium kewense</i>72
5.2	The amount of TTX adjusted for weight of that particular body region for <i>Bipalium adventitium</i> and <i>Bipalium kewense</i>73
5.3	Elution times and TTX profiles of an authentic TTX standard, <i>Bipalium adventitium</i> and <i>B. adventitium</i> co-injected with a TTX standard.....74

CHAPTER 1

INTRODUCTION

Chemicals are used often in organismal systems for communication, defense, and during predation (Eisner and Meinwald 1995). One such toxin is Tetrodotoxin (TTX), which is a highly potent neurotoxin that acts by blocking the pore region of voltage-gated sodium channels (Narahashi et al. 1964; 1967). This action prevents the propagation of action potentials, inducing paralysis. Death occurs from the paralysis of the diaphragm and subsequent asphyxiation (Brodie 1968). Tetrodotoxin was initially identified in fishes of the family Tetraodontiformes (Miyazawa and Noguchi 2001). Puffer fish are within this family of fish, and are well known as a delicacy in Japanese cuisine. The effects of TTX have been known in certain cultures of people for nearly 5000 years (Fuhrman 1986). *Tetraodon lineatus* was found on the Egyptian tomb from the Fifth Dynasty around 2500 B.C, and there is evidence that they were aware of the toxic nature of these fish. The Chinese emperor Shun Nung wrote about the eggs of puffer fish as a medicine sometimes around 2700 B.C. Europeans did not learn of the toxic nature of puffer fish until 1727, when Engelbert Kaempfer published his book *History of Japan*. And, in 1774 Captain James Cook experienced the toxic effects of TTX when they ate fish of “a poisonous quality” (Cook 1775). It wasn’t until nearly 100 years later, that experiments to understand the mechanisms of action and chemical structure of TTX were underway (Fuhrman 1986). Identifying and understanding TTX was important in the early days because consumption of puffer fish could be fatal if not prepared correctly.

Since TTX was initially described, however, there has been a vast increase in the number of species identified with TTX. Red calcareous algae, dinoflagellates, and bacteria are some of the organisms identified with TTX (Miyazawa and Noguchi 2001). Further, there are more than 12 major animal groups that have been identified as having TTX. These taxa range across both invertebrate and vertebrate groups and cross between marine and terrestrial environments. These groups include fish, horseshoe crabs, xanthid crabs, blue-ringed octopuses, gastropods, starfish, flatworms, ribbon worms, arrow worms, annelids, salamanders/newts, and frogs. Due to this variety of organisms possessing TTX, the importance of understanding the toxin has shifted from solely an issue of human health, to questions regarding the ecology and evolution of such a pervasive toxin.

Because TTX is distributed in such a variety of organisms and ecosystems there have become three common avenues of research pursued in ecological studies of TTX: production, predation, and identification of novel taxa with TTX. These lines of research all add to the overall picture of where TTX comes from and how it influences interactions between organisms and both their biotic and abiotic environments.

The mode of production of TTX in natural systems is still unknown, and whether TTX is produced exogenously or endogenously has been debated by scientists studying TTX (Chau et al. 2011). Those who support the idea of exogenous origins of TTX, believe that TTX is produced by bacteria and acquired through one of two modes. First, the bacteria produce TTX, which is then transferred up through the food chain. Secondly, there is a symbiotic relationship between the TTX producing bacteria and the

species acquiring TTX. Most evidence in the literature has been in support of the first hypothesis (Noguchi et al. 2006a, b; Noguchi and Arakawa 2008). For example, TTX producing bacteria have been cultured from species of puffer fish, but these fish no longer have TTX when raised in captivity (Noguchi et al. 2006a, b). However, bacterial cultures have been found to produce very low levels of TTX, further providing support for the idea that TTX is obtained through bioaccumulation (Noguchi and Arakawa 2008). Predators of *Taricha* adults (Williams et al. 2004) and eggs (Gall et al. 2012) have been shown to sequester the toxin, providing further evidence that sequestration through the food chain is possible.

Lehman et al. (2004) tried to determine if rough-skinned newts, *Taricha granulosa*, have TTX producing species of bacteria, but were unable to find any evidence of such. Other data have shown that *T. granulosa* females actually increase in TTX levels over time in the lab despite eating a TTX free diet (Hanifin et al. 2002). Similarly, *Atelopus oxyrhynchus* frogs have been shown to have high levels of TTX for 3.5 years in the lab (Yotsu-Yamashita et al. 1992). These data suggest that TTX may be produced endogenously some how. However, it is also possible that production of TTX varies from species to species. Some may use a form of exogenous production, while others use endogenous production to obtain TTX in their tissues. Many possible pathways for the biosynthesis of TTX have been proposed, however, the pathway has not been fully elucidated for TTX at this time (Chau et al. 2011). Because we don't know the pathway for TTX production, it is difficult to fully understand how and where TTX may be produced/acquired in an organism.

As these questions remain, it has become increasingly important in TTX research to understand the interactions in communities that include TTX-bearing taxa.

Tetrodotoxin has been shown to have a wide variety of ecological roles ranging from a pheromone to a defensive chemical (Williams 2010). Multiple lines of evidence suggest that TTX is often used as a chemical signal as either an attractant or as a warning signal. For example, in species of TTX-bearing marine snails, TTX functions as an attractant with the level of attraction positively correlated with the snail's own TTX levels (Hwang et al. 2004). Similarly, copepods parasitic on puffer fish species utilize TTX in order to find a host (Ito et al. 2006). Larvae of the California newt (*Taricha torosa*) detect TTX in order to avoid being cannibalized by conspecific adults (Zimmer et al. 2006).

Despite the many ecological functions that may exist for TTX, it is most often studied in the context of predator/prey interactions. In some cases, TTX is used during predation to immobilize large or highly mobile prey items. Ritson-Williams et al. (2005) found that TTX in a polyclad flatworm from Guam did not successfully protect individuals from predation, but effectively immobilized prey increasing prey capture success. Several species of TTX-bearing poison arrow worms have been shown to immobilize copepod prey in a lab setting (Nagasawa 1985). Often, the function of TTX is initially elucidated by the location of the toxin. For example, there is not yet any evidence of how effective TTX is against the natural prey of blue-ringed octopuses (Williams 2010). However, this species primarily stores TTX in the posterior salivary glands (Hwang et al. 1989; Yotsu-Yamashita et al. 2007; Williams and Caldwell 2009), which is a common location for octopus venom (Ghiretti 1960).

Most often, however, TTX is considered a defensive toxin. Again, much of the evidence is based on the location of the toxin. In many species such as amphibians, blue-ringed octopuses, and puffer fishes TTX is found in the skin (Noguchi and Arakawa 2008). Further, there are species of flatworm, newt, and puffer fish that have high concentrations of TTX in the ovaries, which is then endowed into their eggs (Williams 2010). However, in regards to *T. granulosa*, there is empirical evidence dating back to 1968 suggesting that TTX is used as a defense from a wide array of potential predators (Brodie 1968). Early life-history stages of *T. granulosa* have also been shown to utilize TTX defensively, which successfully protects them from predation by dragonfly larvae (Gall et al. 2011b). Furthermore, the eggs of *T. granulosa* are highly toxic (Hanifin et al. 2003), and TTX has been indicated as an effective defense against invertebrate egg predators (Gall et al. 2011a).

Though our knowledge of the function of TTX as well as the distribution of TTX in the natural world has grown significantly in the last 50 years, TTX has been difficult to quantify. Tetrodotoxin may be quantified using methods like High Phase Liquid Chromatography (HPLC), Gas Chromatography-Mass Spectroscopy (GC-MS), or Liquid Chromatography-Mass Spectroscopy (LC-MS) (Noguchi and Mahmud 2001). Though, these methods are highly effective for quantification and identification of TTX and its many isomers, they are very expensive and require trained individuals to run them. Other methods such as the mouse bioassay technique commonly employed by Japanese laboratories are difficult to get approval from institutional animal care and use

committees (IACUC), and require housing and feeding for mice. Because of these reasons, TTX research has been challenging for many labs.

The goal of my research has been to refine methodology for quantifying TTX in a manner that is both accurate and inexpensive. This then allowed me to pursue the three main avenues of inquiry regarding TTX: production, predation, and identification of novel taxa with TTX.

Chapter 2. This chapter describes a newly refined methodology for quantifying TTX called a Competitive Inhibition Enzymatic Immunoassay (CIEIA). This procedure is much less expensive, costing about \$0.32 per sample in contrast to \$12.00/sample for HPLC. Other similar techniques have been published for TTX quantification, however, these methods proved to be difficult to replicate as they contained errors, did not report optimal concentrations of reagents, or required detailed knowledge of EIA procedures. This chapter refines those previously published methods in an attempt to aid others in quantification of TTX and amplify our ability to study these systems.

Chapter 3. One of the major questions with TTX research is whether or not the toxin is produced exogenously or endogenously. There is not a consensus one way or another in any system known. A simple method of collecting evidence for one of these two hypotheses is to measure the levels of TTX-bearing animals while in captivity with a controlled diet. Hanifin et al. (2002) found that *T. granulosa* actually increase TTX levels in captivity. However, nobody had asked this question in regards to the eggs of *Taricha*, which are also highly toxic. This chapter examines the TTX levels in *T.*

granulosa eggs over three years in the lab, and provides further evidence to support the hypothesis that newts produce TTX endogenously.

Chapter 4. *Taricha granulosa* have long been known to only have one successful predator, snakes of the genus *Thamnophis* (Brodie 1968; Brodie and Brodie 1990; 1991). These snakes have evolved changes in the gene sequence for the pore region of the voltage-gated sodium channel, which alter its shape, and lowers the binding affinity of TTX to that channel (Geffeney et al. 2002; 2005; Feldman et al. 2009). This renders the snakes resistant to the effects of the toxin, and therefore, able to ingest toxic newts. This predator-prey system is also recognized as a coevolutionary arms race (Brodie and Brodie 1990; Brodie and Brodie 1991). However, there are some documented instances of predation on newts by other species of animal with no ill effects to the predator (Fellers et al. 2008; Gall et al. 2011b). The question in these systems is whether the newts have very low levels of TTX or whether the predator is resistant to TTX in some way. This chapter investigates novel predation on newts by otters in high elevation lakes in Oregon. I also investigated the relationship between elevation and the TTX levels of newts. These data further our knowledge about additional selective pressures on some populations, as well as increase our understanding of TTX in newt populations.

Chapter 5. Despite the array of species identified of having TTX, there has never been a terrestrial invertebrate identified with TTX. In this chapter, I identify two species of terrestrial flatworm that have TTX, *Bipalium adventitium* and *Bipalium kewense*. Further, I show that there is evidence suggesting that both species of *Bipalium* use TTX defensively and during predation. I looked at TTX levels in three body segments of the

flatworms in order to determine relative levels and distribution. I compared these levels as concentration and as the total amount of TTX per unit weight of body segment. These data open up a new area of research in the study of TTX and have great implications for the evolution of TTX bearing species.

LITERATURE CITED

- Brodie, E. D., III and E. D. Brodie, Jr. 1990. Tetrodotoxin resistance in garter snakes: an evolutionary response of predators to dangerous prey. *Evol.* 44:651-659.
- 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 in island populations. *Evol.* 45:221-224.
- Brodie, E. D., Jr. 1968. Investigations on the skin toxin of the adult rough-skinned newt, *Taricha granulosa*. *Copeia* 1968:307-313.
- Chau, R., J. A. Kalaizis, and B. A. Neilan. 2011. On the origins and biosynthesis of tetrodotoxin. *Aquat. Toxicol.* 104:61-72.
- Cook, J. 1775. The journals of Captain James Cook on his voyages of discovery. Cambridge Univ. Press, London.
- Eisner, T. and J. Meinwald. 1995. Chemical Ecology: The Chemistry of Biotic Interaction. Nat. Acad. Press, Washington, D.C.
- Feldman, C. R., Edmund D. Brodie, Jr., E. D. Brodie III, and M. E. Pfrender. 2009. The evolutionary origins of beneficial alleles during the repeated adaptation of garter snakes to deadly prey. *PNAS* 106:13415-13420.

- Fellers, G. M., J. Reynolds-Taylor, and S. Farrar. 2008. *Taricha granulosa* predation. Herpetol. Rev. 38:317-318.
- Fuhrman, F. A. 1986. Tetrodotoxin, tarichatoxin, and chiriquitoxin: historical perspectives. Ann. NY Acad. Sci. 479:1-14.
- Gall, B. G., E. D. Brodie III, and E.D. Brodie, Jr. 2011a. Survival and growth of the caddisfly *Limnephilus flavastellus* after predation on toxic eggs of the rough-skinned newt (*Taricha granulosa*). Can. J. Zoolog. 89:483-489.
- Gall, B. G., A. N. Stokes, S. S. French, E. D. Brodie III, and E.D. Brodie, Jr. 2012. Predatory caddisfly larvae sequester tetrodotoxin from their prey, eggs of the rough-skinned newt (*Taricha granulosa*). J. Chem. Ecol. 38:1351-1357.
- Gall, B. G., A. N. Stokes, S. S. French, E. A. Schlepfforst, E. D. Brodie III, and E. D. Brodie, Jr. 2011b. Tetrodotoxin levels in larval and metamorphosed newts (*Taricha granulosa*) and palatability to predatory dragonflies. Toxicon 57:978-983.
- 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.
- Geffeney, S. L., E. Fujimoto, E. D. Brodie III, E.D. Brodie, Jr., and P. C. Ruben. 2005. Evolutionary diversification of TTX-resistant sodium channels in a predator-prey interaction. Nature 434:759-763.
- Ghiretti, F. 1960. Toxicity of octopus saliva against Crustacea. Ann. NY. Acad. Sci. 90:726-741.

- Hanifin, C. T., E. D. Brodie III, and E.D. Brodie, Jr. 2003. Tetrodotoxin levels in the eggs of the rough-skin newt, *Taricha granulosa*, are correlated with female toxicity. *J. Chem. Ecol.* 29:1729-1739.
- Hanifin, C. T., E. D. Brodie III, and E.D. Brodie, Jr. 2002. Tetrodotoxin levels of the rough-skin newt, *Taricha granulosa*, increase in long-term captivity. *Toxicon* 40:1149-1153.
- Hwang, D. F., O. Arakawa, T. Saito, T. Noguchi, U. Simidu, K. Tsukamoto, Y. Shida, and K. Hashimoto. 1989. Tetrodotoxin-producing bacteria from the blue-ringed octopus, *Octopus maculosus*. *Mar. Biol.* 100:327-332.
- Hwang, P.-A., T. Noguchi, and D.-F. Hwang. 2004. Neurotoxin tetrodotoxin as attractant for toxic snails. *Fisheries Sci.* 70:1106-1112.
- Ito, K., S. Okabe, M. Asakawa, K. Bessho, S. Taniyama, Y. Shida, and S. Ohtsuka. 2006. Detection of tetrodotoxin (TTX) from two copepods infecting the grass puffer *Takifugu niphobles*: TTX attracting the parasites? *Toxicon* 48:620-626.
- Lehman, E. M., E.D. Brodie, Jr., and E. D. Brodie III. 2004. No evidence for an endosymbiotic bacterial origin of tetrodotoxin in the newt *Taricha granulosa*. *Toxicon* 44:243-249.
- Miyazawa, K. and T. Noguchi. 2001. Distribution and origin of tetrodotoxin. *J. Toxicol.-Toxin Rev.* 20:11-33.
- Nagasawa, S. 1985. The digestive efficiency of the chaetognath *Sagitta crassa* Tokioka, with observations on the feeding process. *J. Exp. Mar. Biol. Ecol.* 87:67-75.

- Narahashi, T., J. W. Moore, and R. N. Poston. 1967. Tetrodotoxin derivatives: chemical structure and blockage of nerve membrane conductance. *Science* 156:976-979.
- Narahashi, T., J. W. Moore, and W. R. Scott. 1964. Tetrodotoxin blockage of sodium conductance increase in lobster giant axons. *J. Gen. Physiol.* 47:965-974.
- Noguchi, T. and O. Arakawa. 2008. Tetrodotoxin- distribution and accumulation in aquatic organisms, and cases of Human intoxication. *Mar. Drugs* 6:220-242.
- Noguchi, T., O. Arakawa, and T. Takatani. 2006a. Toxicity of pufferfish *Takifugu rubripes* cultured in netcages at sea or aquaria on land. *Comp. Biochem. Phys. D* 1:153-157.
- Noguchi, T., O. Arakawa, and T. Takatani. 2006b. TTX accumulation in pufferfish. *Comp. Biochem. Phys. D* 1:145-152.
- Noguchi, T. and Y. Mahmud. 2001. Current methodologies for detection of tetrodotoxin. *J. Toxicol. - Toxin Rev.* 20:35-50.
- Ritson-Williams, R., M. Yotsu-Yamashita, and V. J. Paul. 2005. Ecological functions of tetrodotoxin in a deadly polyclad flatworm. *PNAS* 103:3176-3179.
- Williams, B. L. 2010. Behavioral and chemical ecology of marine organisms with respect to tetrodotoxin. *Mar. Drugs* 8:381-398.
- Williams, B. L. and R. L. Caldwell. 2009. Intra-organismal distribution of tetrodotoxin in two species of blue-ringed octopuses (*Hapalochleana fasciata* and *H. lunulata*). *Toxicon* 54:345-353.

- Williams, B. L., E.D. Brodie, Jr., and E. D. Brodie III. 2004. A resistant predator and its toxic prey: persistence of newt toxin leads to poisonous (not venomous) snakes. *J. Chem. Ecol.* 30:1901-1919.
- Yotsu-Yamashita, M., D. Mebs, and W. Flachsenberger. 2007. Distribution of tetrodotoxin in the body of the blue-ringed octopus (*Hapalochlaena maculosa*). *Toxicon* 49:410-412.
- Yotsu-Yamashita, M., D. Mebs, and T. Yasumoto. 1992. Tetrodotoxin and its analogues in extracts from the toad *Atelopus oxyrhynchus* (family: Bufonidae). *Toxicon* 30:1489-1492.
- Zimmer, R. K., D. W. Schar, R. P. Ferrer, P. J. Krug, L. B. Kats, and W. C. Michel. 2006. The scent of danger: tetrodotoxin (TTX) as an olfactory cue of predation risk. *Ecol. Monogr.* 76:585-600.

CHAPTER 2
AN IMPROVED COMPETITIVE INHIBITION ENZYMATIC IMMUNOASSAY
METHOD FOR TETRODOTOXIN QUANTIFICATION¹

Quantifying tetrodotoxin (TTX) has been a challenge in both ecological and medical research due to the cost, time, and training required of most quantification techniques. Here we present a modified Competitive Inhibition Enzymatic Immunoassay for the quantification of TTX, and to aid researchers in the optimization of this technique for widespread use with a high degree of accuracy and repeatability.

BACKGROUND

Tetrodotoxin (TTX) is a low molecular weight neurotoxin that blocks the pore region of voltage-gated sodium channels [1, 2, 3] and is found in a wide array of taxa (reviewed by 4). The diversity of species with TTX raises questions about the ecological functions and evolutionary implications of TTX [reviewed by 5]. Further, TTX is of concern to human health as fugu and marine gastropods are commonly consumed in Asian countries [e.g. 6, 7]. Therefore, quantification of TTX is of high importance for multiple fields of research. Traditional approaches for quantifying TTX have limited efficiency and practicality. For example, one common method of quantifying TTX is High Performance Liquid Chromatography [HPLC; reviewed by 8; 9]. HPLC is an

¹ Coauthored by Amber N. Stokes, Becky L. Williams, and Susannah S. French. Reprinted with permission of Springer from *Biological Procedures Online* Vol. 14, Issue 1, 2012.

effective means of measuring TTX but is costly, time consuming, and requires special training and expensive equipment.

A more efficient method of quantifying TTX is to use an immunoassay specific to tetrodotoxin. Several methods for Competitive Inhibition Enzymatic Immunoassays (CIEIA) or other competitive enzyme immunoassays (EIA) exist [e.g. 10, 11, 12, 13]. However, in our experience previously published immunoassay methods are not replicable without detailed knowledge of EIA procedures, contain errors, or report suboptimal concentrations of reagents. Here, we report a modified CIEIA procedure that employs a commercial monoclonal antibody specific to TTX for identification and quantification. Additionally, we report the repeatability between plates within a lab. This method is flexible and adaptable and could identify and quantify TTX in a range of medical or ecological studies using readily available and more affordable lab equipment and reagents.

RESULTS AND DISCUSSION

This assay is highly repeatable, sensitive, and an accurate means of quantifying TTX (Table 2.1). The minimum limit of detection was 10 ng/ml (13; Figure 2.1a), and the linear range of the standard curve was 10–500 ng/mL with $r^2 = 0.992$ (Figure 2.1b). In the linear range, the average intraplate CV for replicates was 6.38 and 7.72%, while the average interplate CV was 8.88%. The concentration of TTX at which 50% of the anti-TTX was inhibited from binding was ~ 75 ng/mL.

Table 2.1. Comparisons of the calculated concentrations from two different plates run the exact same way. Actual standard concentrations were 100, 75, 50, 25, and 10 ng/mL from top to bottom, and were made for each plate independently.

Concentration 1 (ng/ml)	Concentration 2 (ng/ml)	Mean (ng/ml)	Standard Deviation	CV (%)	Percent Difference
104.37	107.72	106.05	2.37	2.23	3.16
72.77	74.67	73.72	1.34	1.82	2.57
50.22	44.93	47.58	3.74	7.86	11.12
23.87	23.13	23.50	0.52	2.23	3.15
10.31	10.93	10.62	0.43	4.10	5.80

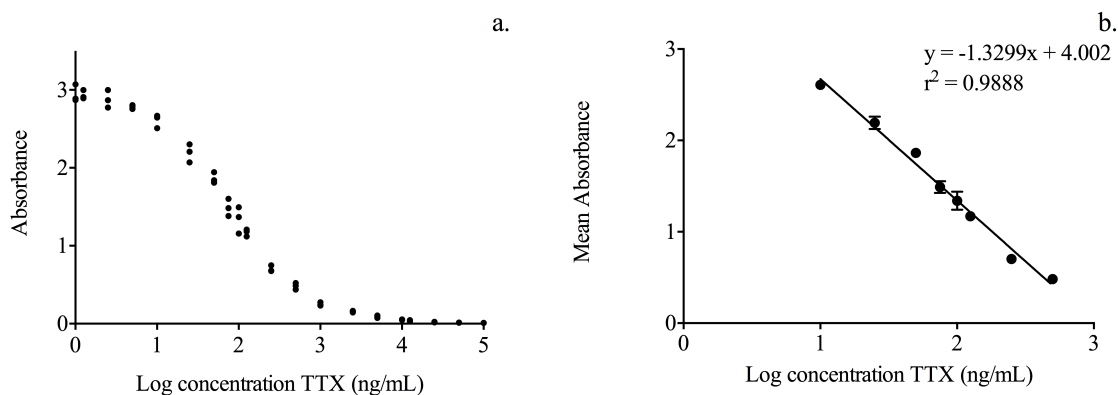


Figure 2.1. Tetrodotoxin standard curves. (a) Standard curve of tetrodotoxin using concentrations of 100,000 ng/mL through 1 ng/mL. Each sample was run in triplicate and all points are displayed to demonstrate variation between replicates. (b) Linear portion of standard curve (\pm SEM). Linear range is between 10 and 500 ng/mL.

This CIEIA procedure allows for broader study of TTX-bearing organisms where a high sample volume can be screened with relatively little expense (\$0.32 per sample vs. \$12.00 per sample with HPLC [excluding equipment and labor]), time (7 hours vs. 48-96 hours for HPLC for 24 samples), and technical expertise. However, because TTX analogs cannot be identified with this technique, we recommend that data be augmented by HPLC or GC-MS services for a few representative samples. Testing of the primary antibodies

with several TTX congeners has shown that they are specific to TTX and do not bind to these congeners [12].

Screening organisms that have yet to be tested for TTX will further our understanding of the role of TTX in ecological systems and evolution. Furthermore, this assay could facilitate rapid pathology tests in human poisoning cases that can be conducted at a wider array of research/medical facilities. Modification of existing methods was necessary to eliminate non-specific binding where possible, which is important for accurate quantification and interpretation of the results. The low variability between plates inherent to this method demonstrates that this technique is sufficiently repeatable to be widely used in a variety of medical and ecological studies.

CONCLUSIONS

The methodology presented here modifies and refines previous methodology (Kawatsu et al. 1997; Lehman 2007; Raybould et al. 1992; Tao et al. 2010) in order to make CIEIA techniques for quantifying TTX more feasible for researchers that do not routinely perform such techniques or have access to specialized equipment (e.g., HPLC). Additionally, this CIEIA technique is more sensitive to TTX detection than HPLC is from previously reported analyses [14, 15]. This immunoassay has been proven useful in quantifying TTX in newts of the genus *Taricha* [16], and has quantified concentrations within the expected range of those quantified using HPLC previously (unpublished data). Furthermore, these methods provide the necessary methodology for eliminating issues with nonspecific binding that may occur with the technique.

METHODS

Conjugate Preparation

Conjugate preparation is significantly modified from previous work [11, 12]. Specifically, TTX binds to Bovine Serum Albumin (BSA; Sigma; A7906-50G) with formaldehyde and the BSA will tether TTX (a small hydrophilic molecule) to the plate. Because the commercial anti-TTX antibodies were created against a keyhole limpet cyanin (KLH) conjugate, the antibodies do not cross-react with BSA in the final assay [12]. Seven-hundred μL TTX (Sigma; T5651) at 1 mg/mL, 300 μL sodium acetate buffer (1 N; adjusted to pH 7.4 using 0.05 N acetic acid; Sigma; S7670), 179 μL of BSA at 33.6 mg/mL, and 41 μL of 37% formaldehyde (Fisher Scientific; AC11969) are added drop-wise to an amber glass vial (conjugate is light sensitive), in that order, and vortexed. TTX is soluble at a pH of 4–5, however previously reported methodology states that TTX should be dissolved at a pH of 7.4 in sodium acetate buffer [11, 12]. We utilized 1 mg TTX lyophilized in 5 mg citrate buffer and dissolved in 1 ml of ddH₂O, which yielded the appropriate pH, with no consequences to the efficacy of the conjugate. The conjugate solution is then incubated in a shaker for three days at 37°C. Following incubation, the solution is transferred to dialysis tubing and dialyzed over a three day period at 4°C against four equally spaced 1L-changes of phosphate buffered saline (PBS; Fisher Scientific; BP665-1). The concentration is then determined by spectrophotometry (NanoDrop ND-1000 Spectrophotometer; at 280 nm). Finished conjugate may be stored at 4°C and does not need to be lyophilized [as in 13].

Conjugate optimization

Optimal conjugate concentration is determined by running plates of standard curves with serial dilutions of the conjugate. Excessively concentrated conjugate results in high variation due to nonspecific binding [17]. Others [11, 12] reported 2 $\mu\text{g/mL}$ concentrations for anti-TTX antibodies (Hawaii Biotech) and 10 $\mu\text{g/mL}$ BSA-TTXF conjugate to coat the plate. We found that using a 2 $\mu\text{g/mL}$ solution of conjugate and consequently, a lower concentration of antibodies, can be used saving materials, eliminating nonspecific binding, decreasing variation, and improving the fit and accuracy of the standard curve. For each new lot of antibody purchased and used, the appropriate concentration of antibodies will have to be optimized using standard solutions of TTX and testing serially diluted anti-TTX antibodies. Both primary and secondary antibodies can be stored at 4°C or -20°C between uses.

Extraction of TTX and Preparation of Standards

TTX is extracted by previously described methods [18]. Briefly, filtrates may be stored at -80°C for up to 5 yr. without degradation of TTX (CT Hanifin pers comm.). Standards are prepared using 1 mg TTX lyophilized in citrate buffer (Ascent Scientific; Asc-055) dissolved in 1 mL of a 1% solution of BSA diluted in PBS. The linear range of the curve is quite large (see results), so we use standard concentrations of 10, 50, 100, 300 and 500 ng/mL diluted in 1% BSA-PBS from the 1 mg/mL stock solution for each assay. In cases where the samples are not diluted by at least 1:2, standards are prepared by diluting in 0.1 M acetic acid rather than the 1% BSA solution. We have found that the absorbance values for acetic acid are slightly different than those of 1% BSA solution.

Using acetic acid as the background for samples that are not diluted compensates for this, and does not alter the accuracy of the standard curve. All standards, samples, and stock solutions should be stored at -80°C between uses with little affect due to freeze/thaw of solutions.

Assay Set-up

Assays are run in 96-well microtiter plates (Nunc MaxiSorp, Fisher Scientific; 439454). The first of three controls is a blank and does not receive any sample, standard, or antibody (Figure 2.2). The second is a positive control that tests the efficacy of the alkaline-phosphatase labeled goat anti-mouse IgG+IgM (H+L) secondary antibodies (Jackson ImmunoResearch; 115-055-044). The third is a negative control, in which 1% PBS-BSA is used as a sample with no TTX. In cases where acetic acid is used to prepare standards, 0.1 M acetic acid is the negative control. This assay is very sensitive to temperature changes and should be run at approximately $25\text{-}30^{\circ}\text{C}$. We also report here, for the first time, that small pigment molecules not excluded during the extraction process can add to the absorbance and thus interfere with TTX quantification. Additionally, there may be non-specific secondary binding in some cases, which may give false positive results. These issues are circumvented by running controls of each extract with no anti-TTX antibody to measure baseline absorbance for each sample, which will be subtracted from the absorbance of the quantified sample.

Negative No 1 ^o No 2 ^o	Negative No 1 ^o No 2 ^o	Negative No 1 ^o No 2 ^o	S1	S1	S1	S1 No 1 ^o	S1 No 1 ^o	S1 No 1 ^o	S9	S9	S9
Positive No 1 ^o	Positive No 1 ^o	Positive No 1 ^o	S2	S2	S2	S2 No 1 ^o	S2 No 1 ^o	S2 No 1 ^o	S10	S10	S10
Negative 0 ng/mL TTX	Negative 0 ng/mL TTX	Negative 0 ng/mL TTX	S3	S3	S3	S3 No 1 ^o	S3 No 1 ^o	S3 No 1 ^o	S11	S11	S11
500 ng/mL TTX	500 ng/mL TTX	500 ng/mL TTX	S4	S4	S4	S4 No 1 ^o	S4 No 1 ^o	S4 No 1 ^o	S12	S12	S12
300 ng/mL TTX	300 ng/mL TTX	300 ng/mL TTX	S5	S5	S5	S5 No 1 ^o	S5 No 1 ^o	S5 No 1 ^o	S9 No 1 ^o	S9 No 1 ^o	S9 No 1 ^o
100 ng/mL TTX	100 ng/mL TTX	100 ng/mL TTX	S6	S6	S6	S6 No 1 ^o	S6 No 1 ^o	S6 No 1 ^o	S10 No 1 ^o	S10 No 1 ^o	S10 No 1 ^o
50 ng/mL TTX	50 ng/mL TTX	50 ng/mL TTX	S7	S7	S7	S7 No 1 ^o	S7 No 1 ^o	S7 No 1 ^o	S11 No 1 ^o	S11 No 1 ^o	S11 No 1 ^o
10 ng/mL TTX	10 ng/mL TTX	10 ng/mL TTX	S8	S8	S8	S8 No 1 ^o	S8 No 1 ^o	S8 No 1 ^o	S12 No 1 ^o	S12 No 1 ^o	S12 No 1 ^o

Figure 2.2. A typical template used for plates. Each sample starts with the letter S. Samples run without primary antibodies are used to eliminate any background noise caused from the sample itself.

Assay Procedures

The assay: (1) Each plate is coated with 100 μ L conjugate diluted in PBS (2 μ g/mL). The plate is incubated for one hour at room temperature (RT), and washed three times with 250 μ L of PBS-T (500 μ L Tween-20 (Fisher Scientific; 23336-2500) per 1 L PBS) buffer using a plate washer (Bio-Rad model 1575; may also be performed by hand). (2) We next block the plate using 200 μ L of 1% PBS-BSA, incubate for one hr at RT, and again wash the plate. (3) Fifty microliters of standards or samples are added to wells in triplicate. Samples should be diluted to within the range of the standard curve (preliminary data may be collected to determine proper dilutions). (4) Fifty microliters of anti-TTX antibodies diluted to the optimal concentration (0.391 μ g/ml in our case) are added to all sample and standard wells except individual extract controls, incubated one hr at RT, and washed. (5) One hundred microliters of anti-mouse IgG + IgM antibodies

(H + L) are added to all wells except the positive and blank controls, incubated one hr at RT, and washed. (6) Fifty microliters of secondary antibodies are added to the positive control wells, and 200 μ L of a 1 mg/mL pNPP solution (diluted in diethanolamine buffer: 400 mL ddH₂O, 52.22 g diethanolamine (Sigma; D8885-500G), adjusted to pH 9.80 with concentrated HCl, 0.051g MgCl₂ (Fisher Scientific; AC41341-0025)) is added to all of the wells. The plate is protected from light and incubated at RT for 10 minutes. (7) The plate is then read in a Bio-Rad xMark Microplate Spectrophotometer (any standard absorbance reader with the appropriate filter is sufficient) at an absorbance of 405 nm. Readings are taken every 5 minutes following the initial 10-minute reading.

Calculations

The mean, standard deviation, and coefficient of variation (CV) for each of the standards are calculated. To back-calculate standard concentrations, mean absorbance values of the standards are plotted against the log of the known concentration for each standard. The time frame with the best standard predictions (least summed variance from known values; usually highest r^2 value of the regression line) is selected for sample quantification. The best time frame is usually 20–45 minutes. Sample values outside the range of the standard curve are diluted and re-run, re-assayed as a more concentrated extract, or reported as either below detection limit (BDL) or above detection limit (ADL) depending on whether they fall above or below the curve. The concentrations for any samples are adjusted via dilution factor. The mean value for the negative control (BSA or acetic acid), or preferably individual extract controls, should be subtracted from the mean values of the unknowns to eliminate background noise for the most accurate final

concentration.

LITERATURE CITED

1. Kao CY: **Tetrodotoxin, saxitoxin, and their significance in the study of excitation phenomena.** Pharm Rev 1966, **18**:997-1049.
2. Narahashi T, Moore JW, Poston RN: **Tetrodotoxin derivatives: Chemical structure and blockage of nerve membrane conductance.** Science 1967, **156**:976-979.
3. Narahashi T: **Pharmacology of tetrodotoxin.** J Toxicol - Toxin Rev 2001, **20**:67-84.
4. Miyazawa K, Noguchi T: **Distribution and origin of tetrodotoxin.** J Toxicol - Toxin rev 2001, **20**:11-33.
5. Williams BL: **Behavioral and chemical ecology of marine organisms with respect to Tetrodotoxin.** Mar. Drugs 2010, **8**:381-398.
6. Islam QT, Razzak MA, Islam MA, Bari MI, Basher A, Chowdhury FR, Sayeduzzaman ABM, Ahasan HAMN, Faiz MA, Arakawa O, Yotsu-Yamashita M, Kuch U, Mebs D: **Puffer fish poisoning in Bangladesh: clinical and toxicological results from large outbreaks in 2008.** Trans R Soc Trop Med Hyg 2011, **105**:74-80.
7. Noguchi T, Arakawa O: **Tetrodotoxin – Distribution and accumulation in aquatic organisms and cases of human intoxication.** Mar Drugs 2008, **6**:220-242.
8. Noguchi T, Mahmud Y: **Current methodologies for detection of tetrodotoxin.** J Toxicol - Toxin Rev 2001, **20**:35-50.
9. Yotsu M, Endo A, Yasumoto T: **An improved tetrodotoxin analyzer.** Agric Biol Chem 1989, **53**:893-895.
10. Kawatsu K, Hamano Y, Yoda T, Terano Y, Shibata T: **Rapid and highly sensitive enzyme immunoassay for quantitative determination of tetrodotoxin.** Jpn J Med Sci Biol 1997, **50**:133-150.
11. Lehman EM: **A simplified and inexpensive method for extraction and quantification of tetrodotoxin from tissue samples.** Herpetological Rev 2007, **38**:298-301.

12. Raybould TJG, Bignami GS, Inouye LK, Simpson SB, Byrnes JB, Grothaus PG, Vann DC: **A monoclonal antibody-based immunoassay for detecting tetrodotoxin in biological samples.** J Clin Lab Anal 1992, **6**:65-72.
13. Tao J, Wei WJ, Nan L, Lei LH, Hui HC, Fen GX, Jun LY, Jing Z, Rong J: **Development of competitive indirect ELISA for the detection of tetrodotoxin and a survey of the distribution of tetrodotoxin in the tissues of wild puffer fish in the waters of southeast China.** Food Addit Contam 2010, **27**:1589-1597.
14. Hanifin CT, Brodie ED, Jr., Brodie ED III: **Phenotypic mismatches reveal escape from arms-race coevolution.** PLOS Biol 2008, **6**:471-482.
15. Yasumoto T, Michishita T: **Fluorometric determination of tetrodotoxin by high performance liquid chromatograph.** Agric Biol Chem 1985, **49**:3077-3080.
16. Gall BG, Stokes AN, French SS, Schlepffhorst EA, Brodie ED III, Brodie ED, Jr.: **Tetrodotoxin levels in larval and metamorphosed newts (*Taricha granulosa*) and palatability to predatory dragonflies.** Toxicon 2011, **57**: 978-983.
17. Deshpande SS: Enzyme Immunoassays from Concept to Product Development. New York: Chapman and Hall. 1996.
18. Hanifin CT, Brodie ED III, Brodie ED, Jr.: **Tetrodotoxin levels of the rough-skin newt, *Taricha granulosa*, increase in long-term captivity.** Toxicon 2002, **40**:1149-1153.

CHAPTER 3

FEMALE NEWTS (*TARICHA GRANULOSA*) PRODUCE TETRODOTOXIN LADEN
EGGS AFTER LONG TERM CAPTIVITY²

We investigated the presence of tetrodotoxin (TTX) in the eggs of wild-caught newts (*Taricha granulosa*) at capture and again after one, two, and three years in captivity. Females initially produced eggs that contained quantities of TTX similar to previous descriptions of eggs from wild-caught adults. After the first year in captivity, the egg toxicity from each female declined, ultimately remaining constant during each of the successive years in captivity. Despite declining, all females continued to produce eggs containing substantial quantities of TTX during captivity. The decline in toxicity can not be attributed to declining egg mass but may be the result of the abbreviated reproductive cycle to which the captive newts were subjected in the lab. Finally, an estimate of the amount of TTX provisioned in the entire clutch from each female is similar to the quantity of TTX regenerated in the skin after electrical stimulation. These results, coupled with other long-term studies on the maintenance and regeneration of TTX in the skin, suggests an endogenous origin of TTX in newts.

² Coauthored by Brian G. Gall, Amber N. Stokes, Susannah S. French, Edmund D. Brodie III, and Edmund D. Brodie, Jr. Reprinted with permission from Elsevier from *Toxicon* Vol. 60, pages 1057-1062, 2012.

INTRODUCTION

One of the most deadly naturally occurring compounds is tetrodotoxin (TTX). This neurotoxin is a non-proteinaceous water-soluble guanidinium ion, consisting of a complex carbon ring-structure with associated amine and aminal groups (Mosher et al., 1964; Narahashi et al., 1967). Organisms in 17 different orders distributed across eight phyla possess TTX (Chau et al., 2011; Miyazawa and Noguchi, 2001), yet the only clear phylogenetic pattern in its occurrence falls within a small number of families (e.g. Salamandridae, Tetraodontidae). This seemingly “random” distribution has made characterizing the mechanism by which organisms acquire the toxin, as well as the biosynthetic pathway by which TTX is produced exceedingly difficult, and little solid evidence has been gathered on either of these fronts.

Three major hypotheses exist to explain the acquisition of TTX in vertebrates and invertebrates; (1) symbiotic bacteria produce TTX, which is then sequestered by the host organism, (2) tetrodotoxin toxicity occurs through bioaccumulation via the food chain, (3) TTX is produced endogenously. The model widely accepted for marine organisms involves the production of TTX by symbiotic bacteria (Simidu et al., 1987; Wang et al. 2010). However, many of the marine organisms found to possess TTX are eaten by larger vertebrates, such as puffer fish, that are resistant to the toxin (Venkatesh et al., 2005). In these cases, it is believed that TTX toxicity occurs through bioaccumulation through the food chain (Noguchi et al., 2006). Finally, some organisms, amphibians in particular, do not appear to fit either of these models and may be able to directly synthesize TTX (reviewed by Hanifin, 2010).

Until the pathway for TTX production is discovered, researchers must utilize indirect evidence on the distribution of TTX to infer where and how this toxin is formed. Regardless of the mechanism by which TTX is acquired, understanding temporal and ontogenetic changes in TTX toxicity would allow a more in-depth assessment of the hypotheses for TTX production. Despite decades of research, ontogenetic or temporal changes in toxicity are poorly understood in most organisms. For example, the puffer fish (Order: Tetraodontiformes) have been intensively studied for over 100 years (Chau et al., 2011; Fuhrman, 1986) and although TTX is present in high concentrations in the liver, ovaries, and skin of wild-collected adult puffer fish (e.g. Jang and Yotsu-Yamashita, 2006), it is virtually unknown if toxicity changes over time in adults or larvae/juveniles (but see Nunez-Vazquez et al., 2012).

Individual and ontogenetic changes in toxicity are probably best understood in the newts (Salamandridae). In newts large quantities of TTX are located in the skin, while minute amounts can be found in other tissues such as muscle and blood (Wakely et al., 1966). There is tremendous within and between population variation in tetrodotoxin toxicity across large spatial scales in *Taricha granulosa*, which is believed to be the result of coevolution with a snake predator (Brodie and Brodie, 1990; Brodie et al., 2002; Feldman et al., 2012; Geffeney et al., 2002; Hanifin et al., 1999, 2008; Williams et al., 2010a). Long-term lab studies indicate that the toxicity of the skin not only increases over time but can be regenerated after excretion, despite being maintained on a diet that does not contain TTX (Cardall et al., 2004; Hanifin et al., 2002).

In newts, high levels of TTX are also found in the ovaries, ova, and recently deposited eggs (Hanifin et al., 2003; Mosher et al., 1964; Wakely et al., 1966). Although several recent studies have expanded our understanding of changes in TTX during early-life history stages (Gall et al., 2011b; Tsuruda et al., 2002), only one study has quantified the toxicity of individual eggs from females (Hanifin et al., 2003). Further, it is unknown if toxicity changes between successive clutches from a female or whether TTX is deposited in the eggs when females are reared in captivity. To better understand the lability of TTX production in female newts, we quantified the amount of TTX in newt eggs immediately after gravid females were collected from the wild and after one, two, and three years in captivity. Additionally, we estimated the amount of toxin produced and provisioned in a clutch by each female and compare this to published reports of TTX regeneration in newts from the same population.

MATERIALS AND METHODS

Animal Collection and Maintenance

We quantified the amount of TTX in a subset of eggs from each of 3 or 4 clutches of eggs deposited in successive years by female *Taricha granulosa*. Gravid female newts (*Taricha granulosa*) were collected in March 2009 (N = 3) and 2010 (N = 5) from Soap Creek ponds in the central Willamette Valley, OR. This population is well studied (Gall et al., 2011a, 2011b; Hanifin et al., 1999, 2002, 2003), and includes the most toxic known newts; a single individual may contain up to 28 mg of tetrodotoxin (Ch. 4), which is the oral lethal dose for as many as 56 humans (Yasumoto and Yotsu-Yamashita, 1996).

After collection, newts were immediately transported to Utah State University where they were housed in 5.7-L containers with 2 L of filtered tap water. Each female was housed in an environmental chamber at 17°C and injected with 2 µl/g LHRH (de-Gly10, [d-His(Bzl)6]-Luteinizing Hormone Releasing Hormone Ethylamide; Sigma #12761) to stimulate egg deposition. In nature *Taricha granulosa* deposit eggs singly on aquatic vegetation over several weeks (Petranka, 1998). In the lab, each female was provided with a small clump of polyester fiber to serve as an oviposition site. This substrate is readily accepted as an oviposition site by newts. A small subset of eggs from each female was collected less than 48 hrs after deposition and frozen at -80°C for TTX quantification. The mass of each egg was recorded prior to freezing (2011 & 2012) or after freezing (2009 & 2010). To account for a change in mass due to freezing, 20 eggs that were weighed prior to freezing were thawed and re-weighed. The change in mass was calculated for each of these re-weighed eggs and the mean change (-7.18%) was added to each egg collected in 2009 and 2010. These adjusted values were used in all analyses.

After a female had deposited all of the eggs from this initial clutch (approximately 3 weeks) the polyester fiber was removed and a small piece of foam was placed in the container to provide a terrestrial refuge. Females were provided blackworms (*Lumbriculus variegatus*) weekly, which were rarely supplemented with earthworms (suborder: Lumbricina). Neither blackworms (Gall et al., 2011b), nor earthworms (ANS, unpublished data) possess TTX. Although we did not standardize the amount of food

given to each female, only rarely were all the blackworms consumed within one week and we therefore assume newts had continuous access to food.

To examine TTX levels in newt eggs after an extended period in captivity, eggs were sampled from these captive females after one, two, and three years in the lab. Females were maintained at 17°C until mid-December at which point the temperature was slowly dropped to 8°C. Newts were maintained at this temperature until early-February, whereupon the temperature was slowly increased to 17°C. Each female was then placed in a 75 L tank with a recently collected male newt that was in reproductive condition. The pair was observed for 72 hrs for signs of amplexus and mating, at which point the female was removed and injected with 2 µl/g LHRH. A small amount of polyester fiber was then added to the container to serve as an oviposition site. A small subset of eggs was frozen for TTX analysis and the husbandry process was repeated (as described above). Females were maintained in this manner for two or three years after initially being collected.

Tetrodotoxin Quantification

Frozen egg samples were extracted for analysis using previously described techniques (Hanifin et al., 2002). Tetrodotoxin was quantified using a Competitive Inhibition Enzymatic Immunoassay (CIEIA) as in Stokes et al. (2012; Ch. 2). This assay is highly specific and works by binding anti-TTX monoclonal antibodies to TTX. In the absence of TTX or in low concentrations of TTX, the antibodies bind to the conjugate on the plate allowing secondary antibodies to also bind to the plate, resulting in a high absorbance reading. This value is then used to calculate the TTX concentration using a

linear standard curve. The assay is able to detect TTX at a minimum concentration of 10 ng/mL, and has a linear range of 10-500 ng/mL (Stokes et al., 2012; Ch. 2). All samples were diluted 1:2, 1:4, 1:8, 1:16, or 1:32 in Bovine Serum Albumin (BSA) to assure they were within the linear range of the standard curve. All plates were read at 405 nm. The average coefficient of variation on each plate was between 5.04 and 11.09%. This immunoassay has been proven useful in quantifying TTX in newts of the genus *Taricha* (Ch. 4), and has yielded concentrations within the expected range of newts quantified using HPLC previously (this study; unpublished data).

Statistical Analysis

We used repeated-measures ANOVA to examine for changes in the total egg toxicity, TTX concentration (ng TTX/mg mass), and egg mass among the four years. Female was treated as a random factor while year was treated as a fixed effect. We fit the model to multiple covariance structures and choose the most appropriate model based on the lowest AIC value (total TTX: autoregressive moving average; TTX concentration: autoregressive; egg mass: autoregressive). Assumptions of normality and homoscedasticity were assessed with graphical analysis of residuals; all assumptions appeared to be adequately met for all response variables. A Kendall's tau rank correlation was used to determine if total TTX, TTX concentration, and egg mass were consistent between females across years. Year three of captivity was excluded from this analysis because only 2 females deposited eggs, which did not permit a complete assignment of ranks. Analyses were obtained using the PROC MIXED procedure in SAS 9.1 (SAS Institute Inc., Cary, NC, USA).

RESULTS

The amount of Tetrodotoxin in newt eggs varied significantly across years, with total TTX (per egg) and TTX concentration (ng TTX/mg egg mass) declining between the first clutch collected from wild-caught females (W) and subsequent clutches in the lab (total TTX: $F_{[3,15]} = 32.7$, $P < 0.0001$, Fig 1a; TTX conc: $F_{[3,15]} = 34.8$, $P < 0.0001$, Fig 3.1b). There was no significant difference in total TTX or TTX concentration among the first, second, and third years in the lab. Despite declining after the initial clutch, newts continued to produce eggs containing large quantities of TTX in all years in captivity (Fig 3.1). There was no significant correlation between total TTX and year (Kendall's tau = 0.356, $P > 0.25$) or TTX concentration and year (Kendall's tau = 0.321, $P > 0.25$) indicating that the females did not produce eggs that were consistently the same toxicity across years (i.e. the most toxic females did not necessarily produce the most toxic eggs in all three years).

The decline in TTX within newt eggs after being collected from the wild can not be accounted for by a reduction in egg mass between this first year and subsequent years in captivity. The average mass of newt eggs remained constant among years ($F_{[3,15]} = 2.15$, $P = 0.136$, Fig. 3.2). There was no correlation between egg mass and year (Kendall's tau = 0.183, $P > 0.75$) indicating that the mass of a females eggs was variable across years (Fig 3.2).

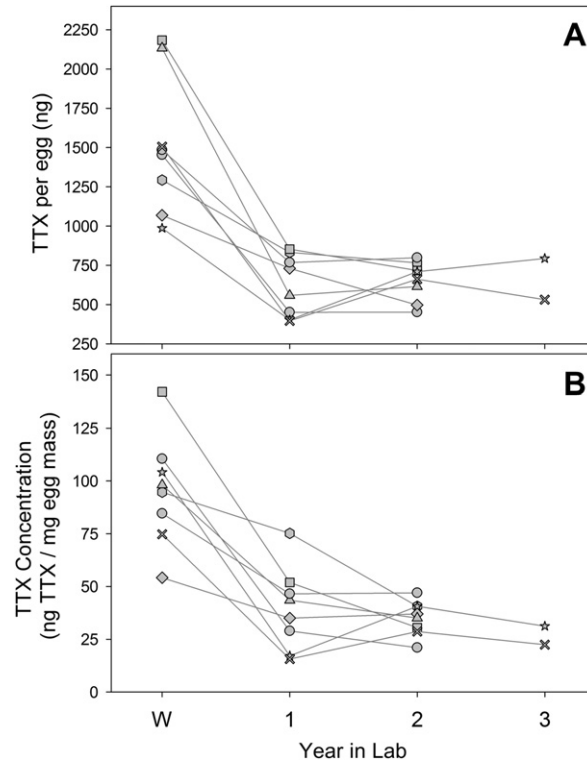


Figure 3.1. Mean total TTX (A) and TTX concentration (ng TTX/mg egg mass) (B) present in eggs from eight different female *Taricha granulosa*. A sample of eggs was taken immediately after collection from these wild-caught females (W), and after one, two, and three years in captivity. There was a significant decline in total TTX ($F_{[3,15]} = 32.7$, $P < 0.0001$) and TTX concentration ($F_{[3,15]} = 34.8$, $P < 0.0001$) after the first year in captivity, yet substantial quantities of toxin were present and remained relatively constant in the following three years.

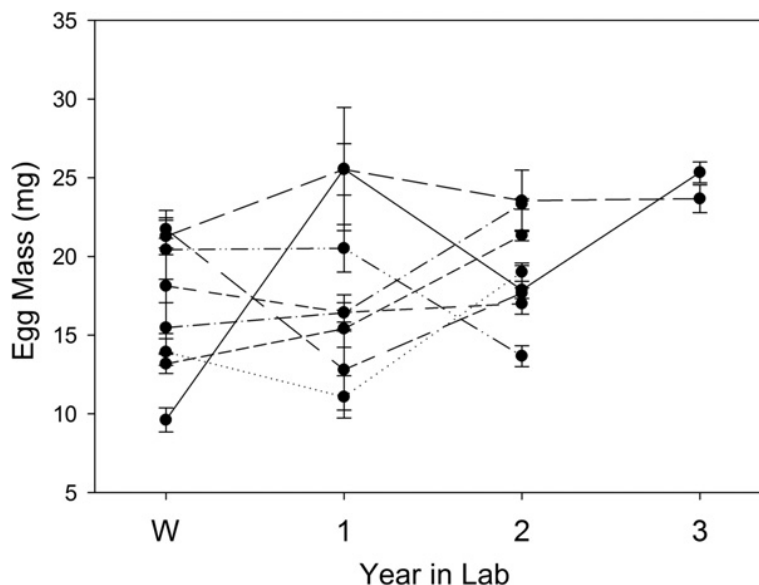


Figure 3.2. Mean (\pm SE) mass (mg) of eggs from eight female newts (*Taricha granulosa*) from initial collection in the wild (W) through three additional years in captivity. There was no significant change in egg mass between years ($F_{[3,15]} = 2.15$, $P = 0.136$).

DISCUSSION

Female newts continued to produce toxic eggs for up to three years in the laboratory, despite being exclusively fed a TTX-free diet. Although the amount of toxin in the eggs declined after the initial clutch was collected, each individual female continued to produce eggs with quantities of TTX that fall within (or slightly below) the naturally occurring range for this population (Hanifin et al., 2003). Given that newts reared in captivity maintain TTX over long periods of time (Hanifin et al., 2002) and are capable of replenishing depleted TTX stores (Cardall et al., 2004), these results are not entirely surprising. It is unknown whether our females synthesized or sequestered their own toxin or mobilized it from another area, such as the skin. Cardall et al. (2004) demonstrated that newts fed a TTX-free diet regenerated an average of 0.76 mg of skin

toxin in nine months in the laboratory (Fig 3.3). This replenishment of TTX in the skin was independent of sex, indicating that the newts were not mobilizing TTX from other tissues and transferring it to the skin (Cardall et al., 2004); other than the skin, the only tissues that contain substantial quantities of TTX are the ovaries in females, which are not available as a source of TTX to males (Hanifin et al., 2004; Wakely et al., 1966). Based on a clutch size of 525 eggs (Hanifin et al. (2003) reported clutch sizes of 400 – 655 eggs from newts from our study site), the females in this study provisioned their entire clutch with an average total of 0.34 mg of TTX (Fig. 3.3). This quantity of toxin, provisioned during years one, two, and three of this study, is slightly less than, but not significantly different from the amount of toxin regenerated in the skin of newts as demonstrated by Cardall et al. (2004) (Mann-Whitney: $U = 223$, $P = 0.25$, Fig. 3.3). These results suggest that females may develop/sequester new toxin rather than transfer it out of the skin.

One major question that remains is why egg toxicity declines after the first year? We hypothesized that the decrease in TTX observed in this study was due to a decrease in egg mass. However, we documented no change in egg mass between clutches (mean egg mass actually increased very slightly) indicating the decline in TTX is not a result of producing smaller eggs. Moreover, the allocation of TTX in newt eggs is under active maternal control and the amount of toxin in each egg is independent of egg volume (Hanifin et al., 2003). More likely, the decrease in egg toxicity is due to the abbreviated reproductive cycle to which these captive newts were subjected. In the wild, female newts do not breed for the first time until six to eight years of age (Petranka, 1998; Twitty 1961, 1966). Eggs are deposited singly over a period of several weeks or months

(Nussbaum et al., 1983; Petranka, 1998), and females eventually leave the pond to overwinter. Twitty (1966) conducted an extensive mark-recapture study on 5587 female *Taricha*. He found that although some individuals breed in successive years (approximately 1.4%), the vast majority of females do not return to breed again for at least two or three years (Twitty, 1966). This extended non-reproductive period may be necessary to acquire sufficient resources to produce eggs or to obtain large quantities of TTX with which to endow their eggs. We hypothesize that the synthesis or sequestration of TTX in newts may be time-limited.

Few studies have attempted to monitor temporal patterns of TTX production in other taxa. Another amphibian, a frog of the genus *Atelopus*, maintained a high level of TTX for more than three years in captivity (Yotsu-Yamashita et al., 1992). Puffer fish may contain large quantities of TTX in the skin, liver, and ovaries, and although cultured individuals have little or no TTX (Ji et al., 2011; Matsumura, 1996; Noguchi et al., 2006; Sasaki et al., 2008), no studies have monitored the toxicity of individuals or their egg clutches over time. Unlike temporal patterns, our understanding of the ontogenetic changes in toxicity during early developmental stages is slowly advancing. Tetrodotoxin toxicity appears to increase during embryonic development in one species of puffer fish (*Fugu niphobles*), as well as the blue-ringed octopus (Williams et al., 2010b). Nunez-Vazquez et al. (2012) examined the toxicity of multiple life-history stages in a cultured Mexican puffer fish and found very small quantities of TTX in juveniles, pre-adults, and adults. No TTX was identified in eggs, larvae, or post-larvae (Nunez-Vazquez et al., 2012).

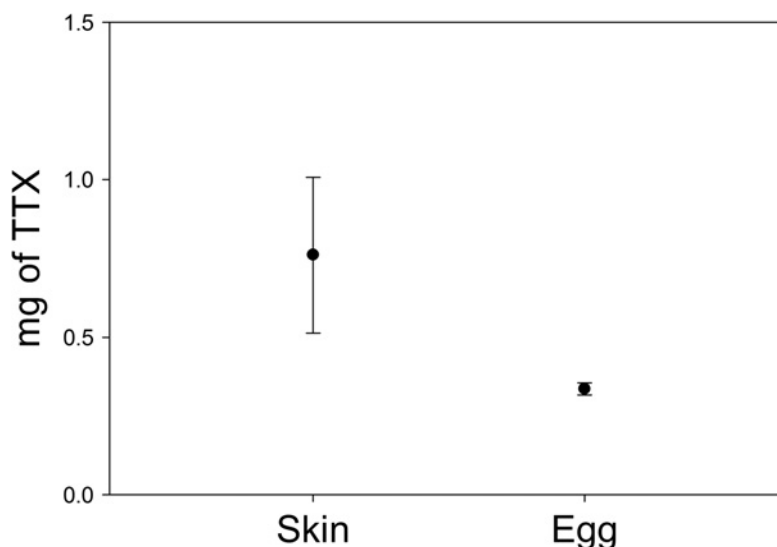


Figure 3.3. Comparison of the mean (\pm SE) amount of TTX regenerated in the skin of adult newts (*Taricha granulosa*) over 9 months (Skin) versus the estimated mean (\pm SE) amount of TTX provisioned by eight female newts in an entire clutch of 525 eggs (Eggs). Regenerated skin TTX was calculated by estimating whole-body TTX from raw data in Cardall et al. (2004) and subtracting post-stimulus TTX from final TTX. An estimate of the total TTX provisioned in a clutch of eggs for each female reared in captivity was calculated by multiplying the average egg toxicity from years one, two, and three for each female by 525 [the average number of eggs produced by females from our study site (Hanifin et al. 2003)]. These values were then averaged to obtain an estimate of the amount of TTX (\pm SE) provisioned in an entire clutch of eggs in the laboratory. In both studies (Cardall et al. 2004, this study) the newts were fed a TTX-free diet. There is no significant difference between the amount of TTX regenerated in the skin and the amount of TTX provisioned in a clutch of eggs by newts in the laboratory (Mann-Whitney: $U = 223$, $P = 0.25$).

The complexity of TTX production in these diverse taxa may be related to the pattern of TTX biogenesis observed in each group. Several groups of marine bacteria have been identified that produce TTX in vitro (Simidu et al., 1987), and some of these bacteria have been isolated from the tissues of TTX-bearing marine organisms (Wu et al., 2005; Yu et al., 2004). The prevailing hypotheses for TTX toxicity in marine organisms are dietary sequestration through the food chain or symbiotic bacteria (see review in

Williams, 2010). However, tetrodotoxin production in amphibians is more controversial. Wild-caught adult *Atelopus* sp. possess TTX (Kim et al., 1975, 2003; Yotsu-Yamashita and Tateki, 2010), yet two *Atelopus varius* reared from eggs in captivity did not possess TTX after 2 and 3 years, respectively (Daly et al., 1997). Nevertheless, Daly et al. (1997) questioned the dietary and bacteria hypotheses in *Atelopus* sp. that overlap in distribution and habitat characteristics yet possess different TTX congeners (Daly, 2004).

In newts, evidence is mounting that bacteria and dietary sequestration may not be involved in TTX production. The eggs of *Cynops pyrrhogaster* are provisioned with small quantities of TTX, which disappears during development (Tsuruda et al., 2002). The larvae are non-toxic, but toxicity begins to increase during the juvenile stage when the granular glands are forming (Tsuruda et al., 2002); the authors did not indicate if these newts were reared in the lab or wild-caught. The bacteria hypothesis in newts was questioned by Lehman et al. (2004) who found bacterial DNA in the intestines of newts (which contain very little TTX), but failed to find evidence for bacteria in extracts from the most toxic tissues. Additionally, when newts are maintained in captivity and fed a diet that does not include TTX, individuals maintain skin toxicity over long periods (Hanifin et al., 2002), continue producing toxic eggs (this study), and rapidly regenerate TTX after electrical stimulation (Cardall et al., 2004).

Because the metabolic pathway by which bacteria, marine organisms, and terrestrial vertebrates produce TTX is unknown, the mechanism by which toxicity is acquired in TTX-bearing organisms remains obscure. Understanding this mechanism requires analyses of ontogenetic and within-individual changes in TTX in wild and

captive populations. The study presented here provides further evidence that TTX toxicity in newts is likely of endogenous origin, and adds to the growing body of literature that suggests newts may be able to directly synthesize TTX due to the large quantities produced in relatively short time-frames and under simple, TTX-free, diets.

LITERATURE CITED

- Brodie, E.D., III., Brodie, E.D., Jr., 1990. Tetrodotoxin resistance in garter snakes: An evolutionary response of predators to dangerous prey. *Evolution* 44, 651-659.
- Brodie, E.D., Jr., Ridenhour, B.J., Brodie, E.D., 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, 2067-2082.
- Cardall, B.L., Brodie, E.D., Jr., Brodie, E.D., III., Hanifin, C.T., 2004. Secretion and regeneration of tetrodotoxin in the rough-skin newt (*Taricha granulosa*). *Toxicon* 44, 933-938.
- Chau, R., Kalaitzis, J.A., Neilan, B.A., 2011. On the origins and biosynthesis of tetrodotoxin. *Aquatic Toxicology* 104, 61-72.
- Daly, J.W., 2004. Marine toxins and nonmarine toxins: Convergence or symbiotic organisms? *J. Natural Products* 67, 1211-1215.
- Daly, J.W., Padgett, W.L., Saunders, R.L., Cover, J.F., Jr., 1997. Absence of tetrodotoxin in a captive-reared riparian frog, *Atelopus varius*. *Toxicon* 35, 705-709.

- Feldman, C.R., Brodie, E.D., Jr., Brodie, E.D., III., Pfrender, M.E., 2012. Constraint shapes convergence in tetrodotoxin-resistant sodium channels of snakes. PNAS 109, 4556-4561.
- Fuhrman, F.A., 1986. Tetrodotoxin, Tarichatoxin, and Chiriquitoxin: Historical Perspectives. Ann. NY. Acad. Sci. 479, 1-14.
- Gall, B.G., Brodie, E.D. III., Brodie, E.D., Jr., 2011a. Survival and growth of the caddisfly *Limnephilus flavastellus* after predation on toxic eggs of the Rough-skinned Newt (*Taricha granulosa*). Can. J. Zool. 89, 483-489.
- Gall, B.G., Stokes, A.N., French, S.S., Schleppehorst, E.A., Brodie, E.D., Brodie, E.D., 2011b. Tetrodotoxin levels in larval and metamorphosed newts (*Taricha granulosa*) and palatability to predatory dragonflies. Toxicon 57, 978-983.
- Geffeney, S., Brodie, E.D., Jr., Ruben, P.C., Brodie, E.D., III., 2002. Mechanisms of adaptation in a predator-prey arms race: TTX-resistant sodium channels. Science 297, 1336-1339.
- Hanifin, C.T., 2010. The chemical and evolutionary ecology of tetrodotoxin (TTX) toxicity in terrestrial vertebrates. Mar. Drugs 8, 577-593.
- Hanifin, C.T., Brodie, E.D., III., Brodie, E.D., Jr., 2002. Tetrodotoxin levels of the rough-skin newt, *Taricha granulosa*, increase in long-term captivity. Toxicon 40, 1149-1153.
- Hanifin, C.T., Brodie, E.D. III., Brodie, E.D., Jr., 2003. Tetrodotoxin levels in eggs of the rough-skin newt, *Taricha granulosa*, are correlated with female toxicity. J. Chem. Ecol. 29, 1729-1739.

- Hanifin, C.T., Brodie, E.D., III., Brodie, E.D., Jr., 2004. A predictive model to estimate total skin tetrodotoxin in the newt *Taricha granulosa*. *Toxicon* 43, 243-249.
- Hanifin, C.T., Brodie, E.D., Jr., Brodie, E.D., III., 2008. Phenotypic mismatches reveal escape from arms-race coevolution. *Plos Biology* 6, 471-482.
- Hanifin, C.T., Yotsu-Yamashita, M., Yasumoto, T., Brodie, E.D., III., Brodie, E.D., Jr., 1999. Toxicity of dangerous prey: Variation of tetrodotoxin levels within and among populations of the newt *Taricha granulosa*. *J Chem Ecol* 25, 2161-2175.
- Jang, J., Yotsu-Yamashita, M., 2006. Distribution of tetrodotoxin, saxitoxin, and their analogs among tissues of the puffer fish *Fugu pardalis*. *Toxicon* 48, 980-987.
- Ji, Y., Liu, Y., Gong, Q.L., Zhou, L., Wang, Z.P., 2011. Toxicity of cultured puffer fish and seasonal variations in China. *Aquaculture Research* 42, 1186-1195.
- Kim, Y.H., Brown, G.B., Mosher, F.A., 1975. Tetrodotoxin: occurrence in atelopid frogs of Costa Rica. *Science* 189, 151.
- Kim, Y.H., Kim, Y.B., Yotsu-Yamashita, M., 2003. Potent Neurotoxins: Tetrodotoxin, Chiriquitoxin, and Zetekitoxin from *Atelopus* Frogs in Central America. *J Toxicol-Toxin Rev* 22, 521-532.
- Lehman, E.M., Brodie, E.D., Jr., Brodie, E.D., III., 2004. No evidence for an endosymbiotic bacterial origin of tetrodotoxin in the newt *Taricha granulosa*. *Toxicon* 44, 243-249.
- Matsumura, K., 1996. Tetrodotoxin concentrations in cultured puffer fish, *Fugu rubripes*. *J Agr Food Chem* 44, 1-2.

- Miyazawa, K., Noguchi, T., 2001. Distribution and origin of tetrodotoxin. *J. Toxicol.-Toxin Rev.* 20, 11-33.
- Mosher, H.S., Fuhrman, F.A., Buchwald, H.D., Fischer, H.G., 1964. Tarichatoxin-tetrodotoxin: a potent neurotoxin. *Science* 144, 1100-1110.
- Narahashi, T., Moore, J.W., Poston, R.N., 1967. Tetrodotoxin derivatives: Chemical structure and blockage of nerve membrane conductance. *Science* 156, 976-979.
- Noguchi, T., Arakawa, O., Takatani, T., 2006. Toxicity of pufferfish *Takifugu rubripes* cultured in netcages at sea or aquaria on land. *Comp. Biochem. Phys. D* 1, 153-157.
- Nunez-Vazquez, E.J., Garcia-Ortega, A., Campa-Cordova, A.I., Parra, I.A., Ibarra-Martinez, L., Heredia-Tapia, A., Ochoa, J.L., 2012. Toxicity of cultured bullseye puffer fish *Sphoeroides annulatus*. *Mar. Drugs* 10, 329-339.
- Nussbaum, R.A., Brodie, E.D., Jr., Storm, R.M., 1983. *Amphibians and Reptiles of the Pacific Northwest*. University of Idaho Press, Moscow, ID.
- Petranka, J., 1998. *Salamanders of the United States and Canada*. Smithsonian Institution Press, Washington, D.C.
- Sasaki, K., Takayama, Y., Tahara, T., Anraku, K., Ito, Y., Akaike, N., 2008. Quantitative analysis of toxin extracts from various tissues of wild and cultured puffer fish by an electrophysiological method. *Toxicon* 51, 606-614.
- Simidu, U., Noguchi, T., Hwang, D.F., Shida, Y., Hashimoto, K., 1987. Marine bacteria which produce tetrodotoxin. *Applied and Environ. Microbiol.* 53, 1714-1715.

- Stokes, A.N., Williams, B.L., French, S.S., 2012. An improved competitive inhibition enzymatic immunoassay method for tetrodotoxin quantification. *Biol. Proced. Online* 14, 1-5.
- Tsuruda, K., Arakawa, O., Kawatsu, K., Hamano, Y., Takatani, T., Noguchi, T., 2002. Secretory glands of tetrodotoxin in the skin of the Japanese newt *Cynops pyrrhogaster*. *Toxicon* 40, 131-136.
- Twitty, V.C., 1961. Second-generation hybrids of the species *Taricha*. *PNAS* 54, 51-58.
- Twitty, V.C., 1966. *Of Scientists and Salamanders*. Freeman, San Francisco, CA.
- Venkatesh, B., Lu, S.Q., Dandona, N., See, S.L., Brenner, S., Soong, T.W., 2005. Genetic basis of tetrodotoxin resistance in pufferfishes. *Curr. Biol.* 15, 2069-2072.
- Wakely, J.F., Fuhrman, G.J., Fuhrman, F.A., Fischer, H.G., Mosher, H.S., 1966. The occurrence of tetrodotoxin (tarichatoxin) in amphibia and the distribution of the toxin in the organs of newts (*Taricha*). *Toxicon* 3, 195-203.
- Wang, J., Fan, Y., Yao, Z., 2010. Isolation of a *Lysinibacillus fusiformis* strain with tetrodotoxin-producing ability from puffer fish *Fugu obscurus* and the characterization of this strain. *Toxicon* 56, 640-643.
- Williams, B.L., 2010. Behavioral and chemical ecology of marine organisms with respect to Tetrodotoxin. *Mar. Drugs* 8, 381-398.
- Williams, B.L., Hanifin, C.T., Brodie, E.D., Jr., Brodie, E.D., III., 2010a. Tetrodotoxin affects survival probability of rough-skinned newts (*Taricha granulosa*) faced with TTX-resistant garter snake predators (*Thamnophis sirtalis*). *Chemoecology* 20, 285-290.

- Williams, B.L., Hanifin, C.T., Brodie, E.D., Jr., Caldwell, R.L., 2010b. Ontogeny of tetrodotoxin levels in Blue-ringed Octopuses: Maternal investment and apparent independent production in offspring of *Hapalochlaena lunulata*. *J. Chem. Ecol.* 37, 10-17.
- Wu, Z., Yang, Y., Xie, L., Xia, G., Hu, J., Wang, S., Zhang, R., 2005. Toxicity and distribution of tetrodotoxin-producing bacteria in puffer fish *Fugu rubripes* collected from the Bohai Sea of China. *Toxicon* 46, 471-476.
- Yasumoto, T., Yotsu-Yamashita, M., 1996. Chemical and etiological studies in tetrodotoxin and its analogs. *Toxin Rev.* 15, 81-90.
- Yotsu-Yamashita, M., Mebs, D., Yasumoto, T., 1992. Tetrodotoxin and its analogues in extracts from the toad *Atelopus oxyrhynchus* (family: Bufonidae). *Toxicon* 30, 1489-1492.
- Yotsu-Yamashita, M., Tateki, E., 2010. First report on toxins in the Panamanian toads *Atelopus limosus*, *A. glyphus* and *A. certus*. *Toxicon* 55, 153-156.
- Yu, C.F., Yu, P.H.F., Chan, P.L., Yan, Q., Wong, P.K., 2004. Two novel species of tetrodotoxin-producing bacteria isolated from toxic marine puffer fishes. *Toxicon* 44, 641-647.

CHAPTER 4

OTTER PREDATION ON *TARICHA GRANULOSA* AND VARIATION IN
TETRODOTOXIN LEVELS WITH ELEVATION

Tetrodotoxin (TTX) is a low molecular weight neurotoxin that is found in a wide variety of taxa. TTX blocks voltage-gated sodium channels, preventing the propagation of action potentials inducing paralysis in susceptible animals. *Taricha granulosa* have been documented to possess TTX in high quantities and only have one positively identified major predator, *Thamnophis sirtalis*. However, recent observations of predation events on *T. granulosa* by otters were documented in a high elevation population just outside of Crater Lake National Park in Oregon. We quantified TTX levels in this population as well as three other populations within Crater Lake National Park using a Competitive Inhibition Enzymatic Immunoassay. We further compared these high elevation populations to a known high toxicity population from Benton County Oregon. We found that the populations within Crater Lake have lower levels of TTX relative to populations outside of the lake, and that all high elevation locations have relatively low levels of TTX. We then analyzed previously published whole newt TTX levels and elevation, and found that there is a significant negative relationship. However, there is a non-significant relationship between whole newt TTX levels and elevation when examining elevations below 300 m.

INTRODUCTION

Tetrodotoxin (TTX) is one of the most potent neurotoxins known (e.g. Brodie 1968; Brodie et al. 1974; Hanifin et al. 1999; Mosher et al. 1964; Wakely et al. 1966). Tetrodotoxin acts by blocking voltage-gated sodium channels in nerve and muscle tissues, thereby preventing propagation of action potentials (Narahashi et al. 1967). A fatal dose of TTX is usually between 0.5 – 2 mg for an adult human (Yasumoto and Yotsu-Yamashita 1996), and death usually occurs due to paralysis of the diaphragm and asphyxiation (Brodie 1968). TTX is unusual in that it is found in a wide array of taxa from marine bacterial species to terrestrial vertebrates (Chau et al. 2011; as reviewed by Miyazawa and Noguchi 2001). One of the most well studied TTX-bearing species is the rough-skinned newt, *Taricha granulosa* (e.g. Brodie 1968; Daly et al. 1987; Hanifin et al. 1999; Mosher et al. 1964; Wakely et al. 1966).

The only well documented predators of adult *T. granulosa* are snakes of the genus *Thamnophis* (Brodie and Brodie 1990, 1991). These two species are involved in a coevolutionary arms race with reciprocal selection acting upon toxicity in newts and resistance in snakes (Brodie and Brodie 1990, 1991; Hanifin et al. 2008). Populations of Thamnophine snakes that are sympatric with newts have evolved varying levels of resistance to TTX via amino acid changes in the pore region of voltage-gated sodium channel (Feldman et al. 2009, 2012; Geffeney et al. 2002, 2005). These adaptations allow snake predation on newts when the predators are resistant enough to tolerate the toxin load present in the local newt population (Hanifin et al. 2008).

Early experiments using *T. granulosa* have shown that TTX is lethal to nearly every potential predator (Brodie 1968), and mortality has been documented following predation attempts on *Taricha* in several species of birds (Mobley and Stidham 2000; Pimentel 1952; Storm 1948). However, there are some more recent examples of successful predation on newts. Great blue herons in northern California have been observed eating newts with no apparent ill effects (Fellers et al. 2008). Additionally, a population of newts in Santa Rosa, CA has been heavily preyed upon over many years (Stokes et al. 2011). In this case, the predator (never positively identified) circumvents the toxin in the skin by eviscerating the newts and only eating the internal organs.

Here we present a new instance of predation on newts by river otters (*Lontra canadensis*) at Lake in the Woods, a small, high altitude lake in Douglas County, Oregon. On August 6, 2007 we observed an otter (*L. canadensis*) eating more than one *T. granulosa*. On October 27-28, 2007 one adult and two juvenile otters were observed eating approximately 20 salamanders in one day. The otters were eating both *T. granulosa* and *Ambystoma gracile* (Fig. 4.1). At no time did any of the otters display any signs or symptoms of TTX intoxication. River otters are opportunistic predators (Kruuk 2006; Melquist et al. 2003), and their diets change depending on the season and availability of food sources (Melquist et al. 2003). In addition to a diet consisting mostly of fish, they have been documented feeding on several amphibian species including Pacific giant salamanders (*Dicamptadon aterrimus*), two-toed amphiumas (*Amphiuma means*), and several frog species (Ranidae). The observation of otters eating toxic prey like *Taricha granulosa* can lead to one of two immediate hypotheses: either the newts in

that locality have little to no TTX, or the otters are resistant to TTX in some way. We investigated the apparent success of this predation event by testing TTX levels in *Taricha granulosa* from the Lake in the Woods population. Furthermore, we tested TTX levels in nearby high elevation lakes just outside of and within Crater Lake National Park and compared them to a well-known high toxicity population from Benton County, Oregon. Given these results, we explored the relationship between elevation of each population and whole newt TTX levels using our dataset and previously published whole newt TTX level data.



Fig. 4.1 *Lontra canadensis* feeding on *Taricha granulosa* (a and b) and *Ambystoma gracile* (c and d) at Lake in the Woods, Oregon

METHODS

Animal Collection/Care

Newts were collected from four populations including Skell Channel (n=19, Klamath County), Phantom Ship (n=5, Klamath County), Spruce Lake (n=19, Klamath County), and Lake in the Woods (n=6, Douglas County), Oregon by hand or using minnow traps (Fig. 4.2). Two of these locations (Spruce Lake and Lake in the Woods) are high elevation lakes outside of Crater Lake, while two (Skell Channel and Phantom Ship) are locations within Crater Lake itself. Thirty-four newts were also collected from Soap Creek ponds in Benton County, Oregon by hand. All newts were transported back to Utah State University, and housed in 5.7-L containers with 2 L carbon-filtered water in an environmental chamber at 6°C. Newts from the high elevation sites were later euthanized with ms222 and skin punches were taken following the methods of Hanifin et al. (2002) for TTX quantification. Newts from Benton County were anesthetized using 1% ms222 to take skin punches, but were not euthanized and used in subsequent experiments.

TTX Quantification

TTX was extracted from skin tissues using the methods of Hanifin et al. (2002). Quantification of TTX was done using a Competitive Inhibition Enzymatic Immunoassay as in Stokes et al. (2012; Ch. 2). We used the linear range of the standard curve, which was between 10 and 500 ng/mL. The minimum level of detection in this assay is 10 ng/mL. Values less than 10 ng/mL are referred to as below detectable limits (BDL) and

considered as zero in our analyses. Newt samples from high elevation sites were not diluted and run against standards diluted in acetic acid. Newt samples from Soap Creek were diluted 1:120, 1:300, 1:500, 1:800 or 1:1000 in a 1% Bovine Serum Albumin-PBS solution (BSA) to get them within the linear range of the standard curve. These samples were run against standards diluted in BSA. The average coefficient of variation on each plate was between 4.72 and 7.03%. Whole newt TTX was calculated using the methods of Hanifin et al. (2004).

Elevation Data

Mean whole newt TTX levels for several populations throughout Oregon were obtained from Hanifin et al. (2008), Ridenhour (2004), and the present study. The average whole newt TTX levels from this study, rather than from previous studies, for Benton County were used in these analyses. We determined the elevation of each collection site for each population by entering latitude and longitude coordinates into Google Earth and National Geographic Topo! software (Version 4.2.8, 2006).

Statistical Analyses

Comparisons of newt TTX levels among populations in the current study were done using non-parametric Wilcoxon/Kruskal-Wallis tests. This was followed with Wilcoxon comparisons for each pair. The relationship between log-transformed elevation and mean population TTX concentrations for populations ≤ 265 m were examined using linear regression. We used studentized residuals to help identify outlier populations following the principle that residuals exceeding ± 2 may be considered

“discrepant observations” and should be evaluated as possible outliers (Fox 1991). Finally, we binned newt populations by 500 m elevation cohorts and compared log-transformed mean population TTX levels from the current and previous studies using one-way analysis of variance and a Tukey HSD test to control for all pairwise comparisons. Analyses were performed using JMP 9 (SAS Institute, Inc) or using the regression wizard feature in SigmaPlot v 10.0 (Systat Software, Inc.).

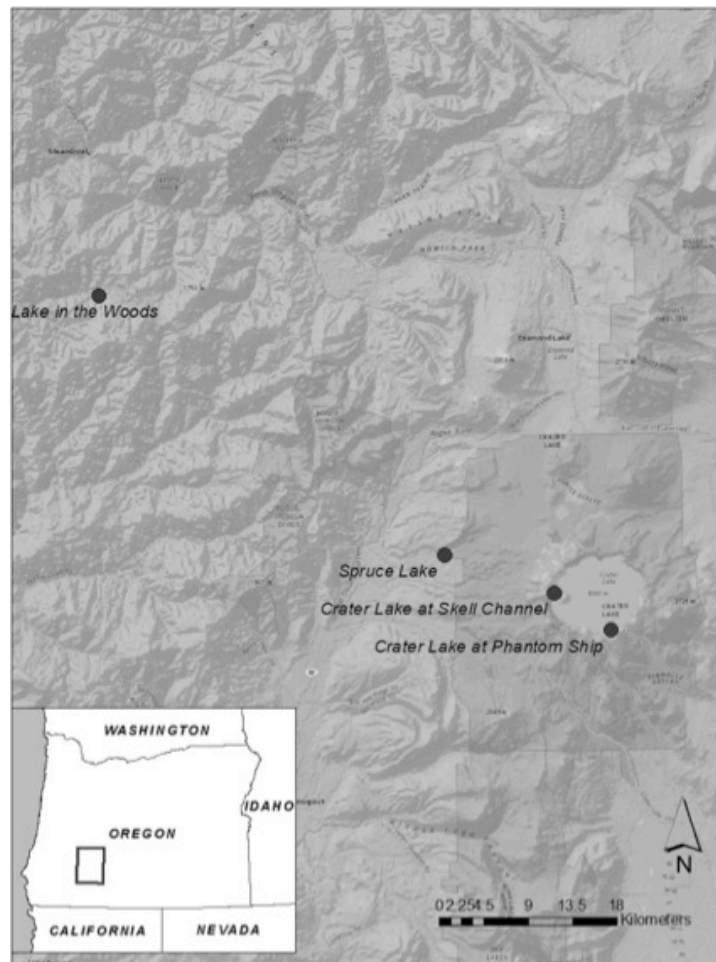


Fig. 4.2 Map of the four high elevation locations in Oregon where *Taricha granulosa* were collected. Note that *Lontra canadensis* predation was observed at Lake in the Woods

RESULTS

Each of the high elevation populations had newts with some TTX, though with very low concentrations (Fig. 4.3). Individuals from Soap Creek have over 1000 times more TTX on average than any of the high elevation populations ($P < 0.001$). There were significant differences in TTX concentrations between several of the populations (Table 4.1). Pairwise comparisons indicated that those populations outside of Crater Lake (Soap Creek, Lake in the Woods, and Spruce Lake) had significantly greater levels of TTX relative to populations within Crater Lake. No differences in TTX levels were detected between Spruce Lake and Lake in the Woods populations (outside Crater Lake) and Skell Channel and Phantom Ship (inside Crater Lake) populations.

The empirical relationship between mean whole body population TTX (mg) and elevation is shown in Fig. 4.4a. The majority (65%) of sampled populations occupied sites at elevations \leq approximately 265 m (Fig. 4.4a hatched box). In addition, there is a relatively large gap in the elevational distribution of sampled populations between sites located at ≤ 265 m and populations sampled from sites > 700 m. A t-test comparing the populations ≤ 265 m and > 700 m was highly significant ($P < 0.001$). However, the lack of sites within the mid elevation range limits our ability to mathematically describe the relationship between elevation and TTX using a single continuous function. For this reason, we restricted our analysis to the 15 populations sampled from sites located at elevations ≤ 265 m and showing measurable concentrations of whole body TTX (Fig. 4.4b). From this restricted analysis, we show that the relationship between elevation and mean population TTX is weakly positive but not significant ($P = 0.209$). Even if we

remove populations from sites that appear to be outliers (e.g., studentized residuals that exceed $|2|$ and circled on Fig. 4.4b) the resulting regression equation is still not significant ($P = 0.093$). The results emphasize that below 250 m, there are no statistical trends in TTX levels that can be explained by elevation alone. Finally, when populations are binned into 500 m elevation cohorts, it is apparent that populations below 500 m have significantly higher mean whole body toxicity levels than populations occupying sites above 1,000 m (Fig. 4.4b; $P = 0.002$, $F = 7.309$).

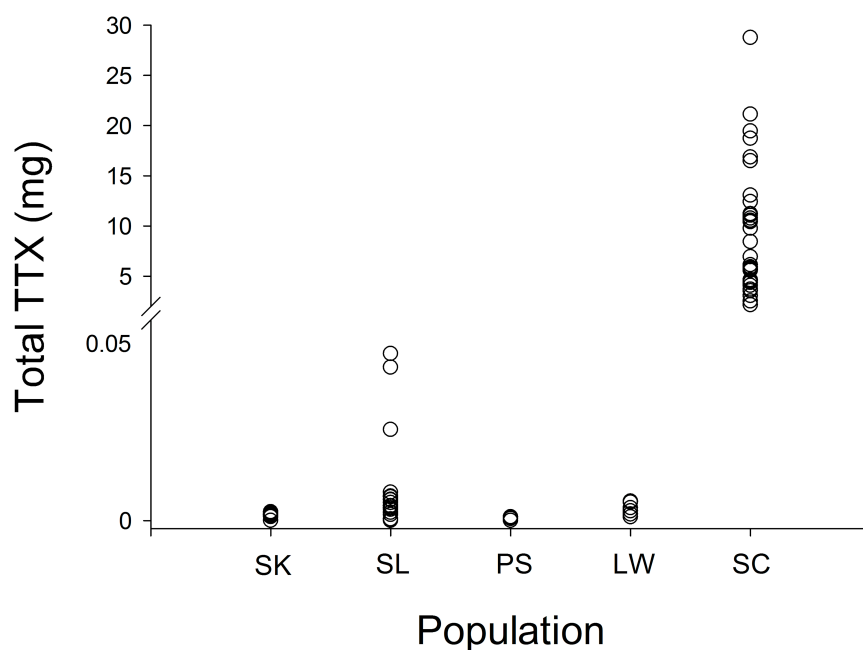


Fig. 4.3 Total whole body TTX (mg) for each individual newt (*Taricha granulosa*) at each of five populations in western Oregon. Population labels are abbreviated as follows: SK= Skell Channel (N = 19; mean = 0.00049 mg; SEM = 0.00018); SL= Spruce Lake (N = 19; mean = 0.00819 mg; SEM = 0.00279); PS= Phantom Ship (N = 5; mean = 0.00053; SEM = 0.00016); LW= Lake in the Woods (N = 6; mean = 0.00819; SEM = 0.00064); SC= Soap Creek (N = 34; mean = 8.55110; SEM = 1.15029). Note that the scale of the y-axis changes beyond the break

Table 4.1 Results from Wilcoxon pairwise comparisons for high elevation populations. Stars indicate significant differences. Populations are abbreviated as follows: SL = Spruce Lake; SK = Skell Channel; PS = Phantom Ship; LW = Lake in the Woods. Populations with “+” superscript indicates that they are within Crater Lake

Comparison	Score Mean Difference	Standard Error Difference	Z	P-value
SL to SK ⁺	15.2632	3.493339	4.36922	<0.0001*
SL to PS ⁺	8.5895	3.551001	2.41889	0.0156*
SL to LW	3.1798	3.445880	0.92279	0.3561
SK ⁺ to PS ⁺	-2.6526	3.183194	-0.83332	0.4047
PS ⁺ to LW	-5.3167	2.008316	-2.64733	0.0081*
SK ⁺ to LW	-10.4167	3.196192	-3.25909	0.0011*

DISCUSSION

Newts from the high elevation regions of Oregon outside of Crater Lake ranged from BDL to 0.042 mg of TTX, while newts within Crater Lake ranged from BDL to 0.00214 mg of TTX. The toxicity levels of newts in the high elevation lakes are low enough that otters, and possibly other predators (e.g. fish; Swanson et al. 2000), likely can eat at least some individuals with very few, if any, side effects. Many predators of toxic prey are able to circumvent the harmful toxin(s) either through changes in behavior (Williams et al. 2003), including development of novel predation strategies (Stokes et al. 2011), or adaptation to the toxin itself (Feldman et al. 2012; Geffeney et al. 2002, 2005; Phillips and Shine 2006). Garter snakes have been shown to “taste” and subsequently reject newts that are excessively toxic (Williams et al. 2003). It is possible that although we did not observe otters rejecting prey, they may also be able to determine whether or not a newt is safe to eat by “tasting” the newt and rejecting ones that are too toxic.

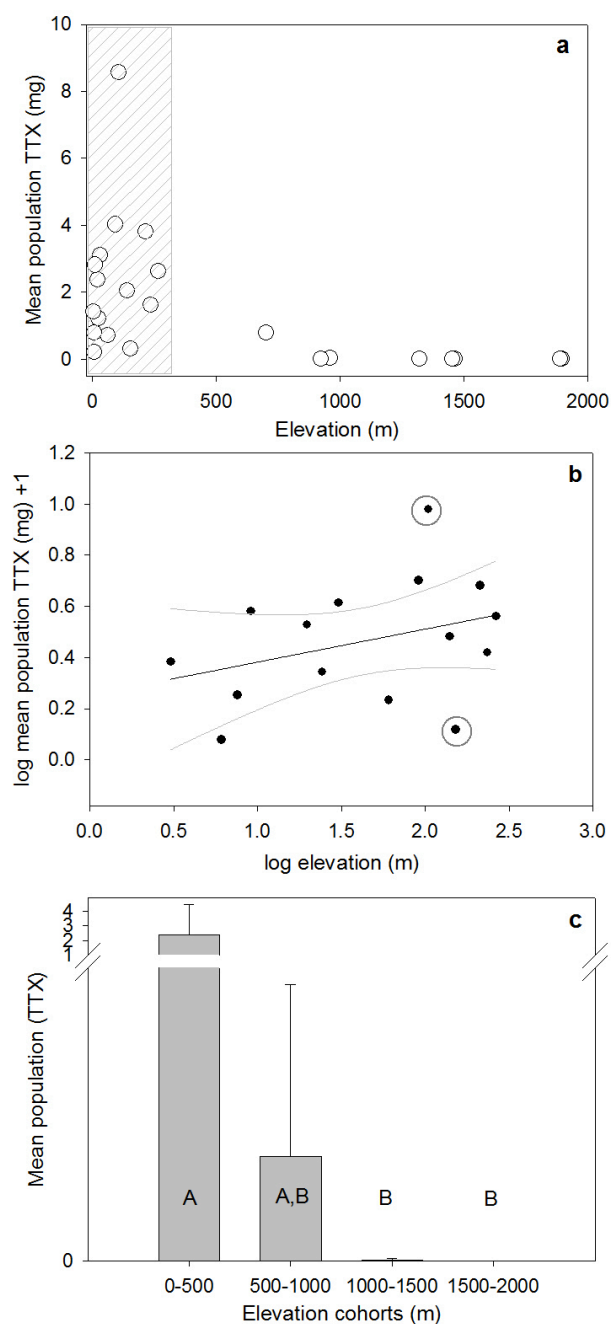


Fig. 4.4 Relationship between elevation and TTX levels across populations from Hanifin et al (2008), Ridenhour (2004), and the present study. (a) The empirical relationship between mean whole body population TTX (mg) and elevation, with the majority of sample sites highlighted with the hatched box. (b) Regression analysis of populations below 265 m ($n = 15$); circles represent outliers that were removed to confirm the analysis. (c) Comparison of binned data in 500 m cohorts

Hanifin et al. (2008) showed that TTX levels vary greatly both within and between populations of rough-skinned newts along the Pacific coast of North America. Specifically, within Oregon they found that newts from the Benton County area of Oregon have very high levels of TTX with a mean of 4.695 mg of TTX. Our results nearly doubled this previously reported mean. Furthermore, Hanifin and colleagues (2008) measured TTX levels in newts from Parsnip Lakes (Jackson Co.) and Scott Lake (Lane Co.), Oregon that were each above 1,200 m in elevation. Both locations were found to have very low average levels of TTX with means of 0.002 and 0.000 mg of TTX, respectively. All other populations from Oregon that were measured in that study were below 300 m, and the lowest mean was 0.787 mg TTX. Similarly, Ridenhour (2004) demonstrated that newts at lower elevations tend to have higher levels of TTX than those at higher elevations. In this study, newts at elevations below 300 m ranged in mean TTX levels from 0.197 to 4.695 mg. Elevations above 300 m had relatively low TTX levels that ranged from 0.025 to 0.776 mg TTX.

It seems possible that the apparent differences in TTX levels in newts from high elevations versus those in lower elevations may be due to relaxed selection from a low abundance of predators. It has been shown that reptile diversity on elevational gradients is most greatly correlated with temperature (McCain 2010). Therefore, snake diversity generally decreases with increasing elevation, and this effect is stronger on wetter mountains than drier mountains. However, *Thamnophis sirtalis* are not uncommon to Crater Lake National Park, and are often found on the lakeshore among other areas (Farner et al. 1953). The elevation on the shore of the lake is approximately 1900 m or

more. Without a detailed measure of relative abundance of snakes in this area, it is unclear how selective pressures may be altered by elevation in these systems.

Furthermore, from the present study, it is clear that other predators are likely to impact selection on newt toxin levels.

The newts from Crater Lake are in contrast to those from Soap Creek, which are some of the most toxic individuals sampled. The most toxic individual in Soap Creek contained 28 mg of TTX (enough to kill up to 56 humans (Yasumoto and Yotsu-Yamashita 1996)). In contrast, the least toxic populations, such as these high elevation populations, have large numbers of individuals that lack detectable levels of TTX. Soap Creek newts are also one of the best examples of variation within a population of newts known to date, ranging from no detectable TTX (Williams et al. 2004) to 28 mg of TTX (present study). Interestingly, the newts within Crater Lake have significantly lower levels of TTX than the populations outside of Crater Lake. We do not yet have conclusive evidence that indicates whether or not production of TTX is endogenous or exogenous (reviewed by Miyazawa and Noguchi 2001). However, studies done thus far on *Taricha granulosa* suggest that TTX may be of endogenous origins (Cardall et al. 2004; Hanifin et al. 2002; Lehman et al. 2004). At this time it is not possible to definitively discern whether the variation between the populations inside Crater Lake and those outside of the lake is due to genetic isolation or dietary differences. This latter point is particularly important to consider, because low productivity in an ultraoligotrophic lake like Crater Lake may limit energy available for the endogenous production of TTX.

Given the lack of evidence for exogenous production of TTX in *T. granulosa*,

however, it can be hypothesized that the variation seen within and between populations may be important to the evolution of this toxin since variation is required for selection to act upon a trait (Fisher 1930). Rough-skinned newts and garter snakes have been one of the most well studied examples of a coevolutionary arms race between predators and their prey (e.g. Brodie and Brodie 1990, 1991; Feldman et al. 2009, 2012; Geffeney et al. 2002, 2005; Hanifin et al. 2008; Williams et al. 2003). Recent work, however, has shown that the evolution of TTX in these newts may be more complicated than a simple two species system. There has been work showing that caddisfly larvae are capable of eating newt eggs, potentially influencing TTX concentrations at a very early life history stage (Gall et al. 2011). Additionally, the evidence in this study and elsewhere (Fellers et al. 2008, Stokes et al. 2011) indicate that garter snakes are not the only predators of rough-skinned newts. Especially in newt populations with low toxicity, novel predators such as river otters have the potential to put selective pressure on these populations of newts, favoring increased levels of TTX.

LITERATURE CITED

- Brodie ED, Jr. (1968) Investigations on the skin toxin of the adult rough-skinned newt, *Taricha granulosa*. *Copeia* 1968:307-313
- Brodie ED, Jr., Hensel JL, Jr., Johnson JA (1974) Toxicity of the urodele amphibians *Taricha*, *Notophthalmus*, *Cynops* and *Paramesotriton* (Salamandridae). *Copeia* 1974:506-511

- Brodie ED III, Brodie ED, Jr. (1990) Tetrodotoxin resistance in garter snakes: an evolutionary response of predators to dangerous prey. *Evol* 44:651-659
- Brodie ED III, Brodie ED, Jr. (1991) Evolutionary response of predators to dangerous prey: reduction of toxicity of newts and resistance of garter snakes in island populations. *Evol* 45:221-224
- Chau R, Kalaitzis JA, Neilan BA (2011) On the origins and biosynthesis of tetrodotoxin. *Aquat Toxicol* 104: 61-72
- Cardall BL, Brodie ED, Jr., Brodie ED III, Hanifin CT (2004) Secretion and regeneration of tetrodotoxin in the rough-skin newt (*Taricha granulosa*). *Toxicon* 44: 933-938
- Daly JW, Meyers CW, Whittaker N (1987) Further classification of skin alkaloids from neotropical poison frogs (Dendrobatidae), with a general survey of toxic, noxious substances in the Amphibia. *Toxicon* 25:1021-1095
- Farner DS, Kezer J (1953) Notes on the amphibians and reptiles of Crater Lake National Park. *Am Mid Nat* 50: 448-462
- Feldman CR, Brodie ED, Jr., Brodie ED III, Pfreder ME (2009) The evolutionary origins of beneficial alleles during the repeated adaptation of garter snakes to deadly prey. *PNAS* 106: 13415-13420
- Feldman CR, Brodie ED, Jr., Brodie ED III, Pfreder ME (2012) Constraint shapes convergence in tetrodotoxin resistant sodium channels of snakes. *PNAS* 109:4556-4561
- Fellers GM, Reynolds-Taylor J, Farrar S (2008) *Taricha granulosa* predation. *Herpetol Rev* 38:317-318

- Fox, J (1991) Regression diagnostics. Sage Publications, Inc., Newbury Park, CA.
- Fisher, RA (1930) The Genetical Theory of Natural Selection. Clarendon Press, Oxford.
- Gall BG, Brodie ED III, Brodie ED, Jr. (2011) Survival and growth of the caddisfly *Limnephilus flavastellus* after predation on toxic eggs of the rough-skinned newt (*Taricha granulosa*). Can J Zool 89: 483-489
- Geffeney SL, Brodie ED, Jr., Ruben PC, Brodie ED III (2002) Mechanisms of adaptation in a predator-prey arms race: TTX-resistant sodium channels. Science 297:1336-1339
- Geffeney SL, Fujimoto E, Brodie ED III, Brodie ED, Jr., Ruben PC (2005) Evolutionary diversification of TTX-resistant sodium channels in a predator-prey interaction. Nature 434:759-763
- Hanifin CT, Yotsu-Yamashita M, Yasumota T, Brodie ED III, Brodie ED, Jr. (1999) Toxicity of dangerous prey: variation in Tetrodotoxin levels within and among populations of the newt *Taricha granulosa*. J Chem Ecol 25:2161-2175
- Hanifin CT, Brodie ED III, Brodie ED, Jr. (2002) Tetrodotoxin levels of the rough-skin newt, *Taricha granulosa*, increase in long-term captivity. Toxicon 40:1149-1153
- Hanifin CT, Brodie ED III, Brodie ED, Jr. (2004) A predictive model to estimate total skin tetrodotoxin in the newt *Taricha granulosa*. Toxicon 43: 243-249
- Hanifin CT, Brodie ED III, Brodie ED, Jr. (2008) Phenotypic mismatches reveal escape from arms-race coevolution. PLOS 6:471-482
- Kruuk H (2006) Otters: Ecology, Behavior and Conservation. Oxford University Press, New York

- Lehman EM, Brodie ED, Jr., Brodie ED III. (2004) No evidence for an endosymbiotic bacterial origin of tetrodotoxin in the newt *Taricha granulosa*. *Toxicon* 44: 243-249
- McCain CM (2010) Global analysis of reptile elevational diversity. *Global Ecol Biogeogr* 19:541-553
- Melquist WE, Polechla PJ, Jr., Toweill D (2003) River Otter in: G.A. Feldhamer, B.C. Thompson, and J.A. Chapman (eds.). *Wild mammals of North America*, Second Edition. The Johns Hopkins University Press. Baltimore, Maryland, pp.708-733
- Miyazawa K, Noguchi T (2001) Distribution and origin of tetrodotoxin. *J Toxicol* 20:11-33
- Mobley JA, Stidham TA (2000) Great horned owl death from predation of a toxic California newt. *The Wilson Bull* 112:563-564
- Mosher HS, Fuhrman FA, Buchwald HD, Fischer HG (1964) Tarichatoxin-tetrodotoxin: a potent neurotoxin. *Science* 144:1100-1110
- Narahashi T, Moore JW, Poston RN (1967) Tetrodotoxin derivatives: chemical structure and blockage of nerve membrane conductance. *Science* 156:976-979
- Pimentel RA (1952) Studies on the biology of *Triturus granulatus* Skilton. Unpublished Ph.D. dissertation. Oregon State University, Corvallis
- Phillips BL, Shine R (2006) An invasive species induces rapid adaptive change in a native predator: can toads and black snakes in Australia. *Proc R Soc B* 273:1545-1550
- Ridenhour BJ (2004) The coevolutionary process: The effects of population structure on

a predator-prey system. Unpublished PhD dissertation. Indiana University, Bloomington

Stokes AN, Cook DG, Hanifin CT, Brodie ED III, Brodie ED, Jr. (2011) Sex-biased predation on newts of the genus *Taricha* by a novel predator and its relationship with tetrodotoxin toxicity. *Am Mid Nat* 165:389-399

Stokes AN, Williams BL, French SS (2012) An improved competitive inhibition enzymatic immunoassay method for tetrodotoxin quantification. *Biol Proced Online* 14:3

Storm RM (1948) The herpetology of Benton County, Oregon. Unpublished Ph.D. dissertation. Oregon State University, Corvallis

Swanson NL, Liss WJ, Ziller JS, Wade MG, Gressell RE (2000) Growth and diet of fish in Waldo Lake, Oregon. *Lake Reserv Manage* 16:133-143

Wakely JF, Fuhrman GJ, Fuhrman FA, Fischer HG, Mosher HS (1966) The occurrence of Tetrodotoxin (tarichatoxin) in Amphibia and the distribution of the toxin in the organs of newts (*Taricha*). *Toxicon* 3:195-203

Williams BL, Brodie ED, Jr., Brodie ED 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* 59:155-163

Williams BL, Brodie ED, Jr., Brodie ED III (2004) A resistant predator and its toxic prey: persistence of newt toxin leads to poisonous (not venomous) snakes. *J Chem Ecol* 30:1901-1919

Yosumoto T, Yotsu-Yamashita M (1996) Chemical and etiological studies on tetrodotoxin and its analogs. *Toxin Rev* 15:81-90

CHAPTER 5

THE FIRST KNOWN CASE OF TETRODOTOXIN IN A TERRESTRIAL
INVERTEBRATE: TERRESTRIAL FLATWORMS *BIPALIUM ADVENTITIUM* AND
BIPALIUM KEWENSE

Tetrodotoxin (TTX) is a potent low molecular weight neurotoxin. It acts by blocking the pore region of voltage-gated sodium channels, inducing paralysis. Though TTX has been documented in a large number of marine invertebrate groups, it has never before been documented in a terrestrial invertebrate. Here, we show that TTX is present in two species of terrestrial flatworm (*Bipalium adventitium* and *Bipalium kewense*). Further, we provide evidence that TTX is used both during predation and defensively in these two species, and that the egg capsules of *B. adventitium* also have TTX.

INTRODUCTION

Chemical defenses are common throughout the natural world. Within predator-prey systems chemical toxins may be used either defensively (to prevent predation) or during predation (to subdue and/or kill prey) (Eisner and Meinwald 1995). It has long been documented in the literature that the terrestrial flatworms *Bipalium kewense* and *B. adventitium* appear to use a toxin to aid in subduing large earthworm prey items (Dindal 1970; Ogren 1995; Ducey et al. 1999; Zaborski 2002). *Bipalium adventitium* has been found to attack earthworms 100 times their own mass, and are highly successful at killing earthworms that are 10 times their own mass (Ducey et al. 1999). Research on *B. adventitium* has also shown that they are active hunters of earthworms, capable of

following earthworm trails in order to find their prey (Ducey et al. 1999; Fiore et al. 2004).

Feeding in these two species involves a behavior referred to as capping (Ducey et al. 1999). When the flatworm encounters earthworm prey, the flatworm will crawl up the earthworm's body towards the head. The flatworm will then commence capping, which is a behavior in which the flatworm uses its head and body to cover the anterior end of the earthworm (Ducey et al. 1999). In some cases the flatworm will also rub its head back and forth over this anterior section of the earthworm (personal observation). This behavior has been found to significantly reduce the amount of movement, and ultimately escape behavior, exhibited by the earthworm (Ducey et al. 1999). Approximately one third to one half of the way down the body on the ventral side of the flatworm is its mouth, which is used during both feeding and removal of waste. Shortly after capping, the pharynx is expanded and attaches to the earthworm, releasing enzymes to externally digest the prey. The partially digested meal is then taken up into the flatworm through the pharynx. Earthworms will often struggle violently to escape once the flatworm's pharynx is expanded and attached (Dindal 1970; Ducey et al. 1999; Zaborski 2002). However, many times following capping, and within a minute always following expansion of the pharynx, the movements of the earthworm diminish and the earthworm seems to be at least in a partial paralytic state (Dindal 1970; Ducey et al. 1999; Zaborski 2002, personal observation). These observations have indicated that *Bipalium* may have some toxin that aids in subduing prey.

The presence of a toxin may also be utilized for defense against predators of *Bipalium*. In an effort to determine potential predators of *Bipalium*, Ducey et al. (1999) performed feeding trials using various species of non-TTX bearing salamander species; *Ambystoma laterale-jeffersonianum*, *A. maculatum*, *Desmognathus fuscus*, *D. ochrophaeus*, *Plethodon cinereus*, and *P. glutinosus*. In all cases where contact was made the salamanders rubbed their heads on the substrate following tongue contact or ingestion of the flatworm. Given these observations, and the apparent paralysis in the earthworms, we hypothesized that the toxin in question may be Tetrodotoxin (TTX).

Tetrodotoxin is a low molecular weight neurotoxin that acts by blocking voltage-gated sodium channels in muscle and nerve tissues in many animal phyla, preventing propagation of action potentials and resulting in paralysis (Narahashi et al. 1964; 1967). In mammals, death often occurs in response to paralysis of the diaphragm and subsequent asphyxiation (Brodie 1968). One of the most interesting aspects of TTX is that it is found in a wide array of taxa ranging from bacterial species to vertebrates (Miyazawa and Noguchi 2001; Chau et al. 2011). Though this toxin has been found in many invertebrate species including planarians, annelids, and molluscs, to our knowledge they have included only marine taxa. Among vertebrates, TTX is found in species of salamanders and frogs that spend much of their lives terrestrially (Miyazawa and Noguchi 2001). However, there have been no documented instances of TTX in a terrestrial invertebrate, making its presence in a terrestrial Platyhelminth species exceptional.

Studies on taxa with TTX have shown that this toxin has a variety of ecological functions, although, the defensive role of TTX has most commonly been investigated.

This role has been widely documented in the rough skinned newt, *Taricha granulosa*, which is involved in a predator-prey arms race with its snake predators (Brodie and Brodie 1990; 1991). However, there are other documented ecological functions of TTX, demonstrating both the offensive and defensive natures of TTX. For example, California newt (*Taricha torosa*) larvae have been shown to use TTX as a predator cue for cannibalistic conspecific adults, allowing them to change their behavior and avoid predation (Zimmer et al. 2006). In contrast, a species of marine planoceric flatworm has been shown to use TTX during predation in order to disable prey (Ritson-Williams et al. 2005).

In this paper we investigate the presence of TTX in *B. adventitium* and *B. kewense*. We tested whether TTX was present, and then compared levels between the two species. Differences between the two species could later help identify differences in mode of production of TTX or ecological differences between the two species (i.e. stronger selection on one than the other). Additionally, we wanted to determine the location of TTX in the two species of *Bipalium* in an attempt to elucidate the ecological function(s) in these species. It is likely that TTX is used both defensively (against predators) and during predation (against prey) in *Bipalium* sp. Nevertheless, we thought that if TTX were used primarily during predation, it would be more concentrated in one particular area of the body. However, if TTX were used primarily in defense, it may be more evenly distributed. We believed that TTX is likely used during predation to subdue large earthworm prey and would be concentrated either near the head of the flatworm and secreted during capping, or in the mouth region and secreted during digestion.

Furthermore, we tested the egg capsules of *B. adventitium* (*B. kewense* does not generally lay egg capsules) for the presence of TTX. If the eggs are toxic, this also provides evidence for TTX being used as a defensive toxin in these species.

METHODS

Animal care/collection

Bipalium kewense were collected from Santa Clara County, CA and Harris County, TX. *B. adventitium* were collected from Cortland County, NY and Schenectady County, NY. Individuals were shipped to Utah State University in Logan, UT, where they were housed prior to experimentation. Flatworms were housed individually in small (1-L) plastic containers with pinholes punched in the lids to promote airflow, and a damp paper towel for substrate. Flatworms were fed earthworms (*Eisenia fetida*, *Eisenia hortensis*, and *Lumbricus terrestris*) that were cut into pieces about two times the weight of the flatworm every other week. Containers were cleaned regularly with tap water. Between feedings the substrate was sprayed with tap water periodically to keep the environment moist. Containers were stored in larger dark plastic tubs to ensure protection from light.

Extractions and TTX analysis

Bipalium individuals (N = 6 for each species) for whole flatworm analysis were weighed, placed in microcentrifuge tubes and frozen at -80° C. *Bipalium* individuals (N = 6 for each species) for segmented trials were cut into three portions using a scalpel. The head portion was cut just posterior to the head in the “neck region.” The anterior

body portion of the flatworm was from the initial cut of the head posteriorly to the mouth. In cases where it was difficult to see the location of the mouth, a blunt probe was gently rubbed on the ventral side of the flatworm until some of the pharynx protruded. The posterior body region was the remainder of the flatworm beyond the mouth. All three segments were individually weighed, placed in labeled microcentrifuge tubes, and frozen at -80°C . *Bipalium adventitium* that had arrived in the lab produced egg capsules shortly after arrival ($N = 8$). These capsules were also frozen at -80°C and weighed prior to extraction for TTX quantification.

Tetrodotoxin was extracted from flatworms using the methods of Hanifin et al. (2002). Flatworms were homogenized in either 600 or 800 μl of 0.1 M acetic acid and boiled for five minutes. Each sample was then centrifuged at 13,000 RPMs for 20 minutes. The supernatant was then pipetted into centrifugal filter tubes and centrifuged in the same manner as before. All samples were stored at -80°C for quantification. Quantification of TTX was done using a Competitive Inhibition Enzymatic Immunoassay as in Stokes et al. (2012; Ch. 2). Standards were prepared from TTX-citrate purchased from Abcam, and diluted in 0.1 M acetic acid to the linear range of the standard curve, which was between 10 and 500 ng/mL . Values less than 10 ng/mL are referred to as below detectable limits (BDL) and considered as zero in our analyses. Samples were not diluted. The average coefficient of variation on each plate was between 4.05 – 4.51%.

Qualitative analysis and confirmation of the presence of TTX was performed using High Performance Liquid Chromatography (HPLC) with fluorescence detection. Protocols were largely similar to previously published methods (Cardall et al. 2004;

Hanifin et al. 2008; Stokes et al. 2011). Separation of TTX and TTX analogs was performed with mobile phase that consisted of 50mM ammonium acetate and 60 mM ammonium heptafluorobutyrate buffers (pH 5.0) with 1% acetonitrile and a Synergi 4 μ Hydro-RP 80A column (4.6 X 250 mm, Phenomenex, USA) on a Beckman 126 pump system. Samples were injected using a Beckman 508 autosampler. Post column derivitization was achieved using a Pickering CRX 400 post column reactor set at 115 °C in 5N NaOH. Fluorescence was measured using a JASCO 1520 detector (excitation wavelength: 365 nM, emission wavelength: 510 nm). Data acquisition and chromatographic analysis was performed using 32K System Gold software (version 8.0) and an SS420x A/D convertor (Agilent Technologies). Flatworm extracts were compared to commercially sourced authentic TTX standards (see above) to confirm the presence of authentic TTX.

Statistical analyses

For both whole flatworm and segmented flatworm samples we looked at the overall amount of TTX in the sample (concentration), as well as the amount of TTX in the sample relative to body weight of the flatworm or segment. Whole flatworm data did not meet the assumptions of normality and heterogeneity, so were compared using non-parametric Wilcoxon Kruskal Wallis tests using JMP version 10 (SAS Institute). Segmented flatworm samples were analyzed as a split plot design with each individual flatworm as the block, species as the whole plot factor, and segment as the subplot factor. Data for segmented flatworms did not meet the assumptions of normality and heterogeneity. Concentration data were square-root transformed, and data adjusted for

body weight were log transformed to better meet these assumptions. Analyses of segmented flatworms were performed using PROC GLIMMIX in SAS version 9.3 (SAS Institute).

RESULTS

Both *B. kewense* and *B. adventitium* were found to have TTX (Figures 5.1, 5.2, and 5.3). When comparing the concentrations of TTX in whole flatworms, there were no significant differences between the two species ($df = 1$, $P = 0.4712$). The mean concentrations of each sample for *B. adventitium* and *B. kewense* were 40.10 ng/mL TTX (SEM = 14.218, Range = BDL – 81.42 ng/mL) and 62.03 ng/mL TTX (SEM = 5.13, Range = 50.25 – 82.71 ng/mL), respectively. This corresponded with an average of 31.11 and 44.10 ng of TTX per flatworm, respectively. Likewise, there were no significant differences between the species in levels of TTX relative to mass for whole flatworms ($df = 1$, $P = 0.2980$). Relative to mass, whole *B. adventitium* had a mean of 4.64 ng/mg TTX (SEM = 3.26, Range = BDL – 19.89 ng/mg), and *B. kewense* had a mean of 3.72 ng/mg TTX (SEM = 1.22, Range = 0.73– 8.27 ng/mg).

Comparisons of segments indicated no differences between the concentrations of TTX in the two species ($F = 0.00$, $P = 0.9926$, Figure 5.1), or between the segments ($F = 2.76$, $P = 0.0875$). There was, however, a significant interaction between species and segment ($F = 3.94$, $P = 0.0361$), due to slightly different patterns in the distribution of the toxin. *Bipalium adventitium* had higher concentrations of TTX in the anterior and posterior body regions (Figure 5.1a), however, *B. kewense* had a higher concentrations of

TTX in the head (Figure 5.1b). Relative to mass, segmented *B. adventitium* had significantly more TTX than did segmented *B. kewense* ($F = 4.95$, $df = 1$, $P = 0.0364$). There were also significant differences in the amount of TTX in each segment ($F = 89.37$, $df = 2$, $P < 0.0001$, Figure 5.2), with the head having significantly more TTX than the anterior body ($t = 11.57$, $P < 0.0001$) and the posterior body ($t = -8.23$, $P < 0.0001$). The anterior and posterior body segments have similar levels of TTX relative to mass ($t = 1.58$, $P = 0.2761$). Unlike with concentration, there were no significant interactions between species and segment relative to mass ($F = 0.30$, $df = 2$, $P = 0.7444$), as both species had similar patterns of TTX distribution in the body regions.

Bipalium adventitium eggs were also found to have TTX. With a mean of 89.73 ng of TTX total (SEM = 7.14, Range = 49.18 – 110.30) and an average of 10.56 ng TTX/mg weight (SEM = 2.62, Range = 2.06 – 22.33).

The presence of TTX in these species was confirmed using HPLC analysis (Figure 5.3). We compared the peaks from *B. adventitium* to that of a 0.0005 mg/mL TTX standard, and the flatworm sample co-injected with the same TTX standard. The single peak in the flatworm and in the co-injected sample indicates that this toxin is TTX.

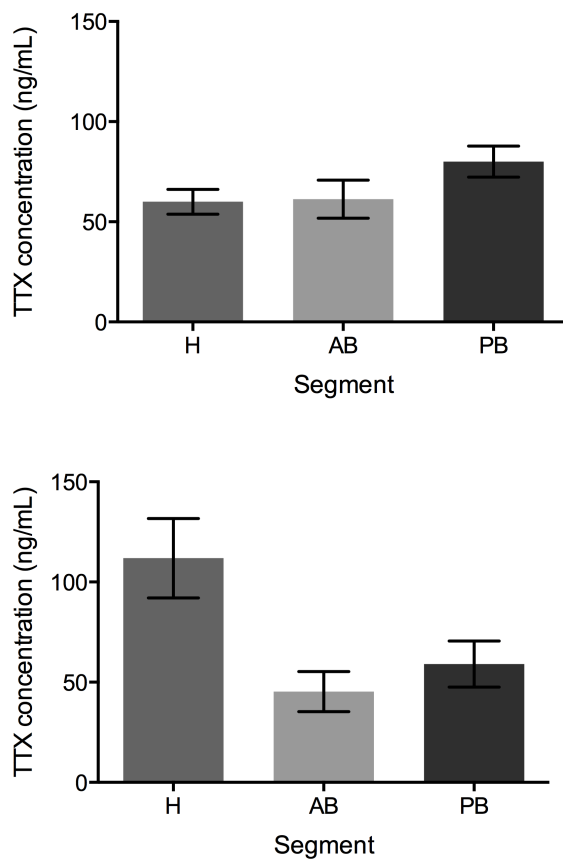


Figure 5.1. Concentration values (ng/mL) for each segment for (a) *Bipalium adventitium* and (b) *Bipalium kewense*. Segments are denoted as H for head, AB for anterior body, and PB for posterior body.

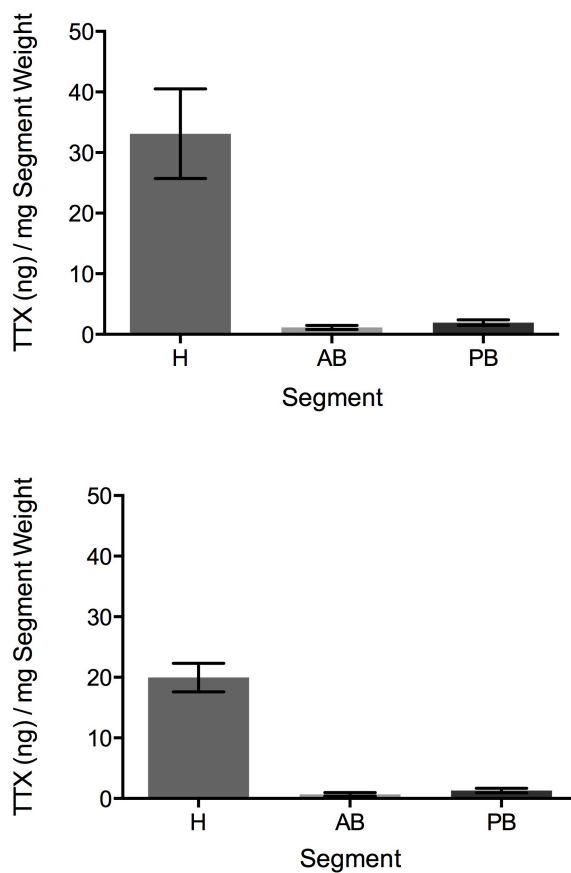


Figure 5.2. The amount of TTX (ng) adjusted for weight of that particular body region (mg) for (a) *Bipalium adventitium* and (b) *Bipalium kewense*. Segments are denoted as H for head, AB for anterior body, and PB for posterior body.

Table 5.1. The amount of TTX (ng) adjusted for weight of body segment (mg) for *Bipalium adventitium* and *Bipalium kewense*.

Body Region	<i>Bipalium adventitium</i>			<i>Bipalium kewense</i>		
	Mean	Range	SEM	Mean	Range	SEM
Head	33.11	14.99-62.66	7.40	19.96	12.28-26.98	2.34
Anterior body	1.15	0.23-2.31	0.33	0.69	0.08-2.10	0.30
Posterior Body	1.94	0.65-1.94	0.47	1.33	0.06-2.34	0.37

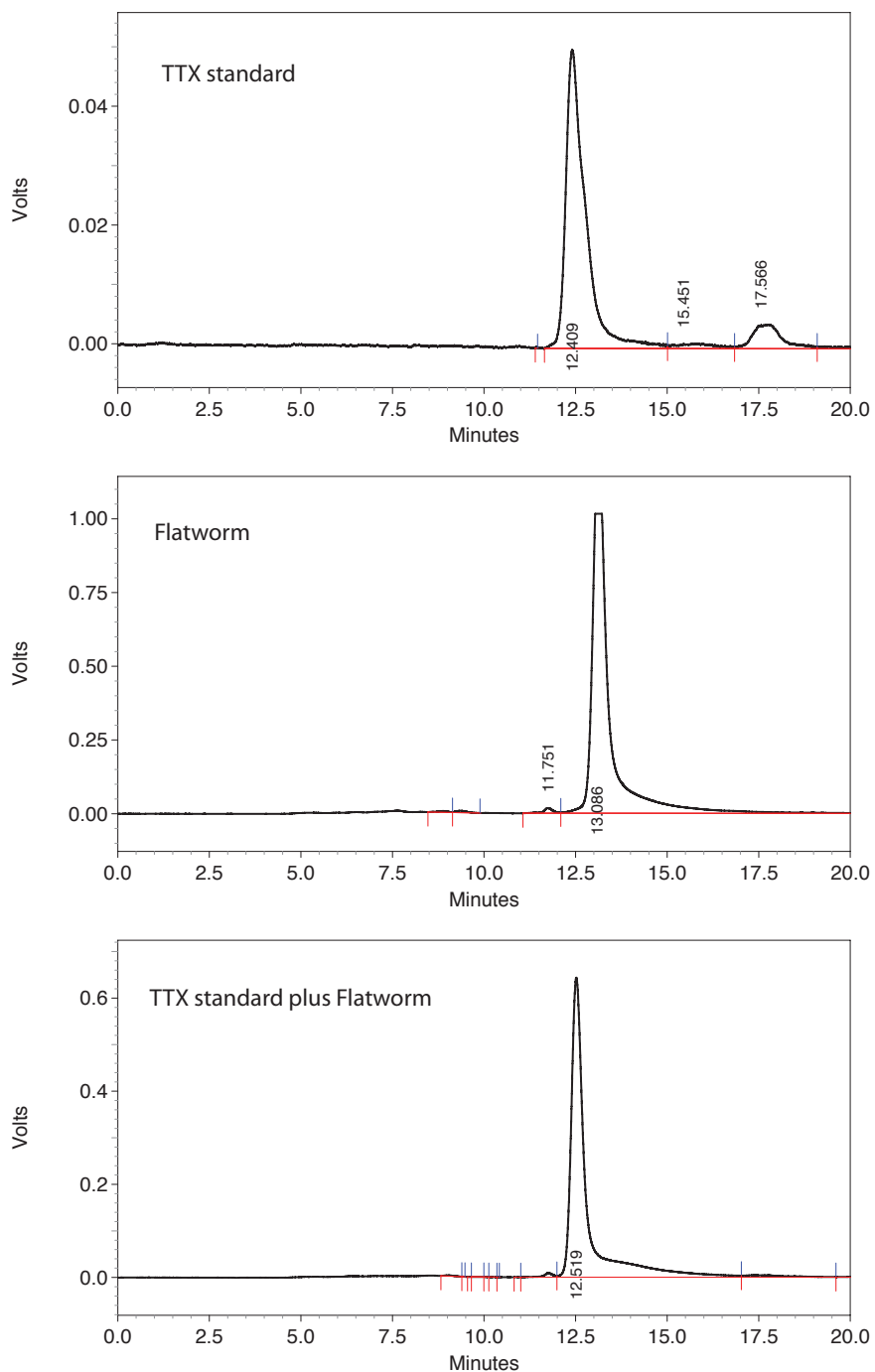


Figure 5.3. Elution times and TTX profiles of an authentic TTX standard (Top), *Bipalium adventitium* (middle), and *B. adventitium* co-injected with a TTX standard (0.0005 mg/ml). The presence of single peak in the flatworm and co-injected sample confirm that the TTX like toxin present in this species is authentic TTX.

DISCUSSION

Tetrodotoxin is most well known from its presence in puffer fish of the family Tetraodontidae, which are commonly eaten in Japan (Miyazawa and Noguchi 2001). However, the number of species that have TTX is quite astounding, and crosses a wide range of taxonomic groups. Although much interest in TTX has been in vertebrate groups, there are a large number of invertebrate groups that have TTX including red calcareous algae, dinoflagellates, and bacteria, to horseshoe crabs, blue-ringed octopuses, and species of gastropods. Notably, there are several groups of worms that bear TTX including annelids and flatworms (*Planocera* species) (Miyazawa and Noguchi 2001; Ritson-Williams et al. 2005). Though this known distribution is wide, this study is the first known to document the presence of TTX in any terrestrial invertebrate species.

Simply identifying the presence of TTX in a novel species has evolutionary implications. However, understanding the ecological uses of this toxin is also very important for understanding the evolution of TTX bearing species, and potentially the mode of TTX production, which currently is unknown (Miyazawa and Noguchi 2001; Chau et al. 2011).

It has been documented many times that earthworms were likely subdued with some sort toxin due to the apparent partial paralysis exhibited by earthworms following attack by *Bipalium* (Dindal 1970; Ducey et al. 1999; Zaborski 2002). These observations support the idea that these flatworms use TTX during predation; though it is unknown currently whether TTX is the only toxin present in these two species. Our study demonstrates some interesting patterns regarding the distribution of TTX in these

flatworms that may further provide support for this idea. We found that there is a large amount of TTX relative to mass found in the heads of these flatworms. The heads of *Bipalium kewense* have been shown to be highly innervated and to possess ciliated sensory organs (Fernandes et al. 2001), therefore, it is possible that some release of TTX occurs from the head during capping. However, the importance of release of TTX from the mouth during feeding cannot be ruled out during this time.

These observations certainly are not sufficient to rule out a defensive use of TTX in these species, as well. We hypothesized that if TTX were used defensively it would be more evenly distributed throughout the body, which it is in both species. Interestingly, however, the pattern of distribution in terms of concentration is different between the two species. *Bipalium adventitium* has more TTX in the posterior body than the head or anterior body (Figure 5-1a), while *B. kewense* has more TTX in the head than the anterior or posterior body (Figure 5-1b). None of these differences are significant individually, but they do contribute to a significant interaction between species and body segment.

The feeding trials conducted by Ducey et al. (1999) using potential salamander and snake predators of *B. adventitium* indicate that predation by these species may be deterred in a couple of ways. First, none of the predators readily identified *Bipalium* as a prey item, with 90% of the salamanders, and all of the snakes failing to strike at the flatworms. Though there is little information regarding the native predators of these flatworms, as they are invasive from Asia they likely have few if any predators here in North America. Both the wild salamander species and snake species showed little interest in eating *Bipalium*, however, ate both earthworms and mealworms readily.

Secondly, many of the flatworms that were struck were rejected. Of those salamanders that did strike at the flatworms two salamanders actually consumed the flatworms, while seven rejected them. Those that rejected the flatworms rubbed the sides of their heads on the substrate following contact with the flatworm, which is a common response to contact with TTX laden prey (Brodie 1968). Repeating these trials using habituated salamanders that were offered *B. adventitium* with forceps, it was found that all of the salamanders struck, and that only three of nine rejected the flatworms (Ducey et al. 1999). Again, all salamanders responded by rubbing their heads on the substrate and gaping their mouths at least once. Though these trials with habituated salamanders indicate that salamanders can eat *B. adventitium* without dying, it is unknown if future offerings of *Bipalium* would be rejected. This indicates that TTX functions well as an antipredator mechanism for *B. adventitium*, and likely functions equally well for *B. kewense*. The two wild salamanders that ate the flatworms may have been offered individuals that do not have TTX as our data in this study indicate is possible.

Further, we documented maternal investment of TTX in the egg capsules of *Bipalium adventitium*. Maternal investment of toxins in young is not uncommon in the animal world. Amphibians commonly have skin toxins, and in many cases endow their eggs with the toxin to protect them (Akizawa et al. 1994; Hanifin et al. 2003), which is sometimes also carried into life stages beyond the egg (Gall et al. 2011b). This type of investment has been well documented in vertebrate species that have TTX such as puffer fish (Kao 1966; Fuhrman et al. 1969) and the rough-skinned newt, *Taricha granulosa* (Hanifin et al. 2003; Gall et al. 2012a). These newts have highly toxic eggs, which

correlate well with the mother's own toxicity (Hanifin et al. 2003), and TTX in these eggs has been shown to be a very successful means of defense against invertebrate egg predators (Gall et al. 2011a). Among TTX bearing invertebrate species it has been shown that in the blue-ringed octopus eggs, paralarvae, and young octopuses all had TTX (Williams et al. 2011). Further, the marine flatworm *Planocera multitentaculata*, also invest TTX into their eggs, and can lay eggs that are 2-50 times more toxic than the parent (Miyazawa et al. 1987). Our study showed that eggs contained approximately three times the amount of TTX on average (89.73 ng TTX) as the average whole flatworm sample (27.89 ng TTX) for the same species. However, we also show that there is quite a bit of variation in the levels of TTX, and many of those sampled had more TTX total than did the eggs. Each *B. adventitium* egg capsule may contain between one and six flatworms, with an average of approximately three flatworms per capsule (Ducey et al. 2005). Egg capsules sit unguarded in the soil for approximately three weeks prior to hatching. Therefore, a defensive toxin would be helpful in warding off opportunistic predation. However, it is as of yet unclear whether or not newly hatched flatworms also have TTX or if the toxin is contained in the egg capsule itself, though it seems likely that the young are also protected.

Bipalium species are of particular interest because they are invasive to North America from various regions of Asia (Hyman 1943, 1954; Winsor 1983; Ogren 1984). Introduced terrestrial flatworms in both North America and Europe have been studied as potential sources of impacts on soil ecosystems (Ogren 1984; Ogren and Kawakatsu 1998; Cannon et al. 1999), and may negatively impact corresponding earthworm

communities as has been shown in Europe (Blackshaw 1990; Boag et al. 1999; Jones et al. 2001). However, there is not a lot known about the ecology of *Bipalium* species, particularly in terms of their relationships with predators and prey. Understanding how they interact with other organisms may aid in conservation efforts. Further, identifying TTX bearing organisms and the uses of TTX in those species will help us to better understand the evolution and production of TTX in such a diverse group of taxa.

LITERATURE CITED

- Akizawa, T., T. Mukai, M. Matsukawa, M. Yoshioka, J. F. Morris, and J. V. P. Butler. 1994. Structures of novel bufadienolides in the eggs of a toad, *Bufo marinus*. Chem. and Pharm. Bull. 42:754-756.
- Blackshaw, R. P. 1990. Studies on *Artioposthia triangulata* (Dendy) (Tricladida: Terricola), a predator of earthworms. Ann. Appl. Biol. 116:169-176.
- Boag, B., H. D. Jones, R. Neilson, and G. Santos. 1999. Spatial distribution and relationship between the New Zealand flatworm *Arthurdendyus triangulatus* and earthworms in a grass field in Scotland. Pedobiologia 43:340-344.
- Brodie, E. D. III and E. D. Brodie, Jr. 1990. Tetrodotoxin resistance in garter snakes: an evolutionary response of predators to dangerous prey. Evol. 44:651-659.
- 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 in island populations. Evol. 45:221-224.

- Brodie, E. D., Jr. 1968. Investigations on the skin toxin of the adult rough-skinned newt, *Taricha granulosa*. *Copeia* 1968:307-313.
- Cannon, R. J. C., R. H. A. Baker, M. Taylor, and J. P. Moore. 1999. A review of the pest status of the New Zealand flatworm in the UK. *Ann. Appl. Biol.* 135:597-614.
- Cardall, B. L., E.D. Brodie, Jr., E. D. Brodie III, and C. T. Hanifin. 2004. Secretion and regeneration of tetrodotoxin in the rough-skin newt (*Taricha granulosa*). *Toxicon* 44:933-938.
- Chau, R., J. A. Kalaizis, and B. A. Neilan. 2011. On the origins and biosynthesis of tetrodotoxin. *Aquat. Toxicol.* 104:61-72.
- Dindal, D. L. 1970. Feeding behavior of a terrestrial turbellarian *Bipalium adventitium*. *Am. Midl. Nat.* 83:635-637.
- Ducey, P. K., M. Messere, K. LaPoint, and S. Noce. 1999. Lumbricid prey and potential herpetofaunal predators of the invading terrestrial flatworm *Bipalium adventitium* (Turbellaria: Tricladida: Terricola). *Am. Midl. Nat.* 141:305-314.
- Ducey, P. K., L.-J. West, G. Shaw, and J. D. Lisle. 2005. Reproductive ecology and evolution in the invasive terrestrial planarian *Bipalium adventitium* across North America. *Pedobiologia* 49:367-377.
- Eisner, T. and J. Meinwald. 1995. *Chemical Ecology: The Chemistry of Biotic Interaction*. National Academy Press, Washington, D.C.
- Fernandes, M. C., E. P. Alvares, P. Gama, and M. Silveira. 2001. The sensory border of the land planarian *Bipalium kewense* (Tricladida, Terricola). *Belg. J. Zool.* 131:173-178.

- Fiore, C., J. L. Tull, S. Zehner, and P. K. Ducey. 2004. Tracking and predation on earthworms by the invasive terrestrial planarian *Bipalium adventitium* (Tricladida, Platyhelminthes). *Behav. Process.* 67:327-334.
- Fuhrman, F.A., G. J. Fuhrman, D. L. Dull, and H. S. Mosher. 1969. Toxins from eggs of fishes and Amphibia. *J. Agr. Food Chem.* 17:417-424.
- Gall, B. G., E.D. Brodie III, and E.D. Brodie, Jr. 2011a. Survival and growth of the caddisfly *Limnephilus flavastellus* after predation on toxic eggs of the rough-skinned newt (*Taricha granulosa*). *Can. J. of Zool.* 89:483-489.
- Gall, B. G., A. N. Stokes, S. S. French, E. D. Brodie III, and E.D. Brodie, Jr. 2012. Female newts (*Taricha granulosa*) produce tetrodotoxin laden eggs after long term captivity. *Toxicon* 60:1057-1062.
- Gall, B. G., A. N. Stokes, S. S. French, E. A. Schlepffhorst, E. D. Brodie III, and E.D. Brodie, Jr. 2011b. Tetrodotoxin levels in larval and metamorphosed newts (*Taricha granulosa*) and palatability to predatory dragonflies. *Toxicon* 57:978-983.
- Hanifin, C. T., E.D. Brodie, Jr., and E. D. Brodie III. 2008. Phenotypic mismatches reveal escape from arms-race coevolution. *PLOS Biol.* 6:471-482.
- Hanifin, C. T., E. D. Brodie III, and E.D. Brodie, Jr. 2002. Tetrodotoxin levels of the rough-skinned newt, *Taricha granulosa*, increase in long-term captivity. *Toxicon* 40:1149-1153.

- Hanifin, C. T., E. D. Brodie III, and E.D. Brodie, Jr. 2003. Tetrodotoxin levels in the eggs of the rough-skin newt, *Taricha granulosa*, are correlated with female toxicity. *J. Chem. Ecol.* 29:1729-1739.
- Hyman, L. H. 1943. Endemic and exotic land planarians in the United States with a discussion of necessary changes of names in the Rhynchodemidae. *Am. Mus. Novit.* 1241:1-21.
- Hyman, L. H. 1954. Some land planarians of the United States and Europe, with remarks on nomenclature. *Am. Mus. Novit.* 1667:1-21.
- Jones, H. D., G. Santoro, B. Boag, and R. Neilson. 2001. The diversity of earthworms in 200 Scottish fields and the possible effect of New Zealand flatworms (*Arthurdendyus triangulatus*) on earthworm populations. *Ann. Appl. Biol.* 139:75-92.
- Kao, C. Y. 1966. Tetrodotoxin, saxitoxin, and their significance in the study of excitation phenomena. *Pharmacol. Rev.* 18:997-1049.
- Miyazawa, K., J. K. Jeon, T. Noguchi, K. Ito, and K. Hashimoto. 1987. Distribution of tetrodotoxin in the tissues of the flatworm *Planocera multitentaculata* (Platyhelminths). *Toxicon* 25:975-980.
- Miyazawa, K. and T. Noguchi. 2001. Distribution and origin of tetrodotoxin. *Journal of Toxicol. - Toxin Rev.* 20:11-33.
- Narahashi, T., J. W. Moore, and R. N. Poston. 1967. Tetrodotoxin derivatives: chemical structure and blockage of nerve membrane conductance. *Science* 156:976-979.

- Narahashi, T., J. W. Moore, and W. R. Scott. 1964. Tetrodotoxin blockage of sodium conductance increase in lobster giant axons. *J. Gen. Phys.* 47:965-974.
- Ogren, R. E. 1984. Exotic land planarians of the genus *Bipalium* (Platyhelminthes: Turbellaria) from Pennsylvania and the academy of natural sciences, Philadelphia. *Proceed. Pennsylvania Acad. Sci.* 58:193-201.
- Ogren, R. E. 1995. Predation behaviour of land planarians. *Hydrobiologia* 305:105-111.
- Ogren, R. E. and M. Kawakatsu. 1998. American Neartic and Neotropical planarian (Tricladida: Terricola) faunas. *Pedobiologia* 42:441-451.
- Ritson-Williams, R., M. Yotsu-Yamashita, and V. J. Paul. 2005. Ecological functions of tetrodotoxin in a deadly polyclad flatworm. *PNAS* 103:3176-3179.
- Stokes, A. N., D. G. Cook, C. T. Hanifin, E. D. Brodie III, and E. D. Brodie, Jr. 2011. Sex-biased predation on newts of the genus *Taricha* by a novel predator and its relationship with tetrodotoxin toxicity. *Am. Midl. Nat.* 165:389-399.
- Stokes, A. N., B. L. Williams, and S. S. French. 2012. An improved competitive inhibition enzymatic immunoassay method for tetrodotoxin quantification. *Biol. Proceed. Online* 14:3.
- Williams, B. L., C. T. Hanifin, E.D. Brodie, Jr., and R. L. Caldwell. 2011. Ontogeny of tetrodotoxin levels in blue ringed octopuses: maternal investment and apparent independent production in offspring of *Hapalochlaena lunulata*. *J. Chem. Ecol.* 37:10-17.

- Winsor, L. 1983. A revision of the cosmopolitan land planarian *Bipalium kewense* Mosely, 1878 (Turbellaria: Tricladida: Terricola). Zool. J. Linn. Soc.-Lond. 79:61-100.
- Zaborski, E. R. 2002. Observations on feeding behavior by the terrestrial flatworm *Bipalium adventitium* (Platyhelminthes: Tricladida: Terricola) from Illinois. Am. Midl. Nat. 148:401-408.
- Zimmer, R. K., D. W. Schar, R. P. Ferrer, P. J. Krug, L. B. Kats, and W. C. Michel. 2006. The scent of danger: Tetrodotoxin (TTX) as an olfactory cue of predation risk. Ecol. Monogr. 76:585-600.

CHAPTER 6

SUMMARY

The goal of my research was to increase our knowledge of the chemical ecology of organisms that have Tetrodotoxin (TTX). Although this toxin has been studied for a long time, the lack of knowledge regarding the evolution, mode of production, and biochemical pathway of TTX leaves many questions to be answered. Therefore, it is important that we continue to understand not only the toxin itself, but also the organisms that have TTX, and their interactions with other organisms.

In order to begin answering some of these questions (Chapter 2), I developed a method of quantifying TTX. Though there are many means by which to quantify this toxin (Noguchi and Mahmud 2001), most were not readily available to me, are expensive, and not feasible within the time limitations of a PhD. Other methods such as the mouse bioassay, are difficult to get approval from the Institutional Animal Care and Use Committee (IACUC), and are not feasible on a large scale. In more recent years, several labs have published Enzymatic Immunoassay (EIA) methodology for quantifying TTX (Raybould et al. 1992; Lehman 2007; Tao et al. 2010). The benefits of using such a technique are many, as EIA procedures are much faster, cheaper, and require less specialized equipment than most other methods utilized to quantify TTX. However, none of these published methods were repeatable in my own lab. Therefore, I worked to modify and refine previously published methods to get a Competitive Inhibition Enzymatic Immunoassay (CIEIA) procedure working. Some of the modifications that I made included using TTX in citrate buffer rather than pure TTX, which did not work for

making the conjugate. Tetrodotoxin is soluble at a pH of around 5, but the procedure requires that the conjugate be prepared at a pH of about 7.4. Using TTX in citrate buffer did not alter the efficacy of the conjugate. Further, I found that each batch of antibodies needs to be optimized, and that using lower concentrations of both sets of antibodies decreased the amount of nonspecific binding. Finally, I reported for the first time, that there are temperature requirements for the incubation steps. If the lab is much below 25 °C, binding will be affected negatively. With this methodology optimized and working I was able to move forward with my research.

Production. One of the major questions regarding TTX research is whether TTX is produced endogenously or exogenously in organisms. Tetrodotoxin is found in a wide array of taxa that are not closely related to one another (Miyazawa and Noguchi 2001). Therefore, it seems difficult to understand how the organism itself might produce TTX, lending support to the idea that TTX is of exogenous origin in most systems. Sequestration has been documented in several taxa (Williams et al. 2004; Gall et al. 2012), and there is evidence that some species like puffer fishes only have TTX when exposed to TTX producing prey (Noguchi et al. 2006a, b). Many of the hypotheses regarding this question suggest that perhaps TTX producing bacterial species are responsible (Chau et al. 2011). However, in the lab these species of bacteria do not produce concentrations of TTX necessary to explain the levels found in species such as puffer fish or newts (Noguchi and Arakawa 2008). Bioaccumulation, where TTX is acquired up the food chain, then seems likely (Chau et al. 2011). However, to our knowledge many species (in particular terrestrial species like *Taricha granulosa*, *T.*

torosa, *Bipalium adventitium* (Chapter 5), *B. kewense*, etc.) do not eat TTX bearing prey. Further, there is no evidence currently that *Taricha granulosa* has any TTX producing bacteria (Lehman et al. 2004). Additionally, TTX stores that have been depleted have been found to be replenished in lab reared *T. granulosa* (Cardall et al. 2004).

Lab studies have been a good way of further understanding this question, as they can be long-term and controlled. For example, Hanifin et al. (2002) found that both male and female newts increased in TTX levels over a one-year period in the lab. *Atelopus oxyrhyncus* have been shown to retain high levels of TTX for more than three years in the lab (Yotsu-Yamashita et al. 1992). I wanted to pursue these questions further by measuring the levels of TTX found in *Taricha granulosa* eggs after a long duration in captivity (Chapter 3). Eggs were measured upon capture, and again after the first, second, and third years in captivity. I found that after the first year in the lab, TTX levels did decline in eggs, though egg size did not differ. However, egg TTX levels remained constant beyond that point, only differing with the initial levels found at capture. I found that the levels of TTX endowed in the eggs of *T. granulosa* are not different from the amount of TTX found to be replenished in other studies of newts. This suggests that female newts may actually produce or sequester TTX rather than just moving TTX from the skin to the eggs. I believe that the decrease in TTX levels after the first year is most likely due to the abbreviated breeding cycle experienced in the lab. These data further support the hypothesis that TTX is produced endogenously in *T. granulosa*.

Predation. Because so many questions remain regarding TTX, it is increasingly important to understand the broad interactions between TTX bearing taxa and both the

biotic and abiotic environments that they encounter. Tetrodotoxin is most often viewed as a defensive toxin; protecting prey from predators (Williams 2010). *Taricha granulosa* have been well documented as utilizing TTX defensively. Potential predators have been shown to succumb to the effects of TTX, except for Thamnophine snakes, or garter snakes (Brodie 1968). Garter snakes have evolved resistance to TTX by genetic changes to the shape of the pore region of their voltage-gated sodium channels (Geffeney et al. 2002, 2005; Feldman et al. 2009). Both TTX levels and resistance, however, are highly variable on a geographic scale, leaving the door open for natural selection to act upon both traits (Hanifin et al. 2008). However, there are a few documented cases of predation on newts by other species. Some species have behaviors that allow them to utilize new food sources. For example, in Annadel State Park in Santa Rosa, CA a novel unidentified predator eviscerated *T. torosa* and *T. granulosa* newts (Gall et al. 2011). Presumably, this was to avoid the toxic skin and eat the nearly non-toxic internal organs. Fellers et al. (2008) documented a great blue heron feeding on newts in northern California. For my study (Chapter 4), otters were observed eating *T. granulosa* newts in high elevation lakes in Oregon. This is likely to be possible if the otters are resistant to TTX in some way, or if the newts have little TTX. I found the latter to be the case. Further, I was able to show that newts in high elevation lakes in Oregon generally have lower levels of TTX than at lower elevations. These data provide insight into interactions that are more complicated than previously thought. There are sources other than snakes of predation and selection on these populations. However, other factors yet to be identified may be in play.

Novel Taxa. Identifying novel taxa with TTX may help us further understand the previous two topics of production and predation. Tetrodotoxin has been found in an increasingly large number of species ranging from bacteria to newts and frogs (Miyazawa and Noguchi 2001). Though these species range from marine environments to terrestrial environments, there have not been any terrestrial invertebrates identified with TTX. However, I identified two species of terrestrial flatworm that have TTX: *Bipalium adventitium* and *B. kewense*. Both species are invasive to North America from regions throughout Asia (Hyman 1943, 1954; Winsor 1983; Ogren 1984), and both are prolific hunters of earthworms (Winsor 1983; Ducey et al. 1999; Fiore et al. 2004). Previous studies documented that earthworms often stopped moving shortly after attack by these flatworms (Dindal 1970; Ducey et al. 1999; Zaborski 2002). This was usually attributed to some sort of venom that the flatworms possessed, but was never identified. Beyond identifying TTX in these two species, I wanted to understand the possible ecological functions of TTX. I wanted to know if TTX is used defensively against predators, during predation like a venom, or possibly both. I found that both species have TTX distributed throughout their bodies. However, the amount of TTX/mg of body tissue is much greater in the head than anywhere else. These two things suggest that TTX may be used both during predation and defensively in these species. These flatworms can provide us with a range of information regarding TTX. Flatworms have the potential to provide information about possible routes of bioaccumulation in terrestrial vertebrate species, and provide further information about complex interactions between predators and prey.

Both within vertebrate and invertebrate systems, there are an array of questions remaining that would help further our understanding of TTX and the organisms that have the toxin. Specifically, there are many populations of newts in California that have yet to be studied intensively. There are also many questions regarding predation and possible sequestration that could span both vertebrate and invertebrate systems. And, finally, there are many questions regarding the gene sequence of these TTX bearing species.

Newts in California. Most of the research regarding *Taricha* species and TTX has been done on *T. granulosa* in Oregon. Not as much is known about *T. torosa*, *T. sierrae* or *T. rivularis*. Though, generally these species have been found to have relatively low levels of TTX, there is not much known about their interactions with other species, nor is there much known about any geographic patterns in TTX levels between populations. I hope to better understand these three species in terms of TTX, which may help us better understand the differences in levels found among the three.

Predation on flatworms. We have yet to clearly identify possible predators on *Bipalium* species. I would like to test whether TTX bearing newt species are more willing than non-TTX bearing salamander species to eat *Bipalium*. Further, I would like to test whether or not newts that eat *Bipalium* acquire increased levels of TTX. Though *Bipalium* are clearly not the source of TTX in newts, it will be interesting to test whether newts can sequester the toxin from a dietary source.

Voltage-gated sodium channel genes. The mechanism of resistance to TTX in garter snakes has been identified as amino acid changes to the gene for the pore region of voltage-gated sodium channels in nerve and muscle tissue (Geffeney et al. 2002;

Geffeney et al. 2005). These data have been supported with similar findings in TTX bearing newts as well as closely related non-bearing species (Hanifin, personal communication). However, not much work has been done in this area for invertebrate species. I would like to pursue this question by sequencing these genes in *Bipalium* as well as other species of invertebrates. Caddisflies, for example, have been shown to eat *T. granulosa* eggs but the mechanism of resistance is unknown. And, it is unknown if any potential changes present in the gene are found in all caddisflies or just in those sympatric with newts.

LITERATURE CITED

- Brodie, E. D., Jr. 1968. Investigations on the skin toxin of the adult rough-skinned newt, *Taricha granulosa*. *Copeia* 1968:307-313.
- Cardall, B. L., J. Edmund D. Brodie, E. D. B. III, and C. T. Hanifin. 2004. Secretion and regeneration of tetrodotoxin in the rough-skin newt (*Taricha granulosa*). *Toxicon* 44:933-938.
- Chau, R., J. A. Kalaizis, and B. A. Neilan. 2011. On the origins and biosynthesis of tetrodotoxin. *Aquat. Toxicol.* 104:61-72.
- Dindal, D. L. 1970. Feeding behavior of a terrestrial turbellarian *Bipalium adventitium*. *Am. Midl. Nat.* 83:635-637.
- Ducey, P. K., M. Messere, K. LaPoint, and S. Noce. 1999. Lumbricid prey and potential herpetofaunal predators of the invading terrestrial flatworm *Bipalium adventitium* (Turbellaria: Tricladida: Terricola). *Am. Midl. Nat.* 141:305-314.

- Feldman, C. R., J. Edmund D. Brodie, E. D. B. III, and M. E. Pfrender. 2009. The evolutionary origins of beneficial alleles during the repeated adaptation of garter snakes to deadly prey. *PNAS* 106:13415-13420.
- Fellers, G. M., J. Reynolds-Taylor, and S. Farrar. 2008. *Taricha granulosa* predation. *Herpetol. Rev.* 38:317-318.
- Fiore, C., J. L. Tull, S. Zehner, and P. K. Ducey. 2004. Tracking and predation on earthworms by the invasive terrestrial planarian *Bipalium adventitium* (Tricladida, Platyhelminthes). *Behav. Process.* 67:327-334.
- Gall, B. G., A. N. Stokes, S. S. French, E. D. Brodie III, and E.D. Brodie, Jr. 2012. Predatory caddisfly larvae sequester tetrodotoxin from their prey, eggs of the rough-skinned newt (*Taricha granulosa*). *J. Chem. Ecol.* 38:1351-1357.
- Gall, B. G., A. N. Stokes, S. S. French, E. A. Schlepfforst, E. D. Brodie III, and E.D. Brodie, Jr. 2011. Tetrodotoxin levels in larval and metamorphosed newts (*Taricha granulosa*) and palatability to predatory dragonflies. *Toxicon* 57:978-983.
- 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.
- Geffeney, S. L., E. Fujimoto, E. D. Brodie III, E.D. Brodie, Jr., and P. C. Ruben. 2005. Evolutionary diversification of TTX-resistant sodium channels in a predator-prey interaction. *Nature* 434:759-763.
- Hanifin, C. T., E.D. Brodie, Jr., and E.D. Brodie III. 2008. Phenotypic mismatches reveal escape from arms-race coevolution. *PLOS Biol.* 6:471-482.

- Hanifin, C. T., E.D. Brodie III, and E. D. Brodie, Jr. 2002. Tetrodotoxin levels of the rough-skin newt, *Taricha granulosa*, increase in long-term captivity. *Toxicon* 40:1149-1153.
- Hyman, L. H. 1943. Endemic and exotic land planarians in the United States with a discussion of necessary changes of names in the Rhynchodemidae. *Am. Mus. Novit.* 1241:1-21.
- Hyman, L. H. 1954. Some land planarians of the United States and Europe, with remarks on nomenclature. *Am. Mus. Novit.* 1667:1-21.
- Lehman, E. M. 2007. A simplified and inexpensive method for extraction and quantification of tetrodotoxin from tissue samples. *Herpetol. Rev.* 38:298-301.
- Lehman, E. M., E. D. Brodie, Jr., and E. D. Brodie III. 2004. No evidence for an endosymbiotic bacterial origin of tetrodotoxin in the newt *Taricha granulosa*. *Toxicon* 44:243-249.
- Miyazawa, K. and T. Noguchi. 2001. Distribution and origin of tetrodotoxin. *J. Toxicol. - Toxin Rev.* 20:11-33.
- Noguchi, T. and O. Arakawa. 2008. Tetrodotoxin- distribution and accumulation in aquatic organisms, and cases of Human intoxication. *Mar. Drugs* 6:220-242.
- Noguchi, T., O. Arakawa, and T. Takatani. 2006a. Toxicity of pufferfish *Takifugu rubripes* cultured in netcages at sea or aquaria on land. *Comp. Biochem. Phys. D* 1:153-157.
- Noguchi, T., O. Arakawa, and T. Takatani. 2006b. TTX accumulation in pufferfish. *Comp. Biochem. Phys. D* 1:145-152.

- Noguchi, T. and Y. Mahmud. 2001. Current methodologies for detection of tetrodotoxin. *J. Toxicol. - Toxin Rev.* 20:35-50.
- Ogren, R. E. 1984. Exotic land planarians of the genus *Bipalium* (Platyhelminthes: Turbellaria) from Pennsylvania and the academy of natural sciences, Philadelphia. *Proc. Pennsylvania Acad. Sci.* 58:193-201.
- Raybould, T. J. G., G. S. Bignami, L. K. Inouye, S. B. Simpson, J. B. Byrnes, P. G. Grothaus, and D. C. Vann. 1992. A monoclonal antibody-based immunoassay for detecting tetrodotoxin in biological samples. *J. Clin. Lab. Anal.* 6:65-72.
- Tao, J., W. J. Wei, L. Nan, L. H. Lei, H. C. Hui, G. X. Fen, L. Y. Jun, Z. Jing, and J. Rong. 2010. Development of competitive indirect ELISA for the detection of tetrodotoxin and a survey of the distribution of tetrodotoxin in the tissues of wild puffer fish in the waters of southeast China. *Food Addit. Contam.* 27:1589-1597.
- Williams, B. L. 2010. Behavioral and chemical ecology of marine organisms with respect to tetrodotoxin. *Mar. Drugs* 8:381-398.
- Williams, B. L., E. D. Brodie, Jr., and E. D. Brodie III. 2004. A resistant predator and its toxic prey: persistence of newt toxin leads to poisonous (not venomous) snakes. *J. Chem. Ecol.* 30:1901-1919.
- Winsor, L. 1983. A revision of the cosmopolitan land planarian *Bipalium kewense* Mosely, 1878 (Turbellaria: Tricladida: Terricola). *Zool. J. Linn. Soc.-Lond.* 79:61-100.

Yotsu-Yamashita, M., D. Mebs, and T. Yasumoto. 1992. Tetrodotoxin and its analogues in extracts from the toad *Atelopus oxyrhynchus* (family: Bufonidae). *Toxicon* 30:1489-1492.

Zaborski, E. R. 2002. Observations on feeding behavior by the terrestrial flatworm *Bipalium adventitium* (Platyhelminthes: Tricladida: Terricola) from Illinois. *Am. Midl. Nat.* 148:401-408.

APPENDIX

June 20, 2013

Ms. Amber N. Stokes
Department of Biology
Utah State University
Logan, Utah 84322-5305

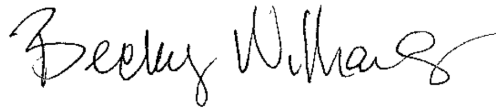
Dear Amber,

You have my permission to use the following manuscript in your dissertation:

Stokes, A. N., B. L. Williams, and S. S. French. 2012. An improved competitive inhibition enzymatic immunoassay method for tetrodotoxin quantification. *Biological Procedures Online* 14.

In my opinion your contribution to these projects constituted clear independent and creative effort in design, implementation, and publication.

Sincerely,

A handwritten signature in black ink that reads "Becky Williams". The signature is written in a cursive, flowing style.

Becky L. Williams
Assistant Professor
USU Uintah Basin

June 20, 2013

Ms. Amber N. Stokes
Department of Biology
Utah State University
Logan, Utah 84322-5305

Dear Amber,

You have my permission to use the following manuscript in your dissertation:

Gall, B. G., A. N. Stokes, S. S. French, E. D. B. III, and J. Edmund D. Brodie. 2012.
Female newts (*Taricha granulosa*) produce tetrodotoxin laden eggs after long
term captivity. *Toxicon* 60:1057-1062.

In my opinion your contribution to these projects constituted clear independent and
creative effort in design, implementation, and publication.

Sincerely,

A handwritten signature in black ink, appearing to read "B. G. Gall". The signature is fluid and cursive, with a large, stylized initial "B" and a long, sweeping underline that extends across the width of the signature.

Brian G. Gall
Assistant Professor of Biology
Hanover College

June 20, 2013

Ms. Amber N. Stokes
Department of Biology
Utah State University
Logan, Utah 84322-5305

Dear Amber,

You have my permission to use the following manuscript in your dissertation:

Gall, B. G., A. N. Stokes, S. S. French, E. D. B. III, and J. Edmund D. Brodie. 2012.
Female newts (*Taricha granulosa*) produce tetrodotoxin laden eggs after long
term captivity. *Toxicon* 60:1057-1062.

In my opinion your contribution to these projects constituted clear independent and
creative effort in design, implementation, and publication.

Sincerely,

A handwritten signature in black ink, appearing to read 'E. D. Brodie III', with a stylized, overlapping flourish.

Edmund D. Brodie III
Director, Mountain Lake Biological Station
Professor, Department of Biology
University of Virginia

**SPRINGER LICENSE
TERMS AND CONDITIONS**

May 25, 2013

This is a License Agreement between Amber N Stokes ("You") and Springer ("Springer") provided by Copyright Clearance Center ("CCC"). The license consists of your order details, the terms and conditions provided by Springer, and the payment terms and conditions.

All payments must be made in full to CCC. For payment instructions, please see information listed at the bottom of this form.

License Number	3156040043835
License date	May 25, 2013
Licensed content publisher	Springer
Licensed content publication	Biological Procedures Online
Licensed content title	An improved competitive inhibition enzymatic immunoassay method for tetrodotoxin quantification
Licensed content author	Amber N Stokes
Licensed content date	Jan 1, 2012
Volume number	14
Issue number	1
Type of Use	Thesis/Dissertation
Portion	Full text
Number of copies	3
Author of this Springer article	Yes and you are the sole author of the new work
Order reference number	
Title of your thesis / dissertation	PRESENCE AND FUNCTION OF TETRODOTOXIN IN TERRESTRIAL VERTEBRATES AND INVERTEBRATES
Expected completion date	Aug 2013
Estimated size(pages)	130
Total	0.00 USD

ELSEVIER LICENSE TERMS AND CONDITIONS

May 24, 2013

This is a License Agreement between Amber N Stokes ("You") and Elsevier ("Elsevier") provided by Copyright Clearance Center ("CCC"). The license consists of your order details, the terms and conditions provided by Elsevier, and the payment terms and conditions.

All payments must be made in full to CCC. For payment instructions, please see information listed at the bottom of this form.

Supplier	Elsevier Limited The Boulevard, Langford Lane Kidlington, Oxford, OX5 1GB, UK
Registered Company Number	1982084
Customer name	Amber N Stokes
Customer address	Utah State University LOGAN, UT 84322
License number	3155550386389
License date	May 24, 2013
Licensed content publisher	Elsevier
Licensed content publication	Toxicon
Licensed content title	Female newts (<i>Taricha granulosa</i>) produce tetrodotoxin laden eggs after long term captivity
Licensed content author	Brian G. Gall, Amber N. Stokes, Susannah S. French, Edmund D. Brodie, Edmund D. Brodie
Licensed content date	November 2012
Licensed content volume number	60
Licensed content issue number	6
Number of pages	6
Start Page	1057
End Page	1062
Type of Use	reuse in a thesis/dissertation
Portion	full article
Format	both print and electronic
Are you the author of this Elsevier article?	Yes
Will you be translating?	No
Order reference number	None
Title of your thesis/dissertation	PRESENCE AND FUNCTION OF TETRODOTOXIN IN TERRESTRIAL VERTEBRATES AND INVERTEBRATES
Expected completion date	Aug 2013
Estimated size (number of pages)	130

CURRICULUM VITAE

Amber Noelle Stokes

Professional Address

Department of Biology
Utah State University
5305 Old Main Hill
Logan, UT 84322-5305

Home Address

115 N 400 W
Smithfield, UT 84335

E-mail: amber.stokes@usu.edu
amnoelle@gmail.com

Education

July 2008-present	PhD candidate	Biology, Utah State University. Advisor: Edmund D. Brodie, Jr. Dissertation: Presence and Function of Tetrodotoxin in Terrestrial Vertebrates and Invertebrates
Expected graduation date:		Summer 2013
July 2008	M.S.	Biology, Utah State University. Advisor: Edmund D. Brodie, Jr. GPA: 3.82 Thesis: Brouillette, A.N. July, 2008. Sex-Biased Predation on <i>Taricha</i> by a Novel Predator in Annadel State Park. M.S. Thesis. Department of Biology, Utah State University, Logan, UT. 46 pp.
June 2004	B.S.	Biology (Premed); Chemistry minor. California State University, Bakersfield GPA: 3.26

Professional Appointments

2006-Present	Research/Teaching Assistant, Department of Biology, Utah State University
2004	Teaching Assistant, Department of Biology, California State University, Bakersfield

Peer Reviewed Publications

Neuman-Lee, L.A., **A.N. Stokes**, S.S. French, L. Lucas, B.G. Gall, G. Hopkins, N.M. Kiriazis, and E.D. Brodie, Jr. *In Review*. Antipredator behavior correlates with corticosterone, but not toxicity, in the Rough-skinned Newt (*Taricha granulosa*). *Physiology and Behavior*.

Brossman, K.H., B.E. Carlson, **A.N. Stokes**, T. Langkilde. *In Review*. Eastern Newt (*Notophthalmus viridescens*) larvae alter morphological but not chemical defenses in response to predator cues. *Chemoecology*.

Stokes, A.N., A.M. Ray, M.W. Buktenica, B.G. Gall, E. Paulson, D. Paulson, S.S. French, E.D. Brodie III, and E.D. Brodie, Jr. *In Review*. Tetrodotoxin levels in high elevation populations of *Taricha granulosa* in Oregon and predation by otters. *Journal of Chemical Ecology*.

- Ferry, E.E., G.R. Hopkins, **A.N. Stokes**, S. Mohammadi, E.D. Brodie, Jr., and B.G. Gall. 2013. Do all portable cases constructed by caddisfly larvae function in defense? *Journal of Insect Science*. 13:5.
- Gall, B.G., **A.N. Stokes**, S.S. French, E.D. Brodie III., and E.D. Brodie, Jr. 2012. Predatory caddisfly larvae sequester tetrodotoxin from their prey, eggs of the rough-skinned newt (*Taricha granulosa*). *Journal of Chemical Ecology*. 38:1351-1357.
- Gall, B.G., **A.N. Stokes**, S.S. French, E.D. Brodie III, and E.D. Brodie, Jr. 2012. Female newts (*Taricha granulosa*) produce tetrodotoxin laden eggs after long term captivity. *Toxicon* 60: 1057-1062.
- Stokes, A.N.**, B.L. Williams, S.S. French. 2012. An improved competitive inhibition enzymatic immunoassay method for tetrodotoxin quantification. *Biological Procedures Online*. 14:3.
- Gall, B.G., **A.N. Stokes**, S.S. French, E.A. Schleppehorst, E.D. Brodie III, and E.D. Brodie, Jr. 2011. Tetrodotoxin levels in larval and metamorphosed newts (*Taricha granulosa*) and palatability to predatory dragonflies. *Toxicon* 57(2011): 978-983.
- Stokes, A.N.**, D.G. Cook, C.T. Hanifin, E.D. Brodie III, and E.D. Brodie, Jr. 2011. Sex-biased predation on newts of the genus *Taricha* by a novel predator and its relationship with tetrodotoxin toxicity. *The American Midland Naturalist* 165(2): 389-399.

Publications in Prep

- Stokes, A.N.**, P.K. Ducey, S.S. French, ED Brodie III, and ED Brodie, Jr. Tetrodotoxin in terrestrial flatworm species, *Bipalium adventitium* and *Bipalium kewense*.

Invited Presentations

- Stokes, A.N.** Snakes, Newts, and Worms...Oh My! Interactions between deadly animals and the animals that love to eat them. Fourth Annual Faraday's Holiday Event. Starhouse Discovery Center, Logan, UT. December 27, 2012. **Keynote Speaker.**

Professional Presentations

***Indicates presenter**

- Stokes, A.N.***, A.M. Ray, M.W. Buktenica, B.G. Gall, E. Paulson, D. Paulson, S.S. French, E.D. Brodie III, and E.D. Brodie, Jr. Tetrodotoxin levels in high elevation populations of *Taricha granulosa* in Oregon and predation by otters. World Congress of Herpetology. Vancouver, British Columbia, Canada. August 9, 2012. **Oral Presentation.**
- Brossman, K.H.* , B.E. Carlson, **A.N. Stokes**, T. Langkilde. Predator-induced chemical and morphological defenses in eastern newt (*Notophthalmus viridescens*) larvae. 97th ESA Annual Meeting. August 2012. **Oral Presentation.**
- Stokes, A.N.***, B.G. Gall, S.S. French, E. Schleppehorst, E.D. Brodie III, and E.D. Brodie, Jr. Tetrodotoxin levels in larval and metamorphosed newts (*Taricha granulosa*) and palatability to predatory dragonflies. Society for the Study of Evolution meetings. Norman, OK. June 18, 2011. **Oral Presentation.**

Stokes, A.N., D. Cook*, C. Hanifin, E.D. Brodie III, and E.D. Brodie, Jr. Sex-biased predation on newts of the genus *Taricha* by a novel predator and its relationship with tetrodotoxin toxicity. California/Nevada Amphibian Population Task Force 2011 Meeting. Yosemite National Park, CA. January 6-7, 2011. **Oral Presentation.**

Stokes, A.N.*, Sex biased predation on *Taricha* in Annadel State Park. Joint Meeting of Ichthyologists and Herpetologists. Portland, OR. July 25, 2009. **Poster.**

Other Research Experience

2004-2005 Dr. Szick-Miranda. Analysis of insertion mutations of *Arabidopsis Thaliana*

Teaching

Fall 2012 Animal Physiology (lecture) instructor (1 section), Utah State University
 Fall 2011 Animal Physiology lab instructor (2 sections), Utah State University
 Spring 2011 Human Physiology lab (3 sections), Utah State University
 Fall 2010 Animal Physiology lab (1 section), Utah State University
 Spring 2010 Animal Physiology lab (1 section), Utah State University
 Fall 2009 Animal Physiology lab instructor (1 section), Utah State University
 Spring 2009 Animal Physiology lab (1 section), Utah State University
 Fall 2008 Animal Physiology lab (1 section), Utah State University
 Spring 2008 Animal Physiology lab (1 section), Utah State University
 Fall 2007 Human Physiology lab (4 sections), Utah State University
 Spring 2007 Biology II lab (2 sections), Utah State University
 Fall 2006 Biology I lab (2 sections), Utah State University
 Fall 2004 Microbiology Lab (3 sections), California State University, Bakersfield.

Grants and Scholarships

2012 Research and Graduate Studies Graduate Student Travel Award, \$400
 2012 Claude E. Zobel Scholarship, Utah State University, \$1000
 2012 Grant-In-Aid of Research, Sigma Xi - \$600
 2012 Dr. Datus M. Hammond Memorial Scholarship, Utah State University - \$500
 2012 Open Access Funding Grant, Utah State University - \$1548
 2009 Diversity Scholarship, Utah State University - \$1000
 2009 Center for Women and Gender Graduate Student Travel Grant, Utah State University - \$500
 2009 Graduate Student Senate Travel Grant, Utah State University - \$300

Awards and Special Recognition

Summer 2011 Nominated for the Hamilton Award, 2011 Society for the Study of Evolution meetings
 Spring 2011 Biology Department Teaching Assistant of the Year, Utah State University
 Spring 2011 Nominated Graduate Teaching Assistant of the Year, Utah State University
 Spring 2004 Nominated Senior Biology Student of the Year, California State University, Bakersfield
 Spring 2004 Nominated for Outstanding Biology Research Paper, California State University, Bakersfield. Title: Transfer of resistance to penicillin between *Streptococcus* species.

Continuing Education

2012 Grant Writers' Seminars and Workshops

University Service

2011-2012 Biology Department Graduate Program Review Committee
 2010-2013 Biology Department Seminar Committee
 Biology Department Curriculum Committee

Leadership Experience

2011-2012 Allies on Campus Steering Committee member. Utah State University
 2010-2011 Biology Graduate Student Association Activities Coordinator. Utah State University
 Allies on Campus Steering Committee member. Utah State University
 2009-2010 Biology Graduate Student Association, president. Utah State University
 Allies on Campus Seminar Coordinator. Utah State University
 Allies on Campus Steering Committee member. Utah State University
 2007-2009 Biology Graduate Student Association, treasurer. Utah State University

Manuscript Review

Marine Drugs
 Journal of Herpetology
 World Journal of Microbiology and Biotechnology

Professional Society Memberships

Society for the Study of Amphibians and Reptiles (SSAR)
 Society for the Study of Evolution (SSE)
 Sigma Xi
 American Association for the Advancement of Science (AAAS)
 International Society of Chemical Ecology

Other Professional Experience

- Air Quality Inspector, Compliance (11/2005-8/2006). San Joaquin Valley Air Pollution Control District, Bakersfield, CA. Inspected facilities to make sure that they were in compliance with District, State, and/or Federal regulations; Reviewed and issued Dust Control Plans (DCP) to facilities under construction and inspected these sites; Taught DCP class to developers; Responded to complaints from the public; Communicated with public regarding rules and regulations.
- Air Quality Specialist, Permit Services (3/2005-11/2005). San Joaquin Valley Air Pollution Control District, Fresno, CA. Reviewed and issued Conservation Management Practices Plans (CMPP) to agricultural operations; Reviewed and issued Permits to Operate (PTO) under District and Title V enforcement for agricultural operations; Reviewed Emissions Inventory Plans for agricultural operations; Communicated with farmers to answer questions, comments, or concerns regarding District/State regulations.

Community Service

2007-present Allies on Campus. Facilitator at Allies seminars. Present material and get attendees involved in discussion.
 2001-2006 Kern County Museum. Taught day-camp students (ages 5-8) at the Lori Brock Children's Discovery Center (children's museum); Participated in fund raising booths to support the museum's education program at various events.
 2004-2005 Kern County Health Department. Performed tests, including:

- Automated DNA tests in order to detect infection of Gonorrhea and/or Chlamydia

- ELISA to detect the presence of HIV, and confirmed positive results with an Indirect Immunofluorescence Assay (IFA).
- Rapid Plasma Reagin (RPR) tests to detect the presence of *Treponema pallidum* in human sera, and confirmed positive results with Fluorescent Treponemal Antibody Absorbed (FTA-ABS) IgG IFA.
- Various tests to detect/confirm/track infections of *Coccidioides immitis* (valley fever) including immunodiffusion, EIA, and *Coccidioides* Complement Fixation.
- IFA procedure to detect the presence of West Nile Virus.