Demographics, Sexual Dimorphism, and Ecological Aspects of Ambystoma annulatum (Ringed Salamander) in Northwest Arkansas, USA

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Demographics, Sexual Dimorphism, and Ecological Aspects of *Ambystoma annulatum* (Ringed Salamander) in Northwest Arkansas, USA

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Biology

by

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December 2023
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This dissertation is approved for recommendation to the Graduate Council.

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ABSTRACT

The need to study and monitor amphibian populations is increasing along with the threats to their population stability and persistence in nature. Northwest Arkansas is one of the fastest growing areas in the nation and with that growth comes rapid changes in land use, massive alterations to habitats, habitat loss, and the introduction of nonnative plants and animals. *Ambystoma annulatum* (Ringed Salamander) is an Ambystomatid endemic to the Ozark and Ouachita Mountains of southern Missouri, northern and western Arkansas, and eastern Oklahoma giving it a relatively small distribution compared to most *Ambystoma*. Therefore, Arkansas constitutes a significant portion of the home range of *A. annulatum*. However, due to the secretive nature of *A. annulatum* little is known about its biology, ecology or population status in the state. Consequently, this has resulted in its designation as a *species of special concern* in the state of Arkansas. In this dissertation, I attempted to gather information on various aspects of *A. annulatum* biology and ecology in Northwest Arkansas. I first studied the demographics of an *Ambystoma annulatum* breeding population in an atypical environment in Washington County, Northwest Arkansas, USA. This was done to understand dynamics of a breeding population of *A. annulatum* in a highly disturbed habitat. Next I studied sexual dimorphism in body size, head dimensions (length, width, and depth), limb length, and tail length in *A. annulatum*. Understanding sexual dimorphism in animals helps to shed light on evolutionary drivers such as sexual selection, or variation in ecological requirements between males and females. Then I surveyed 155 natural and manmade ponds in Washington County for *A. annulatum* presence, along with other herpetofauna species, to get a snapshot of its occurrence. Finally, I isolated a *Naegleria* amoebae from the gut of an adult *A. annulatum* to add to the body of information on amphibian-parasite associations.
ACKNOWLEDGEMENTS

I would like to especially thank my advisor Dr. James M. Walker for his guidance, patience, and encouragement throughout my graduate studies and for presenting me with the opportunity to work on this project. I am also greatly appreciative of my committee members; Dr. JD Willson, Dr. David McNabb, and Dr. Christian Tipsmark for their support and invaluable consultation. I also thank Dr. Jeffery Silberman for his guidance and use of his lab, as well as Scott Bingham for DNA sequencing. A very special thanks to Rosa Ainley, University of Arkansas, for granting me access to her pond which started this entire research project. I would also like to thank the Odglen family, the Davenport family, the Ferguson Family, Johnathan McCain, Kayla Rain, and all of the property owners that have been critically important to this project. Salamanders were collected under the authority Scientific Collecting Permit #032720152 granted to Brian Becker by the Arkansas Fish and Game Commission. The Research Protocols have been approved by the University of Arkansas Animal Welfare Committee (IACUC #15059).
DEDICATION

I would like to dedicate this dissertation to my wife, Heather Becker and to my daughters, Leah Becker and Lillien Becker for their love, support, and many sacrifices along the way.
TABLE OF CONTENTS

Introduction

*Ambystoma annulatum* ........................................................................................................1

Project background..................................................................................................................4

Purpose of Research ................................................................................................................5

Literature Cited .......................................................................................................................6

Appendix ................................................................................................................................10

Chapter 1: Demographics of *Ambystoma annulatum* in an atypical environment in Northwest Arkansas..................................................................................................................21

Abstract .................................................................................................................................21

Introduction .............................................................................................................................22

Materials and Methods ..........................................................................................................24

Results ....................................................................................................................................26

Discussion ...............................................................................................................................28

Literature Cited .......................................................................................................................31

Appendix ................................................................................................................................34

Chapter 2: Sexual dimorphism in head shape and limb length in *Ambystoma*

*annulatum* ............................................................................................................................50

Abstract .................................................................................................................................50

Introduction .............................................................................................................................50

Materials and Methods ..........................................................................................................53

Results .....................................................................................................................................55
Chapter 3: Survey for *Ambystoma annulatum* in farm ponds in Northwest Arkansas

Abstract ........................................................................................................... 80
Introduction ..................................................................................................... 81
Materials and Methods ................................................................................ 83
Results ........................................................................................................... 85
Discussion ..................................................................................................... 86
Literature Cited ............................................................................................. 90
Appendix ...................................................................................................... 95

Chapter 4: Isolation of a *Naegleria* from the gut of *Ambystoma annulatum* in Northwest Arkansas

Abstract ........................................................................................................... 114
Introduction ..................................................................................................... 114
Materials and Methods ................................................................................ 116
Results ........................................................................................................... 117
Discussion ..................................................................................................... 117
Literature Cited ............................................................................................. 118
Appendix ...................................................................................................... 122
Conclusion .................................................................................................... 128
Appendix ...................................................................................................... 131
INTRODUCTION

AMBYSTOMA ANNULATUM

*Ambystoma annulatum* (Ringed Salamander) is a large, heavy bodied salamander belonging to the family Ambystomatidae, the mole salamanders. *A. annulatum* was first described by Cope (1886). They range from dark brown to almost black with complete or incomplete off white to yellow rings on the body and tail (Conant and Collins 1998; Figure 1). *A. annulatum* is endemic to the Ozark Plateau and Ouachita Mountains of the central United States (Trapp 1956; Dowling 1956; Petranka 1998). This geographic area includes portions of southern Missouri, western Arkansas, and eastern Oklahoma (Figure 2). Juvenile and adult *A. annulatum* are both terrestrial and typically inhabit mature oak-hickory forests or mixed hardwood-pine forests that are adjacent to ponds used for their breeding (Semlitsch et al. 2008, 2014; Pittman et al. 2013b). *A. annulatum* is typically an autumn breeding species that uses small fishless ponds for annual reproduction (Trapp 1956; Petranka 1998; Hocking et al. 2008; Semlitsch et al. 2014), though Trauth (2000) has reported winter breeding in the species in the Ozark National Forest of northcentral Arkansas. I also observed *A. annulatum* migration and breeding in a small pond in Washington County, Northwest Arkansas in late December 2018 (unpublished observation).

In mid-September adult *A. annulatum* migration is initiated by the onset of consistent heavy rains in which at least 1.27 cm are required to stimulate emergence from their forest retreats to move to small ponds to breed (Spotila and Beumer 1970). It has been shown that *A. annulatum* uses both natural and manmade ponds for reproduction (Trapp 1956; Brussock and Brown 1982), and that the hydroperiods of these ponds play an important role in both the breeding success of adults and the survival of the larvae to metamorphosis (Semlitsch et al. 2014; Figure 3). Migrations occur at night during, or just after, a rain (Briggler et al 2004).
Observations of adult *A. annulatum* migrations have revealed complex patterns and it has been suggested that the adults move to specific breeding ponds and will even bypass other suitable ponds. It has been shown that *A. maculatum* migrate to specific breeding ponds (Shoop, 1968). Briggler et al. (2004) provided evidence that suggests post-breeding *A. annulatum* do not follow the same patterns or pathways they used to arrive at the pond, and that some may even overwinter near the pond. Adult *A. maculatum* use the same route each time for travel to and from their breeding ponds (Shoop, 1965; Douglas and Monroe 1981; Stenhouse 1985; Phillips and Sexton 1989).

Sexually mature salamanders tend to make more directed movements than juveniles, with the average distance of adults from pond edges being 125 meters and the maximum observed migration distance being 439 meters (Semlitsch 1998; Gamble et al. 2007; Pittman et al. 2014). Males are the first to arrive at breeding ponds and are later joined by females (Briggler et al. 2004). Courtship is initiated when males begin nudging the female’s cloaca. If the female is receptive she will pick up one of several spermatophores deposited by the males on vegetation or other pond substrates (Spotila and Beumer 1970; Figures 4 & 5). Eggs are deposited the same night or the following night, and are usually attached to vegetation if present (Figure 6), or deposited directly on the pond bottom (Figure 7). Females lay an average of 390 eggs in a single clutch (Hutcherson et al. 1989; Peterson et al. 1992). Eggs hatch after a developmental period of 9-16 days (Trapp 1956; Hutcherson et al. 1989), although Johnson (2000) reported 14 to 21 days. Larvae then overwinter in the pond and undergo metamorphosis in late April or early May.

Predators of eggs and larvae include fishes, anuran larvae, invertebrate larvae, newts, and other salamanders (Wilbur et al. 1983; Shulse et al. 2013; Drake et al. 2014; Nyman et al. 1993). Larvae overwinter in their natal pond and they feed primarily on zooplankton and other
invertebrates (Hutcherson et al. 1989; Nyman et al. 1993). The rate of development of larvae and size of metamorphs leaving the pond depend upon a variety of environmental factors including intraspecific competition, habitat quality and predation (Wilbur and Collins 1973; Semlitsch 1987; Semlitsch et al. 1988). Larvae collected from a particular site in Madison County on May 14, 1955 averaged 45 mm in length (Trapp 1956). In Missouri Hutcherson et al. (1989) found the SVL of newly transformed individuals to be between 34 and 40 mm with a mean mass between 0.9 and 1.7 g. In Stone County, Missouri, pre-metamorphic mortality of *A. annulatum* was found to be between 99.84% and 99.90% (Peterson et al. 1991). Larvae that survive the winter and develop through metamorphosis emerge the following spring as juveniles and typically head into forested habitats to resume a fossorial lifestyle. Some of these juveniles may return to their natal pond as early as 5 months of age to breed. Primary predators of juveniles and adults include snakes, anurans, birds, and small mammals (Pittman et al. 2013b). Gamble et al. (2007) showed that juvenile *A. opacum* (a close relative of *A. annulatum*) may disperse to distances of up to 1300 meters away from their natal pond.

The ability of juvenile amphibians to disperse between ponds and terrestrial habitats may be determined by local precipitation patterns during dispersal events, the spatial structure and quality of habitat of suitable ponds, and terrestrial habitat as well as the number of metamorphs leaving the ponds (Peterman et al. 2013b). As a result of these factors, populations that become isolated due to inhospitable habitat, such as the building of a road or other structure, may begin to diverge genetically (Peterman et al. 2013a). Reductions in the genetic variation among more isolated amphibian populations are thought to be a product of smaller population sizes and decreased gene flow (Peterman et al. 2013a). Juvenile *A. annulatum* seek out various microhabitats in their terrestrial environments to live until the breeding season returns. These
microhabitats may include the underneath of rocks, logs, or leaf litter, and in many cases inhabit abandoned burrows (Figure 8). *A. tigrinum* actively dig their own burrows but *A. opacum, A. maculatum*, and *A. annulatum* do not actively dig their own burrows but instead will enlarge crevices or cracks created by other animals (Semlitsch 1983). This behavior may present additional conservational challenges for *A. annulatum* in areas where burrowing animals are extirpated and the soil is compacted due to agricultural activities.

*A. annulatum* has a relatively small distribution when compared to other *Ambystoma* species (Trauth et al. 2004), resulting in it being one of the least studied *Ambystoma* species (Briggler et al. 2004). It is listed as a *Species of Special Concern* in the state of Arkansas where little is known about the current population status. As with many amphibian species, *A. annulatum* faces many threats notably climate change, pollution, invasive species, and habitat destruction. Within the range of *A. annulatum* there are many human activities that are directly affecting their habitat that may include timber harvest, agriculture, urban development, encroachment from roads, and introductions of invasive species and predatory fish.

**PROJECT BACKGROUND**

On April 25, 2013, three adult *A. annulatum* were found living under wood mulch in a flower bed on private property in a ranchette style setting located at 1467 South Pianalto Road (36.169915°N, 94.264046, elev. 395 m), Springdale, Washington County, Arkansas (Figure 9). This area of northwest Arkansas is comprised of large, widely spaced houses, large yards, agricultural projects, and cattle pastures. Google Earth revealed a small pond ~120 meters from the flower bed on the Ainley property (Figure 10). Upon further investigation, many larval *A. annulatum* were found, via dip-netting, within this pond documenting that reproduction of *A. annulatum* has taken place in this highly disturbed habitat (Figure 11).
The finding of these three adults and larvae in this particular pond presented an opportunity to identify, map and examine *A. annulatum* occurrences and study ecological aspects of *A. annulatum* in a highly disturbed and atypical habitat. Another interesting aspect of this study site is the fact that the Golden Shiner (*Notemigonus crysoleucas*), a cyprinid fish, was introduced to the pond in 2014 by the land owner. Predatory fish species have been shown to be veracious predators of *A. annulatum* eggs and larvae (Drake et al. 2014). In addition, Crappies (genus *Pomoxis*), a centrarchid fish, were also released into the pond; although their survival is uncertain. Most of the studies investigating *A. annulatum* have been conducted in Missouri in areas with large tracks of forest. Fewer studies have been conducted in Arkansas but again these were also conducted in landscapes with abundant forest coverage. The purpose of this research was to study ecological aspects of *A. annulatum* in a highly disturbed area of northwestern Arkansas lacking large tracks of forest. The initial focus was on the Ainley Pond with additional work conducted in neighboring ponds and other ponds scattered throughout Washington County, Arkansas, in an attempt to bridge gaps in the current understanding of *A. annulatum* biology and ecology the latter of which could be highly beneficial for conservation and management practices.

**PURPOSE OF RESEARCH**

The purpose of this research had several goals including:

1) Study the demographics of *A. annulatum* at an atypical breeding pond (Ainley pond) in Northwest Arkansas (Chapter 1).

2) Investigate sexual dimorphism in head size and limb length between male and female *Ambystoma annulatum* (Chapter 2).
3) Conduct a widespread survey for the presence/absence of *A. annulatum* in Washington County, Northwest Arkansas (Chapter 3).

4) Isolate and identify potential intestinal amoebae from *A. annulatum* (Chapter 4).

5) Aid in public outreach and education by contacting land owners in the area to gain access to private property for the purpose of conducting surveys. In doing so, I hope to increase public awareness of *A. annulatum* and the important roles farm ponds play in local ecosystems and conservation of herpetofauna (Chapter 3).

6) Document any additional noteworthy observations while researching *Ambystoma annulatum*.

7) Collect tissue samples and establish additional databases for future studies.

**LITERATURE CITED**


APPENDIX

Figures and Tables

Figure 1. Typical adult *Ambystoma annulatum*. Breeding pond in the background. (Photo by Brian Becker).
Figure 2. US map depicting the distribution of *Ambystoma annulatum* in Missouri, Arkansas, and Oklahoma. (Map produced in JMP Pro 17 and edited in Excel)
Figure 3. Typical breeding pond used by *Ambystoma annulatum*. (Photo by Brian Becker)
Figure 4. *Ambystoma annulatum* spermatophores. (Photo by Brian Becker)
Figure 5. *Ambystoma annulatum* spermatophores deposited on a pond bottom. (Photo by Brian Becker).
Figure 6. *Ambystoma annulatum* egg masses (arrow) attached to aquatic vegetation. (Photo by Brian Becker).
Figure 7. *Ambystoma annulatum* egg masses (arrow) deposited on pond bottom. (Photo by Brian Becker).
Figure 8. Juvenile *Ambystoma annulatum* found under rock among leaf litter in a forested area of Washington County. Note the adult pattern is just starting to come in. (Photo by Brian Becker, May 25, 2023).
Figure 9. Adult *Ambystoma annulatum* found in a flower bed on private property in atypical habitat. Washington County, Northwest Arkansas. (Photo by Rosa Ainley)
Figure 10. Google Earth image of the Ainley pond in Springdale, Washington County, Arkansas.

Note the lack of forested habitat and extensive agricultural land use.
Figure 11. *Ambystoma annulatum* larvae found in the Ainley pond via dipnetting. (Photo by Brian Becker)
Chapter 1:

Demographics of *Ambystoma annulatum* in an atypical environment in Northwest Arkansas

ABSTRACT

I report a detailed account of the demographics of a breeding population of *Ambystoma annulatum* (Ringed Salamander) at an atypical breeding pond in Northwest Arkansas, USA. The breeding pond is a farm pond located in open pasture in a widely deforested area predominated by agricultural activities. The pond also had a predatory fish introduction in the months prior to this study. Using a drift fence paired with pit fall traps, I conducted a complete census of the pond starting with the larvae emigration that took place during May of 2015, and the adult migration event that took place during October of the same year. A total of 68 metamorph *A. annulatum* with a mean snout-vent-length (SVL) of 39.26 mm were captured in pitfall traps between May 5th and May 11th of 2015 following heavy rain events. The majority of metamorph individuals (51) emigrated on a single night. Seventy-five percent of the metamorphs left from the shallow side of the pond. Thirty-eight adult *A. annulatum* were captured in pit traps between October 22nd and November 5th, 2015. The largest male had a SVL of 90 mm and a mass of 18.64 g. The smallest male had a SVL of 68 mm and a mass of 7.2 g. The largest female had a SVL of 100 mm and a mass of 16.65 g. The smallest female had a SVL of 81 mm and a mass of 16.3 g. Overall, females were significantly larger than males (t-test, P=0.0037). This small number of breeding adults puts this population at significant risk of local extirpation; particularly if the surrounding environment is not capable of supporting the adult stage and the population experiences low recruitment due to introduced predatory fish.
INTRODUCTION

*Ambystoma annulatum* (Ringed Salamander) is a member of the Mole Salamander family (Ambystomatidae) and is endemic to the Ozark and Ouachita mountains of southern Missouri, western Arkansas, and eastern Oklahoma (Dowling 1956; Trapp 1956; Anderson 1965; Conant and Collins 1998). Like other *Ambystoma* species, *A. annulatum* annually migrate en masse from their terrestrial habitats to small fishless ponds to breed (Trapp 1959; Spotila and Beumer, 1970; Brussock and Brown 1982; Peterson et al. 1991; Briggler et al. 2004). However, unlike other *Ambystoma*, *A. annulatum* is one of two species, the other being *A. opacum*, which migrate in fall as opposed to late winter or early spring (Trapp 1959; Spotila and Beumer 1979; Briggler, 2004; Semlitch et al. 2014). Winter migrations have also been documented for *A. annulatum* (Trapp 1956; Trauth 2000; Becker 2018 unpublished data). In northwest Arkansas, *A. annulatum* generally breeds from mid-September to early November.

*A. annulatum* migration is initiated by the onset of heavy rains of at least 1.27 cm in late September during the night (Spotila and Beumer 1970). Larger males usually arrive at the breeding pond first followed by females and smaller males (Trapp 1959; Briggler et al. 2004; Semlitch et al. 2014). Males will court females and if the female is receptive, she will pick up one of several spermatophores deposited by the male to fertilize her eggs (Hutcherson 1989). Over the next few days, the fertilized eggs will be deposited on the pond bottom, or attached to submerged vegetation. Adult *A. annulatum* will then migrate back to their terrestrial habitats leaving the eggs to hatch after 9-16 days (Trapp 1956; Hutcherson et al. 1989) or 14 to 21 days (Johnson 2000) depending on geographic location. Larvae overwinter in the pond until the following spring when metamorphosis takes place between April and June.
Due to *A. annulatum*’s restricted distribution and fossorial lifestyle, it is one of the least studied *Ambystoma* species. Most studies of population demographics of *A. annulatum* have focused on breeding ponds, because this is where they are most easily detected, that are adjacent to large tracks of forest (Trapp 1959; Spotila and Beumer 1970; Brussock and Brown 1982; Hutcherson et al. 1989; Briggler et al. 2004; Semlitch et al. 2014). Furthermore, both juveniles and adults migrate to or from breeding ponds making them relatively easy to census (Semlitch et al. 1996). The reproductive habits of *A. annulatum*, notably the need for fishless ponds for eggs and larvae, and intact terrestrial habitat for adults, may make *A. annulatum* particularly susceptible to various land use practices, roads, and introduced aquatic predators.

Understanding population structure is essential to understanding population persistence and the various factors that may affect population persistence. To date few studies have described variation in *A. annulatum* breeding populations and juvenile recruitment (Briggler et al. 2004; Semlitch et al. 2014). In April 2013, I found *A. annulatum* larvae in a farm pond (via dipnetting) on private property (Ainley) in a ranchette style setting located at South Pianalto Road (36.169915°N, 94.264046, elev. 395 m), Springdale, Washington County, Arkansas (Figure 1). This area of northwest Arkansas consists mostly of large, widely spaced houses, large yards, agricultural projects, and cattle and horse pastures (Figure 2). Additionally, the Golden Shiner (*Notemigonus crysoleucas*), a common baitfish, was introduced to this pond by the landowners in the months before my detection of the larvae. This finding of an *A. annulatum* breeding pond presented an opportunity to study the demographics and population structure of *A. annulatum* in a highly disturbed environment that also had a recent predatory fish introduction.
The purpose of this study was to obtain information on the migratory movements and recruitment of juvenile *A. annulatum*, determine the demographics of the adult breeding migration to and from the pond, and compare the results to other studies.

MATERIALS AND METHODS

STUDY AREA

The primary study area (Figure 2) consisted of the pond located on the Ainley property. This pond has a perimeter of approximately 150 meters and sits within an open field surrounded by cattle pasture consisting of mixed grasses, including fescue, and large manicured yards. This pond has a permanent hydroperiod. The three dominate aquatic plants found within the littoral zone of the pond are *Zannichellia* sp., *Eleocharis* sp., and *Ludwigia* sp. The soil consists primarily of chert rock and red clay. This pond also contains an abundance of wildlife including: Red-eared Slider (*Trachemys scripta*), Plain-bellied Water Snake (*Nerodia erythrogaster*), American Toad (*Anaxyrus americanus*), Green Frog (*Lithobates clamitans*), Bullfrog (*Lithobates catesbeianus*), Southern Leopard Frogs (*Lithobates sphenopephalus*), Central Newt (*Notophthalmus viridescens*), introduced Golden Shiner (*Notemigonus crysoleucas*), and an abundance of invertebrates (personal observations). This pond also has a noticeable shallow and deep side, with the deeper side being the side modified so that the pond would hold more water.

LARVAE MIGRATION

Starting in late April 2015, a drift fence and pitfall traps were erected around the entire perimeter of the Ainley pond (Figure 3). To capture the metamorph emigration, a drift fence consisting of a three foot tall construction-grade silt fence with wooden stakes was built around the entire perimeter with the lower 8 inches buried within the soil. A total of 46 pitfall traps (1-gallon nursery plant pots) were buried to the rim every 3 meters around the entire perimeter and
equipped with a cover (plywood with metal legs) to prevent predation and provide shade for captured animals. In addition, each pitfall trap contained a wet sponge for moisture to prevent desiccation. These pitfall traps were checked every one to three days, particularly on rainy nights, for metamorph salamanders. When checking the traps it was noted which traps contained animals, the animals SVL was measured using digital calipers, measured to the nearest 0.1 mm, and a tissue sample was taken for later DNA analysis.

The pond basin slope was measured at each of the 46 pit traps around the entire perimeter of the pond by taking the difference between two water depth measurements one meter apart. The difference in slopes between the shallow and deep sides of the pond were tested using t-tests. This was done to compare differences in slopes to larvae migration patterns.

Additionally, three larvae were captured via dipnetting in early April to be reared in captivity. This was done to observe their skin pattern ontogeny and for additional confirmation of identification. These larvae were kept individually in 1-gallon aquaria. They were fed a diet of frozen bloodworms, and provided substrate to move onto after metamorphosis.

**ADULT MIGRATION**

In September and October 2015, the same fence and traps were again used to catch adults migrating to the pond. However, due to flooding (Figure 4) of the area during the previous spring rains, the fence and traps were pulled and moved back approximately 1.5 meters from the water’s edge. This time the pitfall traps were placed every 4 meters on the inside and outside of the drift fence and were used to assess directionality of adult migrations to and from the pond (Figure 5). Traps were checked every one to three nights but checked every night during rains. Upon capture, adult *A. annulatum* were marked via toe clipping so animals could be uniquely identified and then released in the direction in which they were heading. This toe clipping was
collected and saved for later DNA analysis. Ott and Scott (1999) found no significant effects of toe clipping on the survival of _A. opacum_. All adult animals were released after morphological and weight measurements had been taken using digital calipers and a digital scale, which measure to the nearest 0.1 mm and 0.01 g, respectively. In addition to the drift fence and pitfall traps, plastic minnow traps were also placed in the focal pond (Figure 6). These traps, numbering 21 in total, were placed in three sets of seven approximately 4 meters apart and were aligned with pitfall traps. This was done to compare the methodologies of using minnow traps vs pitfall traps as a sampling means for _A. annulatum_. Additional minnow traps were placed in ponds in the surrounding area in an attempt to sample for adults in other localities. The capture rates of the minnow traps in the focal pond were to be used as a calibration tool to estimate population sizes in other ponds where only minnow traps were placed. The minnow traps in all of the ponds were checked every one to three days depending on weather conditions starting mid-September through December 7. All species captured were recorded.

RESULTS

LARVAE MIGRATION

A total of 68 metamorph salamanders with a mean snout-vent-length (SVL) of 39.26 mm were captured in pitfall traps between May 5th and May 11th following rain events (Figure 7). On May 5th, 7 individuals were captured marking the first night of migration following 0.15 cm of precipitation. The following night, May 6th, had two captures. However, the majority of the metamorphs (n=54) were captured the night of May 7th following a rain event that dumped 2.54 cm of precipitation (Figure 8). The following nights only had six individuals emigrate on two separate nights the last of which took place on May 11th. All animals moved at night with no captures taking place during the day.
There was a significant difference between the slopes of the shallow side compared to the deep side of the pond (t-test, P<0.001). Seventy-five percent (n=51) of metamorphs emigrated from the shallow side of the pond, and the remaining 25% (n=17) from the deep end (Figure 9). Linear regression showed no significant correlation in larvae density and pond slope (P=0.17). However, individuals did appear to cluster within intermediate slopes (figure 10).

The larvae reared in captivity showed a gradual change in skin patterning over several months (Figure 11). All three individuals underwent metamorphosis in early May. Speckling began to appear in early June, and the banding pattern characteristic of *A. annulatum* appeared in late September.

**ADULT MIGRATION**

The adult migration began with the first individual (male) on the night of October 22nd and ended with the last individual (male) captured on November 5th with the majority of the animals (n=23) being captured on October 30th (Figure 12). In total, 38 adult *A. annulatum* were captured in pit traps during the fall 2015 breeding migration (Table 1). The first individual was a male with a SVL of 81.6 mm and a mass of 17.89 g. This individual migrated after 0.73 cm of precipitation. The following night had a rain event of 1.49 cm of precipitation. The rest of the animals migrated over the next few nights. The last individual to show up at the breeding pond was the smallest male with a SVL of 68 mm and a mass of 7.2 g. This individual arrived six days after the main migration event and was recaptured seven days later leaving the pond.

The largest male had a SVL of 90 mm and a mass of 18.64 g. The smallest male had a SVL of 68 mm and a mass of 7.2 g. The largest female had a SVL of 100 mm and a mass of 16.65 g. The smallest female had a SVL of 81 mm and a mass of 16.3 g. Overall, females were significantly larger than males (t-test, P=0.0037, Figure 13).
Three (7.8%) of the adults captured in pit traps were also captured in minnow funnel traps (Figure 14). Previous capture was confirmed via toe clip pattern. Many additional animals were caught in the funnel traps including high densities of the introduced Golden Shiner (Notemigonus crysoleucas) (Figure 15).

DISCUSSION

The pattern of metamorph emigration was similar to that reported for other A. annulatum sites and Ambystoma in general (Spotila and Beumer 1970; Petranka 1998; Briggler et al. 2004; Semlitsch et al. 2014). Additionally, all movements took place at night during or just after significant rain events, with the majority of individuals not emigrating until after 2.54 cm of precipitation, (Figure 7). Spotila and Beumer (1970) found that migrations did not take place unless a rain event of 1.27 cm takes place. A total of 68 metamorphs were captured emigrating from the Ainley pond during the spring 2015 migration event. This figure falls within the range reported by Semlitsch et al. (2014) that documented between six and 704 metamorphs emigrating out of five different fishless ponds in Missouri between the years 2004 and 2007. This fits the reported pattern of low but consistent recruitment observed in A. annulatum unlike high levels of recruitment and reproductive failures seen in other species (Semlitsch et al. 1996; Taylor et al. 2005).

A mean SVL of 39.26 mm for transformed individuals at the Ainley pond is also similar to reports from other studies. Again, Semlitsch et al. (2014) reported a mean SVL for newly transformed individuals to be 39.2 mm for five different ponds studied which is nearly the exact same as the Ainley pond. Although, Trapp (1956) found newly transformed individuals to have a mean SVL of 45 mm. A. annulatum size at metamorphosis appears to be fairly conserved across
geographic regions. Sites with smaller mean SVL for newly transformed individuals is correlated with early pond drying (Semlitsch et al. 2014).

Seventy-five percent of the metamorphs left from the shallow side of the pond. This side of the pond had a much shallower slope compared to the deeper side. A shallow pond basin slope has been shown to play a critical role in structuring vegetation communities which in turn influence amphibian communities (Peterman et al. 2014). The fact that the majority of the larvae were concentrated in this area is likely a result of the shallow slope and more abundant vegetation. Additionally, this pond had bait minnows (Notemigonus crysoleucas), introduced in the months prior to this study, which also likely reduced larval numbers in the deeper water thereby concentrating individuals in the shallow side of the pond (Drake et al. 2014).

The skin pattern characteristic of A. annulatum develops over a period of several months. Larvae will resorb their external gills and their skin begins to darken around the time of metamorphosis. At this stage, they may have light speckling that will slowly develop over a period of several months into the characteristic rings seen in adults. Fully developed rings don’t become apparent until September/October when the animals are ready to return to their natal pond to breed although most don’t until 1 or 2 years of age.

The pattern of adult migration to the Ainley pond was very similar to that reported from other sites with large males tending to show up first followed by the females. Additionally, the sex ratio was male biased as typically seen in Ambystoma where male-female ratios of 1:1 and 2:1 have been observed. However, the male-female ratio at the Ainley pond was strongly male biased with a ratio of 3:1. Furthermore, the total number of adults captured at the Ainley pond was not typical for adult A. annulatum counts reported from other breeding ponds in Missouri or
Arkansas (Briggler et al. 2014; Semlitsch et al. 2014). Most of these other studies have reported hundreds to thousands of adults per breeding pond.

Of the 38 adult *A. annulatum* captured at the Ainley pond, 7.89% (n=3) were also captured via plastic funnel traps. These individuals were identified by their unique toe clipped pattern to confirm they had already been captured via pit traps. Exploring additional survey methods is important due to the need to survey and monitor amphibians in a time of amphibian declines. Drift fences and pit traps are labor intensive and can’t always be utilized in places that also have large agricultural animals, unsuitable substrates, or in areas prone to flooding. Funnel traps also capture animals that can’t be captured via pit traps such as various anurans and snakes. Funnel traps offer a relatively easy and passive way to sample amphibians (Willson and Dorcas, 2003).

The low adult population at the Ainley pond is concerning, and the lack of suitable terrestrial habitat may be a principal variable. A critical threshold of 34% forested habitat within 300 m was identified by Homan et al. (2004) for the presence of *A. maculatum* in disturbed and fragmented landscapes. In addition, soil compaction from cattle and other agricultural practices around the Ainley pond could compound the lack of forested habitat. Semlitsch (1982) conducted a laboratory study that suggested that most *Ambystoma* do not actively dig their own burrows but instead rely on already existing burrows made by other animals. These burrows could be lost from the surrounding area due to agricultural activities.

Before the census of this pond, I surveyed the larvae periodically via dipnetting. The average catch-per-unit-effort (CPUE) in April 2015 was four larvae per 5 minutes of dipnetting. Subsequent trips to the pond (2018, 2019, 2020) during prime larvae months (April/May) have yielded zero larvae captures. Introduced predatory fish have been shown to significantly impact
egg and larval survival of *Ambystoma* (Drake et al. 2014; Perterman et al. 2014). It appears this study may have caught a Ringed Salamander population that is on a trajectory for local extirpation due to habitat loss and introduced predatory fish.

**LITERATURE CITED**


APPENDIX

Figures and Tables

Table 1. Summary of adult *A. annulatum* captured at the Ainley pond between October 22\textsuperscript{nd} and November 5\textsuperscript{th}, 2015.

<table>
<thead>
<tr>
<th>Sex</th>
<th>Total</th>
<th>mean SVL (mm)</th>
<th>mean weight (g)</th>
<th>Percent of total captures</th>
</tr>
</thead>
<tbody>
<tr>
<td>Female</td>
<td>8</td>
<td>88.6 ± 6.09</td>
<td>17.9 ± 2.9</td>
<td>21%</td>
</tr>
<tr>
<td>Male</td>
<td>30</td>
<td>80.7 ± 5.21</td>
<td>14.8 ± 3.1</td>
<td>79%</td>
</tr>
</tbody>
</table>
Figure 1. *A. annulatum* larvae dipnetted in the Ainley pond. (Photo by Brian Becker)
Figure 2. Google Earth image showing the primary study pond (Ainley) within a highly disturbed landscape in northwest Arkansas. Image also shows neighboring ponds that I sampled for adult *A. annulatum*.
Figure 3. Drift fence and pitfall traps at the Ainley pond. Note the large homes and mowed yards, and pastures in the background. (Photo by Brian Becker)
Figure 4. Ainley pond flooded during the spring 2015 rains. All pitfall traps and most of the drift fence was submerged. The fence and traps had to be pulled and replaced in time for the 2015 fall adult migration. (Photo by Brian Becker)
Figure 5. Drift fence with pitfall traps placed both on the inside and outside of the fence. (Photo by Brian Becker)
Figure 6. Drift fence and pitfall traps with additional funnel trap. (Photo by Brian Becker)
Figure 7. *A. annulatum* metamorph migration and precipitation. Weather data obtained from Northwest Arkansas regional Airport (XNA). Red line is precipitation, blue line is metamorph counts.
Figure 8. 54 *A. annulatum* metamorphs that migrated on a single night comprising the bulk of the metamorphs emigrating during the spring 2015 migration event.
Figure 9. Google Earth image of the Ainley pond. Yellow line delineates the deep side (A) from the shallow side (B). 75% of migrating metamorphs left from the shallow side of the pond.
Figure 10. Bivariate fit of larvae density by pond slope. Larvae density is the number of individuals captured at each pit trap. Pond slope (measured at each pit trap) is the difference between two water depth measurements (cm) measured one meter apart.
Figure 11. Pattern development of *A. annulatum* from larvae to juvenile. Larvae were housed in 1-gallon aquaria and fed a diet of frozen bloodworms. Substrate was provided for transformed individuals to move out of water. All photos are of the same individual. (Photos by Brian Becker)
Figure 12. Fall 2015 breeding migration of *A. annulatum*. Blue line is animal counts and the red line is precipitation in cm. Weather data taken from the Northwest Arkansas Regional Airport (XNA).
Figure 13. Plot of male and female *A. annulatum* snout-vent-length. N=38. T-test, P=0.0037
Figure 14. Adult *A. annulatum* captured in a minnow funnel trap.
Figure 15. The Golden Shiner (Notemigonus crysoleucas) was introduced into the Ainley pond a few months prior to the 2015 *A. annulatum* census.
Chapter 2: Sexual Dimorphism in Head Size and Limb Length in *Ambystoma annulatum*

ABSTRACT

Many species of salamander show pronounced sexual dimorphism. However, there appears to be extensive variation in the pattern of sexual dimorphism across different taxa. In some species of salamanders the females are bigger than the males but in others the males are larger. There is also variation in sexual dimorphism of head sizes and shapes as well as limb morphology among different salamander taxa. In the genus *Ambystoma*, females tend to have a longer snout-vent-length (SVL) than males. However, few studies have looked at sexual dimorphism in head size or limb lengths in *Ambystoma*. In this study, I measured the SVL, head dimensions (length, width, and depth), ulna, tibia and tail length of 42 of female and 49 male *Ambystoma annulatum* captured in funnel traps from three breeding ponds located in Washington County, Northwest Arkansas in October of 2018. Females were significantly larger than males, but males had longer, wider, and deeper heads for their size than females. Males also had significantly longer tails. There was no significant difference in limb lengths between males and females. Sexual dimorphism in head size may be the result of sexual selection favoring larger heads in males for courtship, dietary differences, or male-male interactions.

INTRODUCTION

Sexual dimorphism are the physical differences in body size, shape, or coloration between males and females of a given species. Sexual dimorphism is widespread among animals (Shine 1989). It has been extensively studied in mammals (Glucksmann 1974; Lindenfors et al. 2007; Karp et al. 2017), birds (Owens and Hartley 1998; Selander 2017), reptiles (Shine 1994; Olsson et al. 2002; Cox et al. 2007; Liao et al. 2013) and to a lesser extent in amphibians.
One of the most common explanations for sexual size dimorphism (SSD) in animals is sexual selection in which larger bodies or heads are favored in males that engage in male-male interactions (Darwin 1874; Vitt and Cooper 1985). Fecundity selection has also been proposed for animals in which the females have a larger body size than the males, because a larger body size will accommodate larger clutch sizes (Tilley 1968; Olsson et al. 2002). Ecological causes such as differences in diet to reduce intraspecific competition have also been proposed (Shine 1989; Anderson and Vitt 1990).

A common pattern seen in lizards where sexual dimorphism occur are that males tend to have larger heads than females. This has been documented in cases where males have a larger SVL and head size than females (Olsson et al. 2002), where males and females have the same SVL but males have larger heads (Schwarzkopf 2005), and in cases where the female has a larger SVL but the male still has a proportionally larger head (Becker and Paulissen 2012).

Amphibians make for an interesting group to study sexual dimorphism because they exhibit a high degree of variation in morphologies, habitats, and life-history characteristics. Unlike lizards, there seems to be more variation in sexual dimorphism among salamanders. Reinhard et al. (2015) studied sexual dimorphism in two Mediterranean salamanders (Salamandra algira and Mertensiella caucasica) and found no significant difference in body size between the sexes but did find a significant difference in limb length with males having larger limbs. The authors attributed this difference in limbs to reproductive behavior involving amplexus.

In a study investigating sexual dimorphism in two plethodontid salamanders (Eurycea aquatica and Eurycea cirrigera), Alcorn et al. (2013) found Female-biased sexual dimorphism in body size in E. cirrigera but not in E. aquatica. Male-biased sexual dimorphism in head width
occurred in both species. They also found significant differences in head shape between male and female *E. aquatic*, but male and female *E. cirrigera* did not differ in head shape. Another interesting study (Zhang et al. 2014) looked at sexual dimorphism in the Wushan Salamander, *Liua shihi* and found that the males had a longer snout-vent length and longer limb size than females, but the females had proportionally larger heads. Adding to the variation in sexual dimorphism seen among different species of salamander, Serro-Cobo et al. (2000) investigating sexual dimorphism in the Pyrenean salamander, *Euproctus asper*, not only found sexual dimorphism in the species (head size, limb length, and body weight) but also found the degree of sexual dimorphism varied between two geographically distinct populations.

Species belonging to another group of salamanders, the Ambystomatidae, also show sexual dimorphisms, the most commonly cited being the larger SVL of females. This has been shown for *Ambystoma opacum* (Pokhrel et al. 2013), *Ambystoma tigrinum* and *Ambystoma texanum* (Williams et al. 2009), *Ambystoma maculatum* (Semlitsch and Anderson 2016; Homan et al., 2018) and *Ambystoma annulatum* (Briggler et al. 2004; Semlitsch et al. 2014). Other aspects of sexual dimorphism have also been studied in various *Ambystoma* species (Arnold, 1976; Morgan et al. 2014; Finkler 2013). However, additional aspects of sexual dimorphism in *Ambystoma annulatum*, the Ringed Salamander, have not been thoroughly studied.

*Ambystoma annulatum* (Ringed Salamander) is a member of the Ambystomatidae, the mole salamanders. It is a large heavy-bodied salamander with a gray belly, black dorsum and whitish to yellow rings along the body and tail (Conant and Collins, 1998). *A. annulatum* is endemic to the Ozark and Ouachita Mountains of southern Missouri, northern Arkansas, and eastern Oklahoma giving it a relatively small distribution compared to other *Ambystoma* (Trapp 1956). *A. annulatum* is one of two fall breeding *Ambystoma*, the other being *A. opacum*. 

52
Breeding takes place from mid-September to early November when adults migrate en masse from their terrestrial habitats to small fishless ponds to breed (Spotila and Beumer, 1970; Briggler et al. 2004; Semlitsch et al. 2014).

Male *A. annulatum* tend to show up to the breeding pond first, followed by the females. Upon arrival of the females, a courtship begins in which males will nudge females with their snout and then swim off to deposit a spermatophore (Spotila and Beumer 1970). Receptive females will then pick up the spermatophore to fertilize her eggs. Eggs are deposited on vegetation or the pond bottom. Eggs usually hatch after 9-16 days (Trapp 1956; Hutcherson et al. 1989). Hatched larvae will overwinter and undergo metamorphosis and emigrate from their natal pond to terrestrial habitat in late April or early May. This study was an investigation of sexual dimorphism in head size and limb length in *A. annulatum*, the Ringed Salamander.

**MATERIALS AND METHODS**

Three previously identified *A. annulatum* breeding ponds were selected to trap adult *A. annulatum* for this study. All three ponds were on private property. Two of these sites were the Roadside and Erwin properties located approximately 4.15 kilometers (2.5 miles) east of Fayetteville, Washington County, Arkansas (Figure 1). The third site was on the Odglen property located approximately 8 kilometers (4.9 miles) west of Fayetteville, Washington County Arkansas (Figure 2). Permission was obtained from all three landowners to set up traps in these ponds for the purpose of capturing *A. annulatum* adults during the breeding migration that took place in October 2018.

Plastic funnel traps were used to trap adult *A. annulatum* (Figure 3). Previous studies have shown plastic funnel traps to be a reliable way to passively trap adult salamanders (Fronzuto and Verrell 2000; Willson and Dorcus 2003; Willson and Dorcus 2004; Willson and
Seven funnel traps were deployed in each of the three ponds spaced 3 meters apart in mid-September. Because the Roadside and Erwin ponds were seasonal ponds with shallow basin slopes and abundant vegetation, the traps were simply placed loose in the pond basin (Figures 4 and 5). These ponds stayed relatively shallow even during the fall rains. However, the Odglen pond has a permanent hydroperiod with very steep basin slopes and little aquatic vegetation to support the traps so they were tied off to nearby trees to maintain their position (Figure 6). Traps were repositioned as needed to avoid total submersion and the potential drowning of animals.

Traps were checked every one to three days, and every day during rain events starting in mid-September to early November. Traps stayed deployed until a suitable number of A. annulatum adults were captured for analysis. All species captured were recorded. Captured A. annulatum were sexed via cloacal examination (Figure 7). The toe clipping was kept for future studies. During the breeding season males have noticeably swollen cloacal glands that are not prominent in females. Salamanders were also toe-clipped to avoid repeated measurements. Morphological measurements consisted of taking the SVL and tail length (measured to the nearest mm) via a scientific ruler, and head dimensions and limb lengths (ulna and tibia) were taken via digital calipers to the nearest 0.1 mm (Figure 8). Animals with noticeably damaged or missing tails were excluded from the analysis. Head dimensions included length measured from the tip of the snout to the gular fold, width was measured at the widest point of the head, and depth was measured at the same point as width. The ulna was measured from the tip of the olecranon process to the end of the flexed wrist. The tibia was measured from the knee to the joint of the flexed foot. An F-test was conducted to check for equal variance in SVL between males and females (α=0.05), and
ANOVA was used to evaluate head dimensions with SVL as the covariate and sex the factor being tested. All statistical analyses were conducted using JMP Pro 17.

RESULTS

Forty-two of the females and forty-nine of the males captured between the three sampling sites were used for analysis. Female *A. annulatum* had a significantly longer SVL than males (mean ± SD of SVL: males = 81.4 ± 6.6 mm; females = 85.5 ± 6.4 mm; df = 89, P= 0.0036, Figure 9). ANCOVA showed that males had significantly larger heads than females in all three head dimensions after accounting for differences in SVL (Figure 10; Figure 11; Figure 12; Table 1). There was no significant difference in the length of the ulna or tibia between males and female (Figure 13; Figure 14; Table 1). There was a significant difference in tail length between males and females (Figure 15; Table 1).

DISCUSSION

The sexual dimorphism in SVL with female *A. annulatum* having larger SVL than males matches what has been previously reported by other studies looking at *A. annulatum* (Briggler 2004; Semlitsch 2014). In amphibians, female body size is a trait often correlated with selection for increased fecundity such as having a larger clutch size or larger egg dimensions (Bruce 1969; Scott and Fore, 1995). At the same time, smaller adult male size may result from the selection for greater mobility to facilitate locating mates during courtship (Shine 1979).

Male *Ambystoma annulatum* have overall larger (longer, wider, deeper) heads than females after accounting for differences in SVL. Differences in head size and shape could be due to differences in diet. Anderson (1968) found dietary differences between male and female *Ambystoma macrodactylum sigillatum* during the breeding season after analyzing stomach contents, in which males apparently ate aquatic prey that the females did not. This was attributed
to the fact females leave breeding ponds shortly after oviposition whereas males stay in the pond the entire breeding season. Several studies have investigated aspects of diet in larval *A. annulatum* (Kluhsman 1991; Nyman et al. 1993; Heuring et al. 2017), but few have examined the stomach contents of adults (Trapp 1959; Hutcherson et al. 1989) with no existing comparisons between adult male and female diets.

Larvae cannibalism has been observed for multiple *Ambystoma* species (Pierce et al. 1983; Nyman et al. 1993; Wildy et al. 2001; Becker 2018 personal observation). *Ambystoma tigrinum* (Tiger Salamander) larvae naturally occur in nature as two distinct morphs, the typical morph that feeds mainly on invertebrates and a cannibal morph that also feeds on conspecifics (Pierce et al. 1983; Pfennig 1997). These two larval morphs have significant differences in head size and bone and tooth structures (Pierce et al. 1983; Pederson 1991; Larson et al. 1999). Of the 24 cannibal morph larvae analyzed by Larson et al. (1999) 23 were male and one was female. The authors noted a difference in tooth morphology between the males and the female. However, it is difficult to draw conclusions from a single female. Additionally, the authors do not cite a reason for the highly skewed male bias. Male biased sex ratios are commonly reported for *Ambystoma* breeding populations (Briggler et al. 2004; Semlitsch et al. 2014; Semlitsch and Anderson, 2016; also see chapter 1). Larval cannibalism paired with larger head sizes in males could, at least in part, explain the male biased sex ratios seen in many *Ambystoma* breeding populations.

Sexual dimorphism in head size as it relates to male-male interactions as well as courtship behavior is well documented in lizards (Vitt and Cooper 1985; O'Bryant and Wade, 2002; Nguyen et al. 2020). Larger head or body sizes seem to give males a competitive advantage and aids in reproductive success. Similar patterns of male-male combat and female
courtship have been observed in salamanders. Park et al. (1996) studied sexual dimorphism, male-male combat, and courtship behavior in *Hynobius leechii* (Korean Salamander). They found that males and females were dimorphic in multiple physical aspects of body and head size. They also reported that males would actively fight other males, via biting, for access to females (smaller males would flee), as well as actively court females by snout rubbing, tail undulation, and amplexus. Houck (1988) reported a higher mating success of larger males in *Desmognathus ochrophaeus*. Although this was attributed to large body sizes among males, not head size. Wang et al., (2023) found a positive relationship between head size and mate guarding in *Pseudotriton ruber*.

Noble (1931) suggested that courtship in amphibians is highly conserved evolutionarily. Indeed courtship in the genus *Ambystoma* seems to be very similar among species (Anderson 1961; Storez 1969; Wyman, 1971; Arnold 1976). The general pattern of courtship in *Ambystoma* begins when a male becomes aware of a female and initiates contact by nudging her with his snout. The next phase consists of amplexus in some species or guarding in others. The male then moves away from the female to deposit a spermatophore on the pond substrate. It has been noted that the male spends more time swimming around the pond than the female does (Anderson, 1961). The female will follow the male and pick up the spermatophore with her cloaca. Some variation on this theme include *Ambystoma macrodactylum croceum* in which there doesn’t appear to be the nudging phase and the addition of amplexus (Anderson 1961). Sexual dimorphism in limb size is often found in species that engage in amplexus during mating. Spotila and Beumer (1970) described the courtship of *A. annulatum* and reported a similar pattern seen in other *Ambystoma*. However, unlike some species of *Ambystoma*, *A. annulatum* do not seem to
engage in amplexus. This may be why I found no significant difference in limb length between males and females (see results).

Another interesting result of this study was the difference in tail size between males and females (see results) where males had significantly longer tails than females. One interesting reason for this may be the use of the tail by males for swimming during the courtship behavior (see above). Having a longer tail may aid in the swimming behavior of males seen during courtship. Another interesting pattern that may explain the longer tail in males may involve the migration pattern observed in *A. annulatum* during the breeding season. Multiple studies looking at the demographics of *A. annulatum* breeding migrations document a tendency for males to show up first at breeding ponds (Briggler et al. 2004; Semlitsch et al. 2014; also see chapter 1). Possible reasons for early male predominance given for *A. maculatum* by Phillips and Sexton (1989) include males being lighter and able to travel more rapidly, males might seek retreats closer to breeding ponds, and males might respond more quickly to environmental cues. Briggler et al. (2004) suggested that older males that are repeat breeders might leave chemical trails that females and younger males will follow. However, when considering the lateral undulation movements seen in migrating *A. annulatum* it is possible that their early arrival at the breeding pond may be a combination of smaller bodies and longer tails that allow them to move faster across the landscape utilizing lateral undulation. Additional studies will be needed in order to tease apart causes for these observed behaviors.

LITERATURE CITED


Table 1. Summary of ANCOVA results for head and limb comparisons between male and female *A. annulatum* (α = 0.05). Significant p-values are bolded.

<table>
<thead>
<tr>
<th></th>
<th>Males (n=49) Least Squares mean</th>
<th>Females (n=42) Least Squares mean</th>
<th>F-ratio</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Head Length (mm)</td>
<td>17.998</td>
<td>17.492</td>
<td>8.123</td>
<td>0.0054</td>
</tr>
<tr>
<td>Head Width (mm)</td>
<td>12.534</td>
<td>12.118</td>
<td>8.813</td>
<td>0.0039</td>
</tr>
<tr>
<td>Head Depth (mm)</td>
<td>7.22</td>
<td>6.807</td>
<td>10.150</td>
<td>0.0020</td>
</tr>
<tr>
<td>Ulna Length (mm)</td>
<td>7.104</td>
<td>7.142</td>
<td>0.109</td>
<td>0.7413</td>
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<tr>
<td>Tibia Length (mm)</td>
<td>8.401</td>
<td>8.446</td>
<td>0.123</td>
<td>0.7258</td>
</tr>
<tr>
<td>Tail Length (mm)</td>
<td>101.918</td>
<td>95.447</td>
<td>13.703</td>
<td>0.0004</td>
</tr>
</tbody>
</table>
Figure 1. Google Earth image of the Roadside and Erwin trapping sites (yellow pins) located approximately 4.15 kilometers (2.5 miles) east of Fayetteville, Washington County, Arkansas.
Figure 2. Google Earth image of the Odglen trapping site (yellow pin) approximately 8 kilometers (4.9 miles) west of Fayetteville, Washington County Arkansas.
Figure 3. Plastic funnel traps used to capture adult *A. annulatum*. (Photo by Brian Becker)
Figure 4. The Roadside pond with plastic funnel trap. This *A. annulatum* breeding pond is a seasonal pond that dries up in late summer. (Photo by Brian Becker)
Figure 5. The Erwin pond. The *A. annulatum* breeding pond is a seasonal pond that dries in late summer. (Photo by Brian Becker)
Figure 6. The Odglen pond with funnel traps anchored to nearby trees to maintain their position. This *A. annulatum* breeding pond has a permanent hydroperiod. (Photo by Brian Becker)
Figure 7. Male *A. annulatum* displaying swollen cloacal glands that can be used to distinguish between males and females. (Photo by Brian Becker)
Figure 8. Tools used to take morphology measurements of *A. annulatum* (Photo by Brian Becker)
Figure 9. Plot of SVL by sex (t-test, $\alpha = 0.05$, $P = 0.0036$). Diamonds encapsulate means.
Figure 10. Relationship between head length and SVL for males and females. Blue line (top) is for males, and the red line (bottom) is for females.
Figure 11. Relationship between head width and SVL for males and females. Blue line (top) is for males, and the red line (bottom) is for females.
Figure 12. Relationship between head depth and SVL for males and females. Blue line (top) is for males, and the red line (bottom) is for females.
Figure 13. Relationship between ulna and SVL for males and females. Blue line (top) is for males, and the red line (bottom) is for females.
Figure 14. Relationship between tibia and SVL for males and females. Blue line (top) is for males, and the red line (bottom) is for females.
Figure 15. Relationship between tail length and SVL for males and females. Blue line (top) is for males, and the red line (bottom) is for females.
Chapter 3:

Survey for *Ambystoma annulatum* in farm ponds in Northwest Arkansas

**ABSTRACT**

Currently there is much concern about accumulating studies documenting amphibian declines all over the world. Thereby creating an increased need to survey, document, and monitor amphibian populations. In Northwest Arkansas, natural wetlands are becoming scarcer due to land use, development, and habitat alterations. Manmade farm ponds represent important alternative breeding habitats for amphibians. *Ambystoma annulatum* (Ringed Salamander) is an Ambystomatid endemic to the Ozark and Ouachita Mountains of southern Missouri, northern Arkansas, and eastern Oklahoma. It has one of the smallest distributions of the *Ambystoma* and is considered a *species of special concern* in the state of Arkansas. *A. annulatum* is a fall breeding salamander that relies heavily on small, fishless ponds to breed. In this study, I surveyed 155 natural and manmade ponds in Washington and Benton Counties in Northwest Arkansas for *A. annulatum*. I also kept notes on the presence/absence of other herpetofauna with special emphasis on *Ambystoma* and recorded data on habitat variables (pH, TDS, water temp, presence of agricultural animals, and vegetation). I calculated the catch-per-unit-effort (CPUE) for *A. annulatum* larvae by taking the mean captures of three 5-minute intervals of dipnetting. The CPUE for *A. annulatum* larvae along with presence/absence data for other herpetofauna, fish, and agricultural animals was consolidated into tables for comparisons. Relationships between *A. annulatum* larvae, fish, cattle, and select habitat variables were explored using Principal Components Analysis (PCA). Out of the 155 ponds surveyed for *Ambystoma*, 45 ponds had at least one species of *Ambystoma*, 28 of these ponds contained *A. annulatum*. *A. annulatum* larvae were positively correlated with aquatic vegetation, and negatively correlated with fish, cattle,
high pH, and high TDS. I also observed one complete reproductive failure for *A. annulatum* due to low dissolved oxygen that I describe in detail. These findings support additional studies that show the importance of land use considerations especially the prevention of fish colonization of *Ambystoma* breeding ponds and limiting cattle use of sensitive aquatic environments.

**INTRODUCTION**

Global amphibian declines are an increasing concern among scientists (Alford and Richards 1999; Young et al 2001; Stuart et al. 2004). Causes of amphibian declines can be complex (Blaustein et al. 2011), but may include loss of suitable habitat, habitat fragmentation, climate change, invasive species, or disease such as chytrid fungus (Pounds, 2001; Kats and Ferrer, 2003; Titus et al., 2014; O’hanlon et al. 2018; Cosentino and Brubaker 2018). However, many amphibian declines likely include a combination of aforementioned factors. Loss of habitat, habitat fragmentation, and invasive species can encompass a wide range of human land use practices including: agriculture, timber harvest, or the accidental or intentional release of invasive animals, such as stocking fish.

It is estimated that there are over 300,000 private ponds and lakes in the state of Arkansas (Seldon, UARK School of Agriculture, Fisheries, and Human Science). Many of these ponds are natural but a significant portion are manmade or modified (Brussock and Brown 1982). Many of these ponds can be found dotting the landscape of Northwest Arkansas (Figure 1). These ponds have a wide range of uses including: water sources for livestock, fish production, recreation, and ornamentation.

Many amphibians rely on small ponds for habitat and reproduction (Knutson et al., 2004). This is especially true for members of Ambystomatidae, the mole salamanders. The genus *Ambystoma* are pond breeding salamanders that migrate annually en masse from terrestrial
habitats to small fishless ponds to breed (Conant and Collins 1998). Some common species include *Ambystoma tigrinum*, (tiger salamander), *Ambystoma maculatum*, (spotted salamander), and *Ambystoma opacum* (marbled salamander). The ecology of *Ambystoma* make them particularly vulnerable to negative land use practices because they require both forested habitat as adults and fishless ponds for reproduction and larval development.

*Ambystoma annulatum* (ringed salamander) has one of the smallest distributions of the *Ambystoma*. *A. annulatum* is endemic to the Ozark and Ouachita Mountains of southern Missouri, northern Arkansas, and eastern Oklahoma (Trapp 1956). Due to this relatively small distribution, *A. annulatum* is considered a species of special concern in the state of Arkansas and is one of the least studied *Ambystoma* species. Unlike other *Ambystoma* that breed in late winter or early spring, *A. annulatum* is one of two fall breeding species, the other being *A. opacum*. Depending on the region, *A. annulatum* breeding takes place from mid-September to early November with some reports of winter breeding (Spotila and Beumer 1970; Trauth 2000; Briggler et al. 2004; Semlitsch et al. 2014).

Heavy rains of at least 1.27 cm are needed to initiate the migration event (Spotila and Beumer, 1970). During which adult *A. annulatum* leave their forested habitats at night to gather in small ponds. Males tend to show up first followed by females (Briggler et al. 2004; Semlitsch et al. 2014). Males and females engage in courtship in which the males nudge the females with their snout and then swim off to deposit a spermatophore on the pond substrate. Receptive females will follow the male and pick up one of the spermatophores with her cloaca, subsequently fertilizing her eggs. Egg are deposited on vegetation or the pond bottom (Spotila and Beumer, 1970). Larvae will hatch after 9–14 days and undergo metamorphosis in late April or early May (Hutcherson et al., 1989). This means *A. annulatum* larvae can spend upwards of 9
months in their natal ponds. Newly transformed individuals will leave the pond at night during spring rains to seek retreats in forested habitats.

The secretive and fossorial nature of adult *A. annulatum* make them particularly difficult to survey. This difficulty is compounded by the fact the adults are only active for a few days to a few weeks out of the year. Their larvae on the other hand are constrained to their breeding ponds for several months making them much easier to survey. The purpose of this study consisted of two parts. The first, was to survey a wide range of ponds in Washington County, Northwest Arkansas for *A. annulatum* in an attempt to identify active breeding ponds and add to the information on the abundance and distribution of *A. annulatum* in Arkansas. Other species of herpetofauna were also documented with a special emphasis on additional *Ambystoma* species as well as potential predators and livestock presence. Additional information was also collected when possible on habitat variables including: hydroperiod, presence/absence of vegetation, and water chemistry (pH, TDS, and temperature). I also describe a reproductive failure event for *A. annulatum*. The second part of this study was an effort to increase public awareness about the importance of small ponds for amphibian biodiversity and garner support for the conservation of these important aquatic habitats in Northwest Arkansas.

**MATERIALS AND METHODS**

Potential *A. annulatum* breeding ponds were searched for using Google Earth (Figure 2). This consisted of first identifying a pond and then looking for the nearest house in order to identify the likely property owner. This process involved driving to the house and knocking on the door to inquire about the pond ownership and then seek permission to survey the pond for herpetofauna. Once permission was granted, I then surveyed the pond for animals.
Upon initial arrival to a pond, I would first conduct a visual survey for animals such as livestock and anything that may flee or hide once disturbed such as turtles or snakes. Then before disturbing the water I would measure pH with a portable handheld ATC pH meter (Figure 3), and measure total dissolved solids (TDS) and water temperature using a TDS-3 handheld meter. Both handheld meters were calibrated before each field trip.

Aquatic sampling consisted of standardized dipnetting using a 5-foot aquaculture dipnet with a metal guard and 3 mm mesh (Figure 4). Dipnetting is one of the most common methods for sampling amphibian larvae (Skelly and Richardson 2009; Denton and Richter 2012). Using a stopwatch, I would dipnet for 15 minutes counting how many larvae were captured per each 5-minute interval. The average of the three timed intervals was taken to calculate a catch-per-unit-effort (CPUE) for *A. annulatum* larvae at each site. Although many ponds were sampled more than once throughout the larval developmental period, only a single sampling event for each site during the months of April and May was selected to use in CPUE comparisons for consistency (Table 3). Additionally, this is the period most stable for *A. annulatum* larvae populations because mortality of larvae is greatest just after hatching and during metamorphosis (Peterson et al., 1991). Larvae were identified based on location, time of year, and costal groove counts (Conant and Collins, 1998; Trauth et al. 2004; Altig and McDiarmid 2015). *A. annulatum* larvae counts were tallied along with newts. Presence/absence was noted for all other animals recorded. All animals captured during dipnetting were recorded.

Ponds were surveyed during the months of March through May from 2017 to 2019. The Ainley Pond dipnetted in 2015 (Chapter 1) was also included in the larval survey. Care was taken to include a wide range of pond types and variation in pond uses (Figure 8). Most pond hydroperiods varied from permanent to semi-permanent; holding water most of the year but
drying in late summer. Patterns in species cohabitation and correlation of habitat variables (Table 3) were explored using Principal Components Analysis (PCA) in JMP Pro 17. Hydroperiod was excluded from the PCA due to unconfirmed hydroperiods for some of the ponds.

RESULTS

A total of 155 ponds were surveyed in Washington and Benton counties between March and May during the years 2017, 2018, and 2019. The majority of which (150) were in Washington County (Figure 5). Ninety-nine of these ponds are located to the west side of the Springdale-Fayetteville metropolitan area (Figure 6). Fifty-one ponds are located on the east side of the Springdale-Fayetteville metropolitan area (Figure 7). Out of the 155 ponds surveyed for *Ambystoma*, 45 ponds (29%) had at least one species of *Ambystoma* (Table 1). Twenty-six of these ponds had *A. annulatum* alone, 11 had *A. maculatum* alone, and two had *A. annulatum* and *A. maculatum* together. Five ponds contained *A. texanum*, although all five of these were clustered together in Woolsey Wet Prairie, a known *A. texanum* location. One pond contained *A. tigrinum* located in Benton County, and 110 ponds (71%) had no *Ambystoma* detections.

The general survey for all species recorded is summarized in Table 2. The catch-per-unit-effort (CPUE) for each pond containing *A. annulatum* along with other important habitat variables is summarized in Table 3. Two ponds that were identified as breeding sites for *A. annulatum* were excluded from the CPUE comparisons because one of the Ponds (McCain #3) did not have a CPUE calculated, presence of larvae was only confirmed, and the other pond (McCain #1) experienced a reproductive failure.

The first three principle components from the PCA explained 41% of the variation seen in the survey data (Table 4). Aquatic vegetation, grass, and newts were positively correlated with PC1 (Table 5; Figure 9). The presence of cattle, high pH, and high TDS were negatively
correlated with PC1 (Table 5; Figure 9). The presence of fish and *Chelydra serpentine* were positively correlated with PC2 and PC3, respectively.

Of the 155 ponds surveyed for this study, 125 were on private property, 13 belonged to the University of Arkansas Agricultural Division, six were found in city parks or other public spaces, six belonged to George’s Farms Inc, and five were on Woolsey Wet Prairie.

**DISCUSSION**

This study represents one of the largest surveys for *Ambystoma annulatum* in Arkansas to date. Out the 155 ponds sampled, *A. annulatum* larvae were found in 18% (28) of the ponds. This seemingly low number could be a result of various land use practices. Peterman et al. (2014) surveyed 169 ponds for *A. annulatum* in an 80% forested area of Missouri and found them in 44% (75) of the ponds, more than double what I found. In all ponds with *A. annulatum* detections the larvae were usually found within the first 15 minutes of dipnetting suggesting fairly easy detectability, although ponds without detections were dipnetted longer to ensure an adequate survey.

Based on my observations and PCA analysis there was a fairly consistent pattern in ponds that contained *A. annulatum* larvae. Larvae were positively correlated with aquatic vegetation and grass and negatively correlated with cattle (Figure 9). This observation is likely due to the fact that cattle are well known to obliterate macrophyte beds around small ponds (Declerck et al. 2006). Grass (Poaceae) is defined as any terrestrial species growing around the margin of the ponds found in open fields. During heavy rains many of these ponds would spill out of their banks and into the surrounding grassy area. Aquatic vegetation is defined as plants adapted to aquatic environments (Keddy 2010). Knutson et al. (2004) recommended limiting cattle to small ponds because of their effect on vegetation in order to increase amphibian biodiversity. The
highest densities of *A. annulatum* larvae were found in ponds that were adjacent forest, lacked fish and cattle, had abundant aquatic vegetation, and maintained a semi-permanent hydroperiod (Table 3; Figures 10 and 11). Interestingly, these two ponds were also the only two ponds surveyed that contain both *A. annulatum* and *A. maculatum* together.

Unsurprisingly, *A. annulatum* larvae were largely absent from ponds containing fish (Table 3). Most studies investigating *Ambystoma* note their study sites were largely fishless ponds (Trapp 1956; Spotila and Beumer 1970; Brussock and Brown 1982; Hutcherson et al. 1989; Briggler et al. 2004; Semlitsch et al. 2014; Peterman et al. 2014). The five fish genera I came across while dipnetting were *Gambusia* sp., *Lepomis* sp., *Pimephales* sp., *Micropterus* sp., and *Carassius* sp. All of which have been documented to have direct negative effects on *Ambystoma* larvae survival and recruitment (Sexton and Phillips 1998; Kats and Sih 1992; Monello et al. 2001; Drake et al. 2014). *Lepomis* sp. were the most numerous occurring in 24% (37) of the ponds, followed by *Gambusia* sp. 8% (13). *Lepomis* sp. are often introduced into water bodies as a sport fish and food fish for other fish. *Gambusia* sp. are often introduced as a food fish but also as a mosquito control agent. Interestingly, Watter et al. (2018) suggested that larval salamanders can be just as effective at controlling mosquitos as *Gambusia* sp.

Two of the ponds included in the larval survey were the Ainley pond (Chapter one) and the Atwood pond. Both of these ponds had fish introductions (Ainley pond= cyprinids; Atwood pond= *Lepomis* sp, *Pimephales* sp, *Gambusia* sp, and *Micropterus* sp) in the months prior to sampling. The Ainley pond was initially sampled in 2015 and the CPUE is included in Table 3. However, subsequent surveys for larvae at this pond (2018, 2019, and 2020) during the months of April and May have yielded zero larvae. The Atwood pond also resurveyed in April of 2020 yielded zero *A. annulatum* larvae.
The most frequently encountered amphibians were *Lithobates sphenoecephalus* and *Acris blanchardi* that were detected in 25% (39) and 23% (36) of the ponds, respectively (Table 2). Some studies have shown that amphibians that normally breed in habitats that are also naturally occupied by fish are less palatable to predators (Gunzburger and Travis 2005). These two species were also the ones most often encountered in ponds that lacked other herpetofauna such as heavily used cattle ponds. Although cattle abundance was not quantified, only recorded as presence/absence, there was a pattern in general herpetofauna biodiversity and the presence of cattle (Table 3). Multiple amphibian species, including *Ambystoma*, were found in ponds with low numbers of cattle or horses. However, ponds with heavy cattle use like George’s Farms Inc. had very low levels of herpetofauna detections and no *Ambystoma* detections.

For reptiles, the most commonly encountered turtles were *Trachemys scripta* and *Chelydra serpentine* which occurred in 18% (29) and 14% (22), respectively (Table 2). *Nerodia erythrogaster* was detected in 12% (19) of the ponds surveyed. Although both *T. scripta* and *N. erythrogaster* were often found dead from injury such as beheading or projectile injury.

One confirmed *A. annulatum* breeding pond (McCain #1) appeared to have experienced a complete reproductive failure November 20, 2020. This was a semi-permanent pond measuring 8 meters by 5 meters with a max depth of 18 cm. This pond had a heavy load of rotting Cattail (*Typha sp.*.) and a thick surface biofilm (Figure 12). Dissolved oxygen (DO) was found to be 0.5 mg/L as measured with a Milwaukee MW 600 Dissolved Oxygen Probe. Due to the ponds small size, the entire pond was able to be surveyed and all eggs masses appeared dead and rotting (Figure 13). For comparison, another nearby breeding pond (McCain #3, same property) less than 100 meters away, had a DO of 6.8 mg/L and thriving *A. annulatum* embryos, most likely from the same breeding population (Figure 14).
The low DO measured in the McCain #1 pond was most likely due to the decaying vegetation load (Jewell, 1971; Belova 1993; Dodson 2004; Mnaya et al. 2006). In turn, the low DO is most likely responsible for the *A. annulatum* reproductive failure at this site. A few studies have investigated the DO requirements for hatching success in some *Ambystoma* species. Sacerdote and King (2009) looked at DO requirements for *Ambystoma maculatum* and *Ambystoma laterale* using both mesocosms placed in wetlands and laboratory treatments. They found that *A. laterale* hatched across all laboratory treatments of 2.0, 4.0, and 6.0 mg/L. *A. maculatum* hatched in all treatments greater than 4.0 mg/L. Kenney and Rose (1974) investigated the effect of DO on activity of *Ambystoma tigrinum*. However, their study used already hatched larvae instead of eggs. *Ambystoma* larvae are capable of gulping air from the water surface during times of low DO. Therefore, the DO requirements of larvae are likely different from that of eggs. Large scale reproductive failures have been reported for *A. opacum* (Taylor and Gibbons 2006). However, due to differences in oviposition, egg mass structure, and symbioses (*A. maculatum*), no one study on DO requirements for hatching success can be representative of *Ambystoma*, therefore additional studies are required.

The second part of this study was to increase public awareness and promote conservation of amphibian population in Northwest Arkansas. Out of 155 total ponds surveyed, 125 representing 80%, were located on private property. All *Ambystoma* detections were found on private property. Therefore the need for private landowners to be aware of and involved in amphibian conservation is vital (McKinley et al. 2017). The vast majority of landowners that had *Ambystoma* on their property were unaware of their presence. With the exception of three private landowners (and two golf courses), all contacted landowners for surveys said yes showing a high level of public interest in wildlife. Ten percent of landowners allowed repeated surveys,
including coming at night, and two landowners designated their ponds as amphibian ponds and changed their plans to stock them with fish. Although not quantified, a considerable portion of my time spent at individual properties during surveys involved impromptu talks on *Ambystoma* ecology.

As human populations continue to grow, land use in Northwest Arkansas is changing more rapidly and invasive species issues are increasing (McKee et al. 2004; Gitau et al. 2010; Falaschi et al. 2020). Most large scale studies looking at factors that affect amphibian abundance and distribution suggest limiting access to small ponds from cattle and fish (Knutson et al. 2004; Semlitsch et al, 2015). The results of this survey also support this. Small, privately owned ponds are a prominent feature of the Northwest Arkansas landscape and consideration of their uses will be vital for amphibian conservation in the future.

**LITERATURE CITED**


APPENDIX

Figures and Tables

Table 1. Summary of *Ambystoma* detections in 155 Northwest Arkansas ponds surveyed during the years 2017, 2018 and 2019.

<table>
<thead>
<tr>
<th>Total Ponds Surveyed</th>
<th>Ponds with only <em>A. annulatum</em></th>
<th>Ponds with only <em>A. maculatum</em></th>
<th>Ponds with both <em>A. annulatum</em> and <em>A. maculatum</em></th>
<th>Ponds with only <em>A. tigrinum</em></th>
<th>Ponds with only <em>A. texanum</em></th>
<th>Ponds with no detections</th>
</tr>
</thead>
<tbody>
<tr>
<td>155</td>
<td>26</td>
<td>11</td>
<td>2</td>
<td>1</td>
<td>5</td>
<td>110</td>
</tr>
<tr>
<td>16%</td>
<td>7%</td>
<td>1%</td>
<td>0.6%</td>
<td>3.2%</td>
<td>71%</td>
<td></td>
</tr>
</tbody>
</table>
Table 2. General survey results for ponds surveyed in Northwest Arkansas in April/May during the years 2017, 2018, and 2019.

<table>
<thead>
<tr>
<th>Taxa</th>
<th>A. annulatum</th>
<th>A. maculatum</th>
<th>A. tigrinum</th>
<th>A. texanum</th>
<th>N. viridescens</th>
<th>Eurycea sp.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total Ponds with detections</td>
<td>28</td>
<td>13</td>
<td>1</td>
<td>5</td>
<td>19</td>
<td>1</td>
</tr>
<tr>
<td>Percentage</td>
<td>18%</td>
<td>8.3%</td>
<td>0.6%</td>
<td>3.2%</td>
<td>12.2%</td>
<td>0.6%</td>
</tr>
<tr>
<td></td>
<td>Gambusia sp.</td>
<td>Lepomis sp.</td>
<td>Pimephales sp.</td>
<td>Micropterus sp.</td>
<td>Carassius sp.</td>
<td>Crayfish</td>
</tr>
<tr>
<td>Total Ponds with detections</td>
<td>13</td>
<td>37</td>
<td>8</td>
<td>4</td>
<td>2</td>
<td>36</td>
</tr>
<tr>
<td>Percentage</td>
<td>8.3%</td>
<td>23.8%</td>
<td>5.1%</td>
<td>2.5%</td>
<td>1.2%</td>
<td>23.2%</td>
</tr>
<tr>
<td></td>
<td>C. serpentina</td>
<td>T. scripta</td>
<td>S. odoratus</td>
<td>N. erythrogaster</td>
<td>N. sipedon</td>
<td>T. proximus</td>
</tr>
<tr>
<td>Total Ponds with detections</td>
<td>22</td>
<td>29</td>
<td>1</td>
<td>19</td>
<td>0</td>
<td>6</td>
</tr>
<tr>
<td>Percentage</td>
<td>14.1%</td>
<td>18.7%</td>
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<td>3.8%</td>
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<tr>
<td></td>
<td>T. sirtalis</td>
<td>A. blanchardi</td>
<td>P. crucifer</td>
<td>G. carolinensis</td>
<td>L. catesbeianus</td>
<td>L. clamitans</td>
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<tr>
<td>Total Ponds with detections</td>
<td>5</td>
<td>36</td>
<td>9</td>
<td>5</td>
<td>28</td>
<td>2</td>
</tr>
<tr>
<td>Percentage</td>
<td>3.2%</td>
<td>23.2%</td>
<td>5.8%</td>
<td>3.2%</td>
<td>18.0%</td>
<td>1.2%</td>
</tr>
<tr>
<td></td>
<td>L. sphenocephalus</td>
<td>A. americanus</td>
<td>H. versicolor</td>
<td>Cattle present</td>
<td>Horses present</td>
<td>Aquatic vegetation</td>
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<td>17</td>
<td>5</td>
<td>32</td>
<td>10</td>
<td>39</td>
</tr>
<tr>
<td>Percentage</td>
<td>25.1%</td>
<td>10.9%</td>
<td>3.2%</td>
<td>20.6%</td>
<td>6.4%</td>
<td>25.1%</td>
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Table 3. CPUE at ponds with *A. annulatum* larvae detections. CPUE = mean of 3, 5-minute sampling periods. 1/0= presence/absence.

<table>
<thead>
<tr>
<th>Site</th>
<th>Date</th>
<th>CPUE larvae</th>
<th>Newts</th>
<th>Gambusia sp.</th>
<th>Lepomis sp.</th>
<th>Pimephales sp.</th>
<th>Crayfish</th>
<th>C. serpentina</th>
<th>T. scripta</th>
<th>Horses</th>
<th>Cattle</th>
<th>Aquatic vegetation</th>
<th>Grass</th>
<th>Permanent hydroperiod</th>
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<tr>
<td>Erwin</td>
<td>19-Apr-18</td>
<td>104</td>
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<td>0</td>
<td>0</td>
<td>1</td>
<td>0</td>
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<td>0</td>
<td>1</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Rain</td>
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<td>1</td>
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<td>0</td>
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<tr>
<td>Bunch</td>
<td>21-Apr-18</td>
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</tr>
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<td>Knolls #2</td>
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<td>1</td>
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<td>0</td>
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Table 4. Eigenvalues for the Principal Components Analysis.

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Table 5. Eigenvectors for the first 3 principal components. Correlations in bold highlight important interactions.

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<td>Larvae</td>
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Figure 1. Google Earth image of commercial cattle pasture (George’s Farms) and private properties dotted with small ponds just west of Springdale, Washington County, Arkansas.
Figure 2. Google Earth image used to identify potential *Ambystoma* breeding ponds. Ponds that are less obvious can be identified based on other topographical features such as patterns in vegetation. This method can also reveal land use practices such as cattle grazing, and pond hydroperiods.
Figure 3. ATC pH meter. Measurements were taken before surveying for larval amphibians. Meter was calibrated before each field trip.
Figure 4. Aquaculture dipnet with 5-foot wooden handle, metal guard, and 3mm mesh used to survey for larval amphibians.
Figure 5. Total ponds (155) surveyed in Northwest Arkansas (yellow pins). 150 ponds in Washington County and 5 ponds in Benton County (upper left).
Figure 6. Ponds (99) in Washington County, located west of Springdale/Fayetteville (west of I49), Northwest Arkansas
Figure 7. Ponds (51) located east of Springdale/Fayetteville, Washington County, Northwest Arkansas
Figure 8. Examples of ponds surveyed for larval *Ambystoma*. 
Figure 9. Loading plot for PC1 and PC2 from Principal Components Analysis (PCA) showing correlations of various habitat variables.
Figure 10. The Erwin pond. This pond had the highest CPUE of *A. annulatum* larvae (CPUE= 104larvae/5min). This pond is adjacent a heavily forested area, contains aquatic vegetation and is surrounded by grass. This pond dries in late summer as seen here. This pond was one of two ponds that also contained *A. maculatum*. 
Figure 11. The Rain pond. This pond had the second highest CPUE of *A. annulatum* larvae (CPUE= 83 larvae/5 min). This pond is adjacent a heavily forested area, contains aquatic vegetation and is surrounded by grass. This pond dries in late summer as seen here. This pond was one of two ponds that also contained *A. maculatum*. 
Figure 12. McCain pond #1, a known *A. annulatum* breeding pond showing thick surface biofilm. This pond experienced a complete reproductive failure documented on November 20, 2020.
Figure 13. Dead *A. annulatum* egg mass. All egg masses in this small pond were all dead representing a complete reproductive failure in this pond.
Figure 14. Healthy *A. annulatum* egg mass located in a pond less than 100 meters from a pond that experienced reproductive failure.
Chapter 4.

Isolation of a *Naegleria* from the gut of *Ambystoma annulatum* in Northwest Arkansas.

**ABSTRACT**

Host-parasite interactions are complicated by a variety of ecological factors. This relationship is further muddied by human alterations of habitats and the accidental or intentional release of nonnative animals. Understanding these relationships is an important factor when considering land use practices and the conservation of biodiversity. This is particularly true for species that exist in low numbers or have small distributions, *Ambystoma annulatum* is one such animal. *A. annulatum* is restricted to the Ozark and Ouachita Mountains of the interior highlands located in central US and is considered a *species of special concern* in the state of Arkansas. Many of the current amphibian population declines are attributed to invasive parasites of one form or another. These observations highlight the need to survey for and identify potential parasites of *A. annulatum*. In this study, I isolated *Naegleria* taken from a fecal sample of an adult *Ambystoma annulatum* captured from a small farm pond located in Springdale, Washington County, Northwest Arkansas. This finding adds to the information on the host-range of *Naegleria*.

**INTRODUCTION**

Parasites are ubiquitous in the animal kingdom. Recently, there has been an increasing interest in amphibian-parasite associations, primarily in the context of amphibian associated diseases (Daszak et al. 1999; Kilpatrick et al. 2010; Blaustein et al. 2018). Parasites of amphibians include a wide range of taxa including: fungi, protozoans, bacteria, viruses, helminths, and arthropods (Densmore et al. 2007). Fungal and viral associations in particular have dominated current research because of their potential to devastate amphibian populations (Fisher and Maretel 2021). Studies on bacteria associations are also becoming more
commonplace as well as the interactions between bacteria and fungi (Becker et al. 2015; Burkart et al. 2017; Shu et al. 2019). Studies of the parasites of the salamander genus *Ambystoma* are fewer due to the secretive nature of these animals.

*Ambystoma annulatum* (Ringed Salamander) is an Ambystomatid that has one of the smallest distributions of the *Ambystoma* (Trapp 1956; Conant and Collins 1998). It is endemic to the Ozark and Ouachita Mountains of southern Missouri, northern Arkansas, and eastern Oklahoma. *A. annulatum* is a fossorial salamander that lives in forested habitats most of the year and only emerges for a few days to a few weeks during fall rains when it migrates en masse to small fishless ponds to breed (Spotila and Beumer 1970; Briggler et al. 2004, Semlitsch et al. 2014). Due to the restricted distribution and secretive nature of *A. annulatum*, it is one of the least studied *Ambystoma* species, including studies of parasitic associations, and is considered a species of special concern in the state of Arkansas. McAllister et al, (1995) was one of the first studies looking at endoparasites of *A. annulatum*. Although they found one protozoa, *Myxidium serotinum*, all parasites recorded were helminths which occurred in 83% of their sample. Helminths are one of the most commonly reported parasites of reptiles and amphibians. This is likely because they are often larger and easier to isolate and identify compared to microscopic parasites.

Host-parasite interactions are complex and can be influenced by a variety of environmental factors including invasive species, climate change, and habitat alterations (Gillespie and Chapman, 2008; Wolinska and King 2009; Reichard et al. 2012; Orlofske et al. 2012). Northwest Arkansas is a rapidly developing part of the country with unprecedented human population growth and habitat alterations, including the introduction of non-native fish to aquatic environments. The need to understand and document host-parasite interaction is more
important than ever. Especially during current amphibian population declines. The purpose of this study was to isolate and identify potential protozoan parasites from the gut of *Ambystoma annulatum*, the Ringed Salamander, from a small farm pond located in Springdale, Washington County, Northwest Arkansas.

**MATERIALS AND METHODS**

The site used to collect samples for this study was a small farm pond located in Springdale, Washington County, Northwest Arkansas (Figure 1). This pond belonged to the Ainley family and was also used for other studies (Chapter 1). A fecal sample was collected from an adult *A. annulatum* captured from the study site in 2015 during processing. This fecal sample was placed on an agar plate that was inoculated with *E. coli* and allowed to sit until it produced a growth ring (24 hours). Amoebae were pulled from the leading edge of the growth ring with a micropipette and transferred to an s-streak plate containing *E. coli* (Figure 2). The leading edge of each s-streak plate was passed to a new agar plate three times to separate out amoebae. Amoebae were viewed with a phase-contrast light microscope (Figure 3).

Amoebae were pulled from the feeding line of the last agar plate pass using 50 µl saline. This sample was then spun in a centrifuge at 4,000 rpm for 5 minutes. Sample was decanted and the pellet was re-suspended with 50 µl of Quick Extract (QE). The sample was then placed in Biometra (PCR). The SSU rRNA gene was amplified by PCR using universal eukaryotic primers. The cycling conditions included an initial 5 min denaturation at 98°C for 30 sec. Then the SSU rRNA gene was amplified in 25 cycles (98°C/8 sec, 60°C/ 30 sec, and 72°C/2 min) followed by 32 cycles (94°C/8 sec, 60°C/30 sec and 72°C/2 min) and a final extension at 72°C/2 min.
Samples were then ran through gel electrophoresis using 1% agarose gel and viewed under a blue light (Figure 4). Bars were cut out with a razor blade and placed in 1.5 ml tubes and prepared for mailing. Samples were mailed to the University of Arizona DNA lab for sequencing.

RESULTS

The Arizona State University DNA lab sent back the ssu-rRNA gene sequence consisting of 2013 base pairs (Figure 5). BLAST results showed the sequence most closely match a *Naegleria* sp. (Eukaryota; Discoba; Heterolobosea; Tetramitia; Eutetramitia; Vahlkampfiidae; *Naegleria*) sequence submitted by Dylova et al., (2006) accession number DQ768717.1 in GenBank (Figure 6).

DISCUSSION

The genus *Naegleria* consists of free-living amoebae widely distributed in soil and freshwater habitats throughout the world (Marciano-Cabral, 1988; Trabelsi et al. 2012). There are at least 47 *Naegleria* spp. described (De Jonckheere 2014). These single-celled organisms characteristically undergo transformation from cyst to amoebae to a flagellated stage (Roberts and Janovy 2000). Only a few species are known to cause disease in humans including *Naegleria fowleri*, the causative agent of amoebic meningoencephalitis.

Few studies have identified amoebae from salamanders (Lobeck 1940; Densmore and Green 2007). To my knowledge no studies have isolated a *Naegleria* from a salamander. Several studies have however isolated *Naegleria* from various organs taken from fish, most commonly gills (Franke and Mackiewicz 1982; Dyková et al. 2001; Dyková et al. 2006). The relationship between *Naegleria* and fish is not well understood. However, Dyková et al. (2010) found an association of *Naegleria* with nodular gill disease (NGD) outbreaks in *Oncorhynchus mykiss*.
(Rainbow Trout) in fish farms in Germany. Because Naegleria are common organisms in aquatic environments, it is not unlikely that many of the Naegleria isolated from fish may be due to contamination or even commensalism (Padrós and Constenla 2021).

*Ambystoma annulatum* spend most of their life in terrestrial habitats and only migrate to small ponds for breeding in late fall (Spotila and Beumer 1970). Additionally, *A. annulatum* primarily breed in ponds that do not contain fish (Briggler et al. 2004; Knutson et al. 2004; Semlitch et al. 2014; see also chapter 3). The pond from which the salamanders were collected for this study had recently had fish introductions by the landowners in the months prior to sampling. There are several studies that have documented the spread of fish-associated parasites from invading fish as well as increased virulence of an introduced parasite on native animals (Gaither et al. 2013; Lymbery et al. 2014; Simkova et al. 2019). However, it is difficult to know whether the Naegleria isolated from the intestine of *Ambystoma annulatum* was already present in the intestine and brought from the terrestrial habitat, or if it was already present in the pond, or if it was introduced by fish. Nevertheless, this study adds to the current understanding of Naegleria distribution, species interactions, and potential host-ranges. Further studies are needed to tease apart this *A. annulatum–Naegleria* association, especially in a time of unprecedented amphibian declines, nonnative fish introductions, and habitat alterations.

**LITERATURE CITED**


APPENDIX

Figures and Tables

Figure 1. Ainley pond where salamander fecal samples were collected for analysis. (Photo by Brian Becker)
Figure 2. Steak plates inoculated with *E. coli* used to culture amoebae. (Photo by Brian Becker)
Figure 3. *Naegleria* sp. (trophozoite and cyst stages) isolated from a fecal sample taken from *Ambystoma annulatum*. Note: background dots are *E. coli* cells. Phase-contrast microscopy at 40x. (Photo by Brian Becker)
Figure 4. Results from the gel electrophoresis.
Figure 5. SSU-rRNA gene sequence consisting of 1032 base pairs.
**Figures**

**Figure 6.** Blast results of the ssu-rRNA gene sequence sent back from Arizona State University DNA lab.

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CONCLUSION

Northwest Arkansas is considered to be one of the fastest growing regions in the United States and has undergone significant land use changes in the past several decades (Yeynolds et al. 2017). We know very little about amphibian population abilities to adapt to these rapid changes. This is particularly true for *Ambystoma annulatum* because of its secretive habits and its short duration of annual activity. *A. annulatum* appears to be sensitive to heavy cattle use of their breeding ponds, and to the introduction of predatory fish, two factors of increasing prevalence in Northwest Arkansas. A variety of studies have documented varying effects of cattle on amphibian populations, but the general trend is negative (Schmutzer et al. 2008; Burton et al. 2009; Corier et al. 2021). Likewise for studies looking at the effects of fish on *Ambystoma*, including *A. annulatum* (Monello and Wright 2001; Drake et al. 2014).

Looking at an aerial view of Northwest Arkansas, a conspicuous feature that stands out right away are the thousands of farm ponds that dot the landscape. These are highly diverse aquatic ecosystems. Some have abundant animal assemblages and some have no detectable animals at all. Two ponds immediately adjacent each other can have completely different plant and animal communities. Yet, little is known to science about the ecology or suitability of these ponds for *A. annulatum*, because most are located on private property. At the same time, the owners of these ponds are often unaware of their pond’s amphibian biodiversity status or the consequences of various land use practices such as introducing predatory fish into these aquatic habitats. In this dissertation, I employed intensive field-based data collection in an attempt to increase our current understanding of *Ambystoma annulatum* ecology.

In chapter 1, I detailed the demographics of an *A. annulatum* breeding pond in an atypical environment. This pond (Ainley pond) sat in open pasture in an extensively deforested area. This
pond also had a previous predatory fish introduction in the months prior to my survey. These factors together allowed for an opportunity to assess an *A. annulatum* breeding population in a suboptimal environment. I found a very small adult population and documented a sudden drop in the CPUE of *A. annulatum* larvae from an initial CPUE 15 larvae/5 min to zero in subsequent surveys. This study reaffirms the need to prevent predatory fish introductions into *A. annulatum* breeding ponds. Because of the high degree of variation in the ecology of ponds located in northwest Arkansas, *A. annulatum* ponds need to be studied in more detail to understand factors that affect *A. annulatum* abundance and distribution.

In chapter 2, I demonstrate that adult female *A. annulatum* have longer SVL than males, but that males have proportionally larger heads (length, width and depth), as well as tails after accounting for differences in body size. I found no differences in limb lengths between males and females. Sexual dimorphism between sexes can shed light on evolutionary drivers such as sexual selection as well as illuminate ecological factors at play. In future studies, I would like to investigate potential sexual dimorphism variation among geographically isolated populations of *A. annulatum*.

In chapter 3, I conducted a large scale survey of 155 ponds for the presence/absence of *A. annulatum* larvae and calculated a CPUE for larvae detections to compare ponds. A total of 155 ponds that were surveyed in Washington and Benton counties between March and May during the years 2017, 2018, and 2019, and only 28 of these ponds had *A. annulatum*. One of these ponds suffered a complete reproductive failure due to low dissolved oxygen. This observation highlights the need for studies investigating the dissolved oxygen requirements for hatching success in *A. annulatum* and that knowledge is critical for considerations of wetland management and amphibian conservation. Additionally, I present evidence from a PCA analysis that heavy
cattle use and fish can negatively affect *A. annulatum* abundance and distribution. This evidence adds to the growing body of knowledge on *A. annulatum* and general amphibian ecological requirements.

In chapter 4, I isolated a *Naegleria* from the intestine of an adult *A. annulatum*. To my knowledge, this is the first *Naegleria* isolated from an *A. annulatum*. Currently there are high incidences of amphibian population declines due to introduced parasites. This study expands the known host-range for *Naegleria* and emphasizes the need for more investigations into the ecology of small farm ponds and the interactions of native and introduced organisms. This study also identifies small farm ponds as potential sources of *Naegleria*.

**LITERATURE CITED**


APPENDIX

Supporting Documents

MEMORANDUM

TO:          James Walker
FROM:        Craig N. Coon, Chairman
DATE:        5/18/15
SUBJECT:     IACUC Approval
Expiration Date:      May 17, 2018

The Institutional Animal Care and Use Committee (IACUC) has APPROVED your Protocol 15055: "Ambystoma annulatum migration and habitat use in a disturbed and non-forested area of Northwest Arkansas", you may begin immediately.

In granting its approval, the IACUC has approved only the information provided. Should there be any further changes to the protocol during the research, please notify the IACUC in writing (via the Modification form) prior to initiating the changes. If the study period is expected to extend beyond May 17, 2018 you must submit a newly drafted protocol prior to that date to avoid any interruption. By policy the IACUC cannot approve a study for more than 3 years at a time.

The IACUC appreciates your cooperation in complying with University and Federal guidelines involving animal subjects.

CNC/aem

cc: Animal Welfare Veterinarian
MEMORANDUM

TO: John David Willson
FROM: Craig N. Coon, Chairman
DATE: May 6, 2016
SUBJECT: IACUC Approval
Expiration Date: May 5, 2019

The Institutional Animal Care and Use Committee (IACUC) has APPROVED your protocol #16067 "Anthropogenic Impacts on Herpetofaunal Biodiversity in Arkansas".

In granting its approval, the IACUC has approved only the information provided. Should there be any further changes to the protocol during the research, please notify the IACUC in writing (via the Modification form) prior to initiating the changes. If the study period is expected to extend beyond May 5, 2019 you must submit a newly drafted protocol prior to that date to avoid any interruption. By policy the IACUC cannot approve a study for more than 3 years at a time.

The IACUC appreciates your cooperation in complying with University and Federal guidelines involving animal subjects.

CNC/aem

cc: Animal Welfare Veterinarian
The Institutional Animal Care and Use Committee (IACUC) has APPROVED your protocol # 19074: *Anthropogenic Impacts on Herpetofaunal Biodiversity in Arkansas.*

In granting its approval, the IACUC has approved only the information provided. Should there be any further changes to this protocol during the research, please notify the IACUC in writing via the Modification Form prior to initiating the changes. If the study period is expected to extend beyond April 4th, 2023, you must submit a newly drafted protocol prior to that date to avoid any interruption. By policy, the IACUC cannot approve a study for more than 3 years at a time.

The following individuals are approved to work on this study: John Wilson, Chelsea Kroos, Ethan Royal, Meredith Swartout, Mitchell Pruitt, Jennifer Montensen, Brian Becker, Brett DeGregorio, Brett DeGregorio, and Bailey Singleton. Please submit personnel additions to this protocol via the Modification Form prior to their start of work.

The IACUC appreciates your cooperation in complying with University and Federal guidelines involving animal subjects.

CNC/amp
## Scientific Collection Permit

**Permit Number:** 01272013  
**Expiration Date:** 8/27/2016

**Permittee:** Brian Booker  
**Sponsor:** University of Arkansas  
**Location:** 606 E. Johnson Ave.  
**Springdale, AR**

**Purpose:** Scientific Research

**# of Traps:** Up to 60

**Location(s):** Washington County

<table>
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<th>Species Type</th>
<th>Collection Methods</th>
<th>Specimens</th>
<th>Removal</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amphibians:</td>
<td>Hand, Live Traps &amp; Teller</td>
<td>Retrieved at point of capture &amp; surface will be killed for study purposes</td>
<td>9 May take 70 adult amphibians</td>
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</table>

This Permit grants the permittee listed above or the designated sub-permittee listed below the privileges accorded under AGFC Code §509. This permit is issued on the conditions set forth herein and becomes effective on the date of issue. A Federal Permit is also required for Migratory and/or Threatened/Endangered Species.

This permit does not allow collection of Species of Greatest Conservation Need (SGCN). No Alligator Snapping Turtle, Chicken Turtle, Queen Snake, Green Salamander, Ouachita Streambed Salamander, Colored Lizard or IL Chara Fxgs may be collected.

This permit is not valid until signed in ink by the permittee. Signature constitutes acceptance of all rules and requirements pertaining to this permit. This permit is non-transferable. This permit does not authorize the purchase or collection on private or other agency lands. Any illegal violation of all terms mentioned may result in revocation of your collection permit. It is incumbent upon the individual collector to obtain appropriate permission to collect from the landowner, whether private or state/federal government. Upon expiration of the permit, but no later than thirty (30) days after the expiration date, the permittee shall submit to the Arkansas Game & Fish Commission a complete written or electronic report of all collections. This permit may be suspended or revoked at the discretion of the Director of the Arkansas Game & Fish Commission.

---

**Permittee Signature**  
**AGFC Authorization**

Sign here to authorize a copy. Copy Sub-Permittee. If this copy is assigned to them.
Arkansas Game & Fish Commission
#2 Natural Resources Drive       Little Rock, Arkansas  72205

Scientific Collection Permit

Permit Number: 032020182                         Expiration Date: 04/10/2019
Permittee: Bryan Becker                        Sponsor: University of Arkansas
          608 E. Johnson Ave.
          Springdale, AR 72764

Purpose: Scientific Research

Location(s): Washington County

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<tr>
<td>Amphibians: <em>Ambystoma</em> spp</td>
<td>Hand, Net &amp; Seine</td>
<td>Captivity &amp; larvae will be killed for study purposes</td>
<td>50 <em>A. annulatum</em> larvae</td>
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</table>

This Permit grants the permittee listed above or the designated sub-permittee listed below with the privileges accorded under AGFC Code 9.09. This permit is issued on the conditions set forth herein and becomes effective on the date of issue. A Federal Permit is also required for Migratory and/or Threatened/Endangered Species.

This permit does not allow collection of Species of Greatest Conservation Need (SGCN), regulated or threatened / endangered species, except for 50 Ringed salamander larvae (*Ambystoma annulatum*).

This permit is not valid until signed in ink by the permittee. Signature constitutes acceptance of all rules and requirements pertaining to this permit. This permit is non-transferable. This permit does not authorize trespass or collection on private or other agency lands. Any illegal violations on all lands mentioned may result in revocation of your collection permit. It is incumbent upon the individual collector to obtain appropriate permission to collect from the landowner, whether private or state/federal government. Upon expiration of the permit, but no later than sixty (60) days after the expiration date, the permittee shall submit to the Arkansas Game & Fish Commission a complete written or electronic report of all collections. This permit may be suspended or revoked at the discretion of the Director of the Arkansas Game and Fish Commission.

Permit Signature

AGFC Authorization

Sign here to Authenticate photocopy. *Circle Sub-Permittee, if this copy is assigned to them.*
# Scientific Collection Permit

<table>
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**Permittee:** John D. Wilson  
**Sponsor:** University of Arkansas, Dept. of Biological Sciences  
Dr. David McNabb, Department Chair

**Location(s):** Statewide

**Purpose:** Scientific Research; Museum Collection; Educational/Class Exercise

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<tr>
<td>Amphibians</td>
<td>Hand Trap/Live Trap/Hook and Line/Seine, Savage Dead/Net (other than mist or seine)</td>
<td>Released at point of capture/Deposited in museum collection</td>
<td>1 adult per species, per location; 50 adults total; 5000 eggs per species</td>
</tr>
<tr>
<td>Reptiles</td>
<td>Hand Trap/Live Trap/Hook and Line/Seine, Savage Dead/Net (other than mist or seine)</td>
<td>Released at point of capture/Deposited in museum collection</td>
<td>1 adult per species, per location; 50 adults total</td>
</tr>
</tbody>
</table>

This Permit grants the permittee listed above or the designated sub-permittee listed below with the privileges accorded under AGFC Code 9.09. This permit is issued on the conditions set forth hereon and becomes effective on the date of issue. A Federal Permit is also required for Migratory and/or Threatened/Endangered Species.

This permit does not allow collection of Species of Greatest Conservation Need (SGCN), with the following exceptions: Crawfish Frog (Lithobates areolatus) – up to 60 adults captured; 0 permanently removed  
1 per species, per location (up to 100 total) of non-regulated / non-T & E SGCN adult reptiles and amphibians

This permit is not valid until signed in ink by the permittee. Signature constitutes acceptance of all rules and requirements pertaining to this permit. This permit is non-transferable. This permit does not authorize trespass or collection on private or other agency lands. Any illegal violations on all lands mentioned may result in revocation of your collection permit. It is incumbent upon the individual collector to obtain appropriate permission to collect from the landowner, whether private or state/federal government. Upon expiration of the permit, but no later than sixty (60) days after the expiration date, the permittee shall submit to the Arkansas Game & Fish Commission a complete written or electronic report of all collections. This permit may be suspended or revoked at the discretion of the Director of the Arkansas Game and Fish Commission.

**Signature:**

**Permittee Signature**  
**AGFC Authorization**

Sub-permittees: Ethin Royal, Elick Lassiter, Ethan Hollender, Elizabeth Hays, Mitchell Pratt, Brett DeGregorio, Brian Becker

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Sign here to authenticate photocopy. Circle sub-permittee if this copy is assigned to them.
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<th>SVL</th>
<th>Weight</th>
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<th>Wtemp</th>
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</table>

Figure 1. Example data sheet used for field data collection.