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Development of Assisted Reproductive Technologies for Endangered North American Salamanders

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Development of assisted reproductive technologies for endangered North American

salamanders

By

Ruth Marie Marcec

A Dissertation
Submitted to the Faculty of
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Development of assisted reproductive technologies for endangered North American

salamanders

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Amphibians are key health indicator species and important sentinels for ecosystem health. With 32% of amphibians threatened with extinction, and an additional 25% without enough data on record to fully understand their status, amphibians are facing what is being called the Amphibian Extinction Crisis. Of caudate amphibians (salamanders/newts), 49.8% are threatened or endangered, making them currently the most threatened vertebrate taxa. As a result of the Amphibian Extinction Crisis, it is imperative that captive breeding facilities be established as a hedge against future amphibian extinctions. Unfortunately, captive assurance colonies face frequent failure due to lack of natural stimuli, which are necessary for amphibian breeding. Therefore, assisted reproductive technologies (ART) have been employed to reproduce endangered amphibians, increasing the success of captive assurance colonies worldwide. Over the last decade, a great deal of attention has been given to restoring anuran (frog/toad) populations through captive breeding, but little attention has been given to preservation of caudate species. Caudate amphibians are notoriously difficult to breed in captivity, thus it is crucial that ART be developed to help preserve endangered caudates sooner,

rather than later. The following studies highlight some of the first attempts at developing ART protocols in caudate amphibians. Protocols were developed for: hormone induction of gamete production; cryopreservation of salamander sperm; and *in vitro* fertilization. These protocols were developed for the common, model species *Ambystoma tigrinum* in order to assure safety and efficiency for later application in endangered species.

DEDICATION

This research is dedicated to the salamanders. These little creature are deserving of much more recognition than they receive. We hope that these small steps we have achieved with this work will lead to further, greater steps in the preservation of these marvelous creatures.

ACKNOWLEDGEMENTS

the word partner is "part owner," and truly this work is as much yours as it is ours. This work could not have occurred without any of you and we hope you take pride in it. Thank you so very much.

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CHAPTER I

INTRODUCTION AND LITERATURE REVIEW

Crisis and Conservation

The Amphibian Extinction Crisis

Extinction. This is a word that brings to mind prehistoric beasts and rare, almost mythical animals. Very rarely does this word bring to mind the image of a salamander or frog. However, amphibians are currently facing global declines and extinction rates that are greater than any other taxa [Gascon et al, 2005; Hoffmann et al, 2010]. These extinctions and declines have been alarming to scientists as they have characteristics of a potential extinction event. Amphibians of many species started declining rapidly all around the same time, these declines were occurring simultaneously globally, and declines were occurring in areas that should be protected, natural areas [Collins & Storfer, 2003]. With 32% of amphibians threatened with extinction, and an additional 25% without enough data on record to fully comprehend their endangered status, amphibians are facing what is being called the Amphibian Extinction Crisis. Amphibians are broken down into three families: the anurans (frogs and toads), the caecilians, and the caudates (salamanders and newts). Of anurans, 31.6% are known to be threatened or endangered. Of caecilians, only 3.4% are known to be threatened or endangered, but these are a very rare and secretive order of amphibians with 67.0% of species lacking in data sufficient to understand endangered status. Of caudates, 49.8% are threatened or

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endangered, making them currently the most threatened vertebrate taxa [Stuart et al, 2004]. The amphibian extinction rates led scientists to discover that the Earth is likely facing its sixth mass extinction event [Barnosky et al, 2011; Wake & Vrendenburg, 2008]. The last mass extinction event was seen in the Triassic period, and it has been confirmed that the current animal extinction rates are equivalent to, if not faster than, those seen at the time of the previous five mass extinction events [Barnosky et al, 2011]. What is most alarming about this extinction event is that most of the causes of animal extinction are man-driven. Global warming, pollution, and habitat fragmentation are all major contributors to the disappearances of species [Collins & Storfer, 2003; Kolbert, 2014; Wake & Vrendenburg, 2008].

Those who work with amphibians can hardly suppress a shudder when *Batrachochytrium spp* is uttered. The species *B. dendritobates* is infamous for having played a part in declines and extinctions of anurans all over the world, and the recently discovered *B. salamandrovins* has been devastating to salamander populations in Europe [Berger et al, 1999; Fisher et al, 2009; Martel et al, 2013]. Along with viral diseases, such as *Ranaviruses* from the *Iridovirus* family, these diseases have been associated with many of the declines of amphibians [Carey et al, 1999; Daszak et al, 2003; Gray et al, 2009]. As studies continue on these diseases and on the population declines, however, an interesting trend has appeared. *Batrachocytrium spp* and *Iridoviruses* are more virulent at cooler temperatures [Berger et al, 1999; Collins & Storfer, 2003; Daszak et al, 2003; Gray et al, 2009; Wake & Vrendenburg, 2008]. As global warming takes place and the overall climate has become warmer, many amphibians have been waking up earlier from hibernation or going into hibernation later [Blaustein et al, 2001; Corn, 2005; Lips et al,

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2008]. The atmospheric increase in temperature causes a longer autumn and spring season with a shorter winter. While the overall environment is warmer in these circumstances, it results in amphibians staying active longer at cooler temperatures. The temperatures do not drop low enough in order to trigger hibernating behaviors, and so the remain active for longer periods in cool autumn and spring. This longer period of activity has made them more susceptible to the lethal *Batrachocytrium* and *Ranavirus* diseases which are active at cooler temperatures [Blaustein et al, 2001; Corn, 2005; Lips et al, 2008; Wake & Vrendenburg, 2008].

Global warming is most certainly a factor in increasing susceptibility of amphibians to disease, and thus directly declining populations by causing death [Blaustein et al, 2001; Collins & Storfer, 2003; Corn, 2005; Lips et al, 2008; Wake & Vrendenburg, 2008]. However, global warming also plays a role in decline of amphibian populations through decreasing recruitment. Amphibians rely on very specific environmental cues in order to reproduce [Clulow et al, 2014; Kouba & Vance, 2009; Kouba et al, 2009; Roth & Obringer, 2003; Roth et al, 2010; Walls et al, 2013]. These cues trigger release of the hormones, primarily of luteinizing hormone releasing hormone (LHRH) at the head of the hypothalamic-pituitary-gonadal axis, which result in a relay of activity to prepare the body for breeding [Eisthen & Krause, 2012; Kano et al, 2005; Kim et al, 1998]. Many different cues must coordinate in order to trigger these effects. If these cues are not coordinated or do not occur, animals will not breed [Kouba & Vance, 2009; Kouba et al, 2009; Walls et al, 2013; Palis et al, 2006]. Under the circumstances of global warming, frogs and salamanders are coming out of hibernation at inappropriate times [Blaustein et al, 2001; Corn, 2005; Lips et al, 2008]. The temperatures, light cycles, and

barometric pressure are all cues which amphibians rely upon for triggering reproduction. With the temperatures changing earlier, these cues are not coordinated appropriately and many species are suffering poor recruitment or are failing to breed all together [Blaustein et al, 2001; Collins & Storfer, 2003; Corn, 2005; Lips et al, 2008; Wake & Vrendenburg, 2008].

The main cause of global warming has been an increase in man-created air pollution [Allen et al, 2009; Caldwell et al, 1991]. Man-made pollutants in the air, in the water, and in the soil have all been detrimental to amphibian health. This is one of the reasons amphibians have been dubbed an "indicator species." Amphibian skin is highly permeable and either absorbs or is highly affected by the chemicals it comes in contact with. Because of these qualities, amphibians are highly susceptible to chemical changes and toxins in the environment. When something in the environment begins to go awry, amphibians are usually the first group of animals to show signs of illness and death. Scientists tend to use amphibians as sentinels for environmental distress [Beebee & Griffiths, 2005; Collins & Storfer, 2003]. Unfortunately, these qualities that act as a benefit to humans are not a necessarily a benefit to the amphibians. Pollutants are an additional factor in the worldwide declines in amphibians. Pollutants increase amphibian death rates, increase susceptibility to disease, and are a cause for decreased recruitment. Pesticide run off, including pesticides such as atrazine, cause a decreased immune system and increased susceptibility to *Ranavirus* and trematode infection [Forson & Storfer, 2006; Kiesecker, 2002; Rohr et al, 2008]. Water pollutants (i.e. nitrogen waste, coal combustion byproducts, pesticides, heavy metals) are linked to decreased recruitment by

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larval death in multiple species of amphibians [Khan & Law, 2005; Marco & Oritz-Santaliestra, 2009; Roe et al, 2006; Rowe et al, 2001; Wijer et al, 2003].

Pollutants not only have a direct effect on the animal, they have an effect on the animal's environment. Pollutants can make a habitat unlivable or destroy habitats. Habitat destruction and habitat fragmentation, via pollutants, construction, or deforestation, are also causes for amphibian population declines. Habitat destruction can be a cause of both death and recruitment failure. Once amphibians settle into a habitat, they are unlikely to migrate to a new habitat because conditions have become poor. Habitat destruction will not encourage amphibians to leave their homes, like it will with other animal species [Diffendorfer et al, 1995; Wolff et al, 1997]. This leads to death of the animals living within the destroyed habitat. However, many amphibians do migrate annually from their homes to breed. If animals from a destroyed habitat have died and are no longer migrating to meet other populations, recruitment and genetic diversity will decrease for that breeding population [Andersen et al, 2004; Cushman, 2006; Dixo et al, 2009]. Habitat fragmentation can have similar effects on recruitment. Construction of homes and commercial areas can establish physical barriers which amphibians cannot pass during the migratory breeding season [Hitchings & Beebee, 1997]. This decreases opportunities for reproduction, decrease genetic diversity, and decrease overall recruitment [Andersen et al, 2004; Cushman, 2006; Hitchings & Beebee, 1997]. Without recruitment and genetic diversity, populations become smaller and more inbred. They become more susceptible to genetic defects while their numbers decrease. Effectively, they are slowly doomed to face extinction [Dixo et al, 2009].

As important sentinels to the status of the ecosystem, it is crucial to take the dramatic global declines and extinctions of amphibian populations in earnest.

Why study salamanders?

Amphibians are critical components of the ecosystem. As animals that live half of their life in the water and half of their life on land, they contribute to both aquatic and terrestrial habitats in a variety of ways. All of the life stages of caudates have proven incredibly important to forest ecosystems. In the forests of Appalachia, it is estimated that terrestrial populations of salamanders make up almost three times the wet-weight biomass of birds and small mammals [Burton & Likens, 1975; Davic & Welsh, 2014]. Studies confirm that in the woodlands of the northwest, the salamander count is similar to that of the Appalachian forests [Davic & Welsh, 2014]. This tremendous number of salamanders contributes to sustaining the health of both the terrestrial and aquatic components of the forest ecosystem [Davic & Welsh, 2014; Semlitsch et al, 2014].

As either aquatic larvae or terrestrial adults, caudates are top predators in their habitats. Some species are considered "keystone predators," as they are necessary for the management of populations in the trophic chain [Davic & Welsh, 2014; Fauth & William, 1991; Smith, 2006]. By using ambush-predator tactics, and by eating whatever food items will fit in their mouths, caudates regulate the competition between prey species, including benthic macroinvertebrates, detritivores, and pest insects. As salamanders will eat anything that fits in their large mouths, they are also important in regulating populations of small predators. Salamanders are known to be cannibalistic when populations are too high, and they will eat small rodents and fish [Davic & Welsh, 2014; Fauth & William, 1991; Vitt & Caldwell, 2009]. Recently, studies have shown that

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larval salamanders of keystone species will outcompete invasive tadpole species, or ingest invasive tadpoles, when presented with the opportunity. This implies that salamanders are a biological barrier for protecting the environment from invasive species [Smith, 2006].

When salamanders hatch out as aquatic larvae, they have immediately become critical to the health of the water system in which they live. Tiny larvae feed on benthic macroinvertebrates and as they age, they feed on larger insects, fish, and bottom feeders. By feeding on the many levels of aquatic fauna and regulating their populations, salamanders help to maintain appropriate chemical balance of water systems. Salamander behavior and diet help to manage nitrate levels and keep the aquatic system a non-toxic habitat. If larval salamander populations are altered, resulting dramatic changes in trophic chains and in nitrogen cycles can lead to die offs in the water column [Davic & Welsh, 2014].

As salamanders mature from aquatic to terrestrial, the importance they hold in the ecosystem also shifts from aquatic to terrestrial. Terrestrial salamanders are highly important to the health of soil and trees. Terrestrial salamanders burrow in tunnels, both shallow and deep, in order to hide during the daylight hours. These burrowing habits benefit the nitrogen cycle of plants through aeration of soil around plant roots [Davic & Welsh, 2014; Semlitsch et al, 2014; Walton & Steckler, 2005]. As they burrow, salamanders once more consume any food item that will fit into their mouths. Their terrestrial feeding habits, along with balancing the overall trophic levels, help to regulate populations of the micro fauna in the earth. Micro fauna in the soil maintain appropriate levels of leaf litter, detritus, and carrion on the forest floor. This allows for balance in the

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nitrogen cycle, the carbon cycle, and the allowance for appropriate nutrition to reach plant roots [Davic & Welsh, 2014; Walton & Steckler, 2005; Wyman, 1998].

Due to their importance to tree and soil health, salamanders are frequently studied by foresters. Studies are often focused on assurance that the cycling of timber stands will no disrupt the life cycles of the salamanders [Macneil et al, 2014]. More research is warranted in this area, but there is some evidence salamanders could be of economic benefit to the timber industry if cycling of plots is planned appropriately [Macneil et al, 2014; Vanderberg et al, 2012]. There is additional evidence that the presence of salamanders in the ecosystem is important to reducing CO_2 levels emitted. It is hypothesized that, because the burrowing and dietary habits of salamanders result the appropriate levels of detritus and debris in forest floors, it also leads to a lower rate of decay and CO_2 emissions [Wyman, 1998].

As much as they benefit the environment, salamanders are also a benefit to human health through biomedical research. Salamanders are frequently used in biomedical research in order to further the progress and development of human tissue regeneration, human healing, and human immune capabilities. These animals have a notable regenerative capability of tissues which mammals do not commonly regenerate, including nervous tissue, cardiac tissue, and retinal tissue [Godwin, 2014; Islam et al, 2014; Piatkowski & Braun, 2014; Singh et al, 2015; Witman et al, 2011; Zukor et al, 2011]. Culturing and maintaining salamander organ tissues outside the body has also shown to be relatively simple compared to mammals [Piatkowski & Braun, 2014]. Researchers hope that studies of the cell interactions in these tissues can be applied to future replacement of damaged tissue in humans, not only curing disease but extending life

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[Islam et al, 2014; Piatkowski & Braun, 2014; Taylor, 2015]. Caudates have long been used in studies to assess the effects of carcinogens on cell division, cell growth, and cell healing [Keramitsoglou et al, 2002; Koussoulakos, 1992; Tsonis, 1983]. However, a recent graduate student thesis showed greater implications of the newt immune system in humane health. This novel study showed that eastern newt (*Notophthalmus viridiscens*) cells have the ability to fight the effects of these cell stressors and potentially prevent cancer [Linklater, 2011]. Salamander and newt skin cells are also well known to produce antimicrobial secretions to ward off diseases in the immediate environment [Walke et al, 2014]. One study showed that animals of the same species will display different adaptations in their skin secretions, depending on the microbial environment [Brucker et al, 2008; Lam et al, 2011; Qu et al, 2015; Walke et al, 2014]. Additionally, Japanese fire belly newts and the Spanish ribbed newt (*Pleurodeles waltl*) have been sent to space to help us understand the long term effects of space travel on astronauts [Gualandris-Parisot et al, 2001; Yamashita et al, 2001]. Biomedical researchers have learned a great deal from the physiology of salamanders and can learn a great deal more.

A need for salamander studies

Aside from playing crucial roles in the ecosystem, amphibians are important sentinels for disease, toxins, or any sign of disturbance in the environment. These animals are very sensitive to changes, and as such their populations are frequently some of the first to be affected by problems in the health of the ecosystem. This makes amphibians excellent indicator species. Despite caudates having a larger percentage of species verging on extinction, most conservation efforts have focused on anuran repopulation and habitat restoration. Habitat restoration, in many cases, is similar for both species. Many

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species of caudates and anurans breed in vernal, or seasonal, pools. They rely on the wet and dry cycle of these pools for appropriate breeding cues and, later, metamorphosis cues. Efforts have been made to protect and restore these pools, with a focus species such as the common spadefoot toad, crested newts, and wood frogs [Petranka et al, 2007; Rannap et al, 2009]. No doubt caudate species, such as Ambystomids and *Notopthalmus* have benefitted from these efforts. When it comes to the efforts of repopulation, very few efforts have been made on behalf of caudates. Much of the reason for this lack of effort is due to the extreme difficulty in reproduction of caudates in captivity. Assisted reproductive technologies must be developed for caudates in order to increase success of captive assurance colonies and increase the success of reintroductions.

Assisted Reproductive Technologies (ART)

ART as tools in conservation

"The first law of intelligent tinkering is to save all the parts."

-Aldo Leopold

As the earth enters the sixth extinction crisis, it is important for conservationists to maintain populations of animals that are at risk. Preservation of habitat and the environment is also key in order to prevent further loss and to allow animals to prosper. However, if there are no animals left in a population, the efforts of habitat preservation are futile for the purposes of species preservation. With the ecological problems in both the environment and with animal populations being primarily manmade, the question of preserving habitat or populations becomes a chicken and the egg type question. It would be impractical to wait until the habitat was ideal to begin rebuilding populations, as this would cause severe population crashes and genetic disasters [Gascon et al, 2005;

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Pedrono, 2011]. Conversely, focusing only on building populations and ignoring the detriment of the environment would not resolve the population declines. The true answer is that both approaches must be taken for ultimate success [Clulow et al, 2014; Gascon et al, 2005; Kouba & Vance, 2009; Kouba et al, 2009; Langwig et al, 2015].

While captive breeding of animals in order to rescue populations, keep populations safe, and maintain genetics of rare animals is an intuitive process, it is not always straightforward. Many animals are not easy to breed in captivity. Most species rely on external stimuli, such as day length, temperature, or food availability, in order to trigger a hormonal response and cue the breeding season [Hahn, 1995; Hau et al, 2000; Kouba & Vance, 2009; Kouba et al, 2009; Swanson et al, 1996]. Additionally, many species are susceptible to failure of conception, high infant mortality, stillbirths, or rejection of offspring by the mother when bred in captivity [Caro, 1998; Taylor & Poole, 1998; Wildt, 1991]. The reason for these phenomena in most species is still unknown, unstable social groups, diet, and inappropriate behavioral enrichment being some of the hypotheses for cause [Caro, 1998; Debsyer, 1995; Merola, 1994; Silk et al, 1981; Snyder et al, 1996; Taylor & Poole, 1998]. Inability to appropriately determine the timing of estrus, effects of stress and cortisol levels on other hormones, and inbreeding depression are also potential causes [Bahr et al., 1998; Bardi et al, 2004; Carlstead & Brown, 2005; Mason, 2010; Taylor & Poole, 1998].

Because captive breeding can be very challenging to manage naturally, the use of Assisted Reproductive Technologies (ART) are often enlisted. Using these technologies, external stimuli can be bypassed and natural hormone cycles can be simulated [Kouba & Vance, 2009; Kouba et al, 2009; Roth & Obringer, 2003; Watson & Holt, 2001; Wildt,

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1991; Wildt et al, 1995]. In species with small populations, genetics can be shared efficiently across long distances by freezing sperm and utilizing artificial insemination techniques. This way, animals do not need to undergo stressful and potentially dangerous transportation for the sake of breeding [Clulow et al, 2014; Kouba et al, 2009; Wildt et al, 1995]. Gametes can be stored indefinitely to ensure preservation of genetics from aging animals. These stored gametes can be used to help maintain genetic diversity and keep specific bloodlines from dying out [Clulow et al, 2014; Kouba et al, 2009; Watson & Holt, 2001; Wildt et al, 1995].

Assisted reproductive technologies are used commonly in conservation of animals. These techniques were first developed in domestic animals. Artificial insemination (AI) was the first used reproductive technology in animals, and was first reportedly performed in 1784 in the dog [Foote, 2002]. It became widely used in agricultural practice for selection of specific genes throughout the 1930s and is still a technology that is depended on in the industry today [Foote, 2002; Dresser, 1988]. In 1949, it was discovered that sperm of roosters could be frozen and stored at low temperatures using glycerol as a cryoprotectant [Smith & Polge, 1950]. This revolutionized assisted reproductive technologies and the management of genetics in agriculture, as it made genes easy to store and transport long distances.

The application of these technologies became common in wildlife in the late 1900s. Despite techniques such as AI and cryopreservation of sperm being used frequently for the purposes of conservation, these techniques are far from perfected in wild animals and often fail. Due to the difficulty of replicating a perfect environment for animals in captivity, even with the assistance of ART captive breeding is a challenge

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[Snyder et al, 1996; Wildt & Wemmer, 1999]. The idea is to provide what tools and resources that are available to give a species and a population its best chance [Wildt & Wemmer, 1999].

Historical use of ART in captive amphibian facilities

While ART has been commonplace in conservation over the last fifty years, it was not until the last decade that these techniques were applied to amphibians. In general, amphibians rely on reproductive cues from nature in order to begin the breeding season. Cues such as changes in temperature, humidity, day length, or barometric pressure are very common triggers for amphibian reproduction [Duellman & Trueb, 1994; Vitt & Caldwell, 2009]. Examples of how these changes effect the physiology of the animals include light cues. Light cues trigger regulation of melatonin in the pineal gland, which regulates dopamine release. Dopamine inhibits the release of luteinizing hormone releasing hormone (LHRH), so depending on the season LHRH will either be inhibited or increased based on the light cycle [Chowdhury et al, 2008; Norris, 2011; Norris et al, 1985; Norris & Jones, 1987; Ogielska, 2009]. Cues that interacts with light cues are temperature cues. Temperature, which appears to be the stronger cue than light in amphibian species than in other vertebrates, also affects LHRH, mainly by stimulating production. While the cue for stimulation is species specific (i.e. cool temperatures in *Ambystoma tigrinum* and warm temperatures in *Ambystoma laterale*), the temperature, in coordination with the light, must change appropriately in order for the LHRH levels to successfully increase [Kim et al, 1998; Norris, 2011; Norris et al, 1985; Norris et al 1987; Ogielska, 2009].

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Many species of salamanders migrate to breed, typically migrating to a location which has specific requirements, such as grass type, foliage thickness, amount of invertebrate life, or depth of water in order to breed [Caldwell et al, 1991; Means & Baggett, 1996; Palis et al, 2006; Semlitsch, 2008]. Animals migrate to the same location every year and exploratory behavior has not been observed when pond sites become unsuitable for breeding [Semlitsch, 2008]. It is thought that prolactin is involved in stimulating the behavior of migration for reproduction [Duvall & Norris, 1977; Grace, 2003], although some evidence disputes this hypothesis [Hasumi, 2007]. In captivity, the specific cues that trigger the any changes in hormones, leading to the reproductive event, are very difficult to replicate in salamanders. Additionally, the specific requirements for breeding habitat are very difficult to replicate. While some species have been successfully bred by building outdoor captive breeding facilities [Clare, Personal Communication], if specific soil, pH, plant life, or chemical water balance cannot be replicated, breeding will not occur [Caldwell et al, 1991; Horne & Dunson 1, 1994; Horne & Dunson 2, 1994;, Kutka & Bachmann, 1990; Palis, et al, 2006; Rowe et al, 2001; Rowe et al, 1993].

Use of exogenous hormones in amphibians

In anurans, natural cues are as difficult to replicate as in caudates, but this has often been overcome by the use of exogenous hormones to stimulate reproductive behavior or the release of gametes [Clulow et al, 2014; Gascon et al, 2005; Kouba & Vance, 2009; Roth & Obringer, 2003]. Most of these protocols focus on the use of human chorionic gonadotropin (hCG), luteinizing hormone releasing hormone (LHRH), and dopamine antagonists (Ovaprim™: domperidone; Amphiplex ™: metoclopramide)

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[Clulow et al. 2014; Trudeau et al, 2010; Trudeau et al, 2013; Kouba & Vance, 2009; Roth & Obringer, 2003]. These methods have been utilized in salamanders, but frequently are not able to be replicated. Most caudate studies have focused on LHRH, hCG, or arginine vasotocin (AVT) [Figel, 2013; Mansour, et al, 2011; Stoops et al, 2014; Toyoda et al, 2003; Trottier & Armstrong, 1975; Uteshev et al, 2015].

Luteinizing hormone releasing hormone (LHRH), also known as gonadotropin releasing hormone, is a polypeptide which signals from the hypothalamus for the release of luteinizing hormone from the pituitary. In amphibians, LHRH appears to be regulated mostly by external stimuli and environmental cues [Eisthen & Krause, 2012; Stoops et al, 2014; Clulow et al, 2014; Kouba & Vance, 2009; Kouba et al, 2009]. Stimuli such as pheromones and chemosensory cues activate a group of LHRH producing neurons near the Terminal Nerve in the olfactory epithelium, but it has yet to be determined if these receptors are excitatory or inhibitory in amphibians [Kim et al, 2013; Norris & Lopez, 2011; Ogielska, 2009]. Unlike in mammals and avians, LHRH is not pulsatile in amphibians but rather is released continuously [Norris & Lopez, 2011; Norris et al, 1985; Norris & Jones, 1987; Ogielska, 2009]. While amphibians hibernate or bromate during their non-mating seasons, LHRH continues to release at a low rate and luteinizing hormone (LH) and follicle stimulating hormone (FSH) build up in the pituitary for release in large amounts when the breeding season begins. Release of these hormones during hibernation is inhibited by dopamine, which is one of the reasons dopamine antagonists are used in assisted reproductive technologies of amphibians [Kano et al, 2005; Kikuyama et al, 2005; Norris & Lopez, 2011; Norris et al, 1985; Norris & Jones, 1987]. LHRH receptors are present in the gonads as well, and it is hypothesized that

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LHRH acts directly on the gonads to increase steroidogenesis. The steroid hormones, produced by stimulation from the gonadotropins and LHRH, in turn produce a negative feedback loop and inhibit the activity of LHRH [Norris & Lopez, 2011; Norris et al, 1985; Norris & Jones, 1987; Ogeilska, 2009].

The hormone human chorionic gonadotropic (hCG) is one of a family of four related glycoprotein hormones: hCG , follicle stimulating hormone (FSH), luteinizing hormone (LH), and thyroid stimulating hormone (TSH). All four of these glycoproteins consist of one α subunit and one β subunit which come together to form a dimer. The α subunits are identical in each of these hormones; the β subunit is the part of the dimer that gives each hormone its unique quality. The β subunits for these hormones have been found to be highly conserved across all vertebrate species, especially in the receptor reactive sites. Of these four hormones, both LH and hCG share the same receptor [Atger et al, 1995; Moyle et al, 1995]. Because the LH/hCG receptor is shared, it is common to use hCG as substitution, or analog, of LH in medical treatments and in experiments [Zhang et al, 2015]. Human chorionic gonadotropin is created by the human fetus and it functions in human maternal recognition of pregnancy, as well as helps to increase progesterone and maintain the corpus luteum [Senger, 2005]. Even though hCG naturally is a hormone secreted only through the placenta during pregnancy in females, it does have analogous effects to LH in males. In some instances, hCG has a higher affinity for LH/hCG receptors and, because of this, it has been used as a therapeutic replacement for LH [Atger et al, 1995; Moyle et al, 1995; Senger, 2005].

While hCG is only found in primates, LH/hCG receptors are found in most vertebrate species. Across vertebrate species, there is a leucine rich repeat domain

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(LRRD) which is highly conserved that is critical for binding both LH and hCG. While conservation of the binding site does not necessitate binding of the hCG ligand, a study in zebrafish showed that, when given exogenous hCG, the fish LH receptors very specifically bound hCG [Kwok, 2005]. LH, after being released from the pituitary, binds to receptors in the gonads and is responsible for spermiation, ovulation, and testosterone synthesis [Senger, 2005; Norris & Lopez, 2011; Norris et al, 1985; Norris & Jones, 1987].

Additionally, there is evidence that many non-mammalian vertebrate FSH-receptors are not selective for FSH and will bind the other glycoprotein dimers in this family [Bogerd et al, 2005]. Therefore, in these animals, hCG will provide the effects of both FSH and LH. FSH, after being released from the pituitary, binds to receptors on the gonads and is responsible for spermatogenesis, oogenesis, and conversion of testosterone to estrogen [Senger, 2005; Norris & Lopez, 2011; Norris et al, 1985; Norris & Jones, 1987].

Arginine vasotocin (AVT) is a peptide hormone that can act as an analog to the mammalian oxytocin [Norris & Lopez, 2011; Vitt & Caldwell, 2009]. This hormone is typically used in studies of sexual behavior and sexual receptivity [Kim et al, 2013; Propper & Dixon, 1997; Toyoda et al, 2003; Woolley et al, 2004]. The control and production of this hormone is not well understood, but calcitonin, prolactin, and prostaglandin are intricately involved in its regulation [Norris & Lopez, 2011; Norris et al, 1985; Norris & Jones, 1987; Ogielska, 2009]. AVT is known to be involved in mating and courtship behaviors in caudates and it has been shown to have pheromone activity [Rajchard, 2005; Toyoda et al, 2003]. It is also involved in the contraction of the oviduct

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in the female, leading to oviposition, and the contraction of the vas deferens in the male, leading to spermiation [Norris & Lopez, 2011; Norris et al, 1985; Norris & Jones, 1987; Ogielska, 2009].

Historically when using these hormones to exogenously induce reproductive activity in caudates, the species that have been selected have been highly inbred or highly acclimated laboratory species [Mansour et al, 2011; Trottier & Armstrong, 1975; Figel, 2013], or they have been animals freshly caught from the wild during breeding season and are likely to respond to hormone stimulation readily [Uteshev et al, 2015; Stoops et al, 2014].

Use of artificial fertilization techniques in amphibian conservation

Since the first artificial insemination (AI) took place in 1784, humans have been applying this technology to animals in order to manage population genetics and recruitment [Foote, 2002]. It was not until the late 1900s that the application of this technology spread from agriculture into wildlife, and it was not until the last decade that this technology was applied more widely for use in conservation of amphibians. Amphibians were first reproduced by artificial fertilization techniques for laboratory breeding purposes. This had been done as early as the 1930s in *Rana* species and was commonplace in *Xenopus* species in the 1970 and 1980s [Hollinger & Corton, 1980; Rugh, 1934; Wolf & Hedrick, 1971]. Artificial fertilization of this manner was solely for experimental purposes and managing the genetics of laboratory populations. It would be decades later until artificial fertilization would be researched more extensively for purposes of conservation in amphibians. [Clulow et al, 2014; Gascon et al, 2005; Kouba[+] & Vance, 2009; Roth & Obringer, 2003]

The majority of research available on artificial fertilization techniques in amphibians is for anurans, more specifically for *Rana* and *Bufo* species [Clulow et al, 2014; Browne et al, 2006; Browne & Zippel, 2007; Calatayud et al, 2015; Calatayud et al, 2016; Kouba & Vance, 2009; Roth & Obringer, 2003; Roth et al, 2010; Trudeau et al, 2013; Trudeau et al, 2010; Waggener & Carroll, 1998]. More information is becoming available within additional taxa [Sherman et al, 2008; Toro & Michael, 2004; Trudeau et al, 2013; Trudeau et al, 2010; Waggener & Carroll, 1998], however information is still growing. Artificial insemination in most species of anurans must take place via *in vitro* fertilization, as the majority of anurans undergo external fertilization [Duellman & Trueb, 1994; Vitt & Caldwell, 2009]. In the majority of caudates, however, fertilization is an internal process. Therefore, in caudates, artificial fertilization can occur via *in vitro* fertilization or via direct internal AI into the cloaca. While many attempts at *in vitro* fertilization in caudates have followed procedures outlined by anuran protocols [Mansour et al, 2011; Stoops et al, 2014; Trottier & Armstrong, 1975], there have been some reports of attempted internal artificial insemination in aquatic species of caudates with success [Toyoda et al 2003; Watanabe et al, 2003; Watanabe, 2010].

As caudate populations continue to decrease, captive assurance colonies seek tools which allow efficient ways to both help increase numbers and manage population genetics [Kouba & Vance, 2009; Kouba et al, 2009; Clulow et al, 2014; Roth & Obringer, 2003]. *In vitro* fertilization (IVF) is one such tool. Through either by hormonally inducing animals to produce gametes, or by collecting gametes from reproductively active animals, IVF allows the selectively pairing of genetics through selective uniting of gametes [Browne & Zippel, 2007; Kouba & Vance, 2009; Kouba et

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al, 2009; Wildt et al, 1995; Wildt et al, 1999]. Underrepresented genetics in a population can be represented more prominently through the use of IVF. Genetics of aging founders may be preserved within a population where, in the wild, there would be little chance of mating opportunities for aged animals The opportunities provided with IVF help ensure that valuable genetics continue to be maintained within a population [Browne & Zippel, 2007; Kouba & Vance, 2009; Kouba et al, 2009]. IVF allows for efficient and low risk long distance matings; animals in distant populations can be bred by shipping gametes rather than entire animals. By shipping only gametes, stress is decreased, risk to the animal is decreased, and, with ease of shipment of multiple gametes, the opportunity for multiple breeding attempts is increased [Kouba & Vance, 2009; Kouba et al, 2009; Wildt et al, 1995; Wildt & Wemmer, 1999]. The potential to increase fertility also exists with IVF due to the opportunity to control variables affecting the sperm and the eggs. Variables, such as sperm concentration, can be manipulated in order to achieve maximal fertilization [Browne & Zippel, 2007; Kouba & Vance, 2009; Kouba et al, 2009].

The sperm concentration is a critical component to consider when performing an IVF. If the sperm to egg ration is too low, low fertilization will occur. However, if the sperm to egg ratio is too high, there is a danger, particularly in lower vertebrates, of polyspermy. In amphibians and other lower vertebrates, the occurrence of multiple sperm fertilizing the egg, or polyspermy, occurs much more frequently than in higher vertebrates. If too many sperm fertilize the egg, abortion will occur. There are mechanisms in place in all vertebrates and most invertebrates which prevent polyspermy from occurring. These mechanisms differ slightly in lower vertebrates and higher vertebrates. Most animals have what is called a "fast polyspermy block" mechanism. This

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mechanism is activated almost immediately after fertilization when contact of the spermatozoa with the oocyte membrane results in rapid depolarization of the oocyte. Depolarization occurs much faster in lower vertebrates than in mammals. This results in faster repolarization of the egg, as well. It has been determined that, due to the rapid repolarization of the egg, many lower vertebrates can be re-fertilized sooner than mammals. [Harada et al, 2011; Iwao, 2012; Toro & Michael, 2004]. In order to ensure that too many sperm do not re-fertilize the egg and cause abortion, it is important to finely balance the sperm to egg ratio when performing an IVF so that enough fertilization is achieved without causing polyspermy.

Due to the nature of their environmentally hardy sperm and eggs, IVF is rather simplistic in amphibians. Because most anurans, and some caudates, undergo extremal fertilization, it is natural to collect sperm and eggs and combine them in the external environment. For such animals, it IVF can consist of collecting sperm and eggs, either by hormonal induction or in seasonally breeding animals, and combine them in a Petri dish [Browne & Zippel, 2007; Kouba & Vance, 2009; Kouba et al, 2009]. Unfortunately, most caudates do not undergo internal fertilization and this presents some challenges to the typical amphibian IVF model. However, regardless of fertilization method, caudate eggs and sperm are designed to survive in the external environment. While it may not be natural for fertilization to occur externally in most caudates, the sperm and the eggs survive readily in the environment, allowing for external IVF attempts in the same manner as externally fertilizing amphibians. The sperm of internally fertilizing caudates is naturally deposited into the environment in a condensed "packet" which is called a spermatophore. The spermatophore can survive to the outdoor environment, sometimes in

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freezing temperatures, for anywhere from hours to days dependent on species [Russell et al, 1981; Selmi et al, 1997; Sever et al, 1985]. Much like anuran eggs, caudate eggs are anamniotic. Therefore, they must be laid in water or in moist areas so that they can effectively expel waste as they develop exposed to the environment [Duellman & Trueb, 1994; Vitt & Caldwell, 2009]. Because caudate sperm and eggs are designed to survive in an external environment, use of the same protocols for IVF in externally fertilizing amphibians are plausible.

An additional tool which makes IVF even more efficient is cryopreservation of gametes. By banking genetic resources for use in IVF, valuable genetics can be maintained effectively.

Use of genetic resource banking in amphibian conservation

The turning point in cryopreservation science was the discovery of glycerol for use as a cryoprotective agent in rooster sperm in 1949 [Smith & Polge, 1950]. After this point, the understanding of cryopreservation and cryoprotectants increased dramatically. Application in agriculture. Soon after, cryoprotective protocols for bulls were developed, with boars and rams following. It was nearly thirty years before cryoprotective protocols would be successfully used in wildlife [Wildt, 1991].

Cryopreservation of gametes is an invaluable tool for use in wildlife conservation. Developing genetic resource banks for threatened and endangered species is a benefit to the species, to the ecosystem, and to the overall conservation management of a population [Wildt et al, 1995; Wildt & Wemmer, 1999]. The primary benefit of a genetic resource bank is the ability to maintain genetic diversity. Cryopreservation grants the ability to maintain gametes from genetically valuable animals indefinitely at low

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temperatures. Genetics can be stored and utilized long after animals are senescent or have died. The long term storage of gametes is a critical genetic resource to draw from in order to manage dwindling populations appropriately [Clulow et al, 2014; Kouba & Vance, 2009; Wildt et al, 1995; Wildt & Wemmer, 1999]. When breeding animals naturally, there is always a chance that animals will not mate. By having a genetic resource bank, the ability to perform artificial insemination or *in vitro* fertilization is always available in the event a natural mating fails. The use of cryopreserved sperm in breeding events also allows for more breeding events over longer distances. It is impractical, stressful, and unsafe to ship animals over long distances for breeding events. Using stored gametes, multiple packages can be sent over a great distance to multiple institutions, extending the ability to manage genetics more efficiently. Genetic resource banks also allow management of captive populations more efficiently, as they allow for keeping fewer captive animals. The equipment for keeping and maintaining a genetic resource bank is inexpensive and takes up very little space, while animal care is expensive and animal enclosures take up space. By collecting and storing gametes and keeping minimal animals, captive colonies can be more efficient and economic [Gascon et al, 2005; Clulow et al, 2014; Kouba & Vance, 2009; Wildt et al, 1995; Wildt & Wemmer, 1999].

Over the last decade, more cryopreservation protocols have been developed for anurans to help maintain genetic diversity as their populations decline [Clulow et al, 2014; Kouba & Vance, 2009]. Most of these protocols have been based on protocols for freshwater fish, as fish sperm have similar osmolality-related properties to anuran sperm: the spermatozoa are activated when there is a decrease in osmolality [Browne & Zippel, 2007]. A great deal of research has been devoted to the cryopreservation of fish gametes

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for agricultural purposes and interests. Dimethyl sulfoxide (DMSO), methanol, and glycerol are the most typical cryoprotectants used in fish cryopreservation, with extenders including Ringer's solution, phosphate buffered saline (PBS), sucrose, egg yolk, and bovine serum albumin (BSA) [Cabrita et al, 2010; Muchlisin, 2005]. Most frog studies have focused on using DMSO, glycerol, sucrose, BSA, or egg yolk extended in Ringer's or PBS [Browne & Zippel, 2007; Clulow et al, 2014; Kouba & Vance, 2009]. There is some evidence that DMFA and trehalose is a winning combination in anurans [Kouba & Vance, 2009; Langhorne, 2015]. Very little information is available in salamander cryopreservation. The only reported cryoprotectants used in salamanders are sucrose [Figel, 2013], DMSO [Marcec et al, 2014; Unger et al, 2013], and glycerol [Unger et al, 2013].

The cryoprotectant is critical in the success of a protocol. However, as it was discovered in the 1950s when glycerol was applied to bovine sperm, the needs of the sperm are species specific [Fickel et al, 2007; Holt-1, 2000; Kouba & Vance, 2009; Suquet et al, 2000; Wildt et al, 1995; Wildt & Wemmer, 1999]. There is evidence that the needs of the sperm are individual specific, as individuals have different populations of sperm in each ejaculate and this must be accounted for in the way the individual reacts to the cryoprotectant [Cabrita et al, 2010; Holt-1, 2000; Holt-2, 2000; Kouba & Vance, 2009; Parks & Graham, 1992]. Cryoprotectants interact with the phosophlipid membrane of the sperm in order to prevent damage during the cooling and thawing periods. During cooling, the membrane hardens and ice crystals form both inside and outside of the cell. During thawing, the membrane becomes fluid again and the crystals inside and outside the cell melt. Damage can be inflicted to the cell during these processes, by tearing or

rupturing of the membrane or organelles [Holt-1, 2000; Holt-2, 2000; Medeiros et al, 2002; Parks & Graham, 1992].

Cryoprotectants protect cells from the stress which is caused by freezing and thawing. There are two major classes of cryoprotectants: permeating and non-permeating. Permeating cryoprotectants (i.e. DMSO or glycerol) enter the cell while non-permeating cryoprotectants (i.e. sucrose or BSA) do not cross the cell membrane [Holt-1, 2000; Holt-2, 2000; Sztein et al, 2001; Suquet et al, 2000]. There are a number of ways in which cryoprotectants help to prevent damage to the cell during freezing and thawing. The main goal of cryoprotectants is to prevent cell shrinking and lysis during the process of freezing and thawing. During freezing, ice crystals form outside cells and form "pockets" of hyperosmolar areas. Cells trapped in these areas are susceptible to severe dehydration. The addition of cryoprotectants help so that less osmotic stress is placed on the cell [Holt-1, 2000; Holt-2, 2000; Parks & Graham, 1992; Sztein et al, 2001]. However, if care is not taken to appropriately balance the cryoprotectants and use an appropriate cooling rate, the cryoprotectants themselves can cause severe cell stress or lysis. Cryoprotectants also are meant to help prevent or protect the cell from ice crystal formation. Many cryoprotectants are thought to increase fluidity of the phospholipid membrane so that it becomes less fragile and less likely to break during the cryopreservation process [Holt-1, 2000; Holt-2, 2000; Parks & Graham, 1992]. Cryoprotectants can also reduce oxidative damage to cells by acting by reducing reactive oxygen species and lipid peroxisome damage [Bansal & Bilaspuri, 2010; Uysal & Bucak, 2007]. After preliminary trials, four cryoprotectants were used in the course of these salamander studies: DMSO, sucrose, trehalose, and BSA.

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The cryoprotectant DMSO is a permeating cryoprotectant. It is a low molecular weight permeating cryoprotectant [Anchordoguy et al, 1987]. While there are some aspects of the mechanism of action of DMSO which are not understood, it is known to make cells more permeable, especially to hydrophobic entities. It does this by forming holes in the phospholipid membrane which allow small molecules to pass through. It also disrupts the formation of ice crystals, making them less destructive to cells [Anchordoguy et al, 1987; Gurtovenko & Anwar, 2007] .

Sucrose and trehalose are sugars that act as non-permeating cryoprotectants. Some animals utilize these sugars as natural cryoprotectants to help prevent damage to their cells when they freeze during the winter [Fuller, 2004]. Both are glucose disaccharides and non-permeating cryoprotectants. They are capable of protecting the cell membrane integrity, proteins, and liposomes [Anchordoguy et al, 1987; Storey et al, 1998; Sztein et al, 2001]. Trehalose also acts as a non-enzymatic antioxidant [Bansal & Bilaspuri, 2010]. Bovine serum albumin (BSA) also acts as a non-enzymatic antioxidant cryoprotectant. It is a small protein that decreases reactive oxygen species and protects the membrane from lipid peroxidation. It also is important in maintaining membrane integrity, and increasing fertility and motility in certain species upon thawing [Bansal & Bilaspuri, 2010; Uysal & Bucak, 2007].].

While cryoprotectants are crucial to successful cryopreservation, the freezing and thawing rates are what is key to success. A freezing rate must be finely balanced so that osmolality is not disrupted to quickly and the cells do not dehydrate. Likewise, thawing must be regulated so that cells do not lyse. It is ideal to have a freezing rate at which vitrification occurs. Vitrification is the formation of a glass-like state in which no ice

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crystals are formed. Vitrification has not been a method commonly used in anurans, and in attempts to vitrify amphibian sperm repeatedly it was found results were variable within the same batch of sperm [Sargent & Mohun, 2005]. Vitrification has not been a reported method used in caudate amphibians. Thus far, only three reports of success in freezing and thawing viable caudate sperm have been made: two in Ambystomid species [Figel, 2013; Marcec et al, 2014], and two in Cryptobranchids [Unger et al, 2013; Peng et al, 2013]. All of these experiments utilized different freezing and thawing methods with variable success. However, there is still much to be learned about the physiology and the effect of cryopreservation on salamander sperm.

Salamander Reproduction

Salamander Sex Organs

The caudate urogenital tract is primitive. In most male caudates, the genital kidney, or the sexual kidney, lies cranially and is connected to the testicle by the vasa efferentia tubules. These tubules empty into the specially adapted nephrons of the genital kidney that transport sperm into the retained Wolffian duct. The Wolffian duct either empties directly into the cloaca or anastomoses with the collecting ducts of the kidney where they empty into the cloaca, dependent upon species [Siegel et al, 2013; Siegel et al, 2012; Siegel et al, 2014; Siegel et al, 2010]. In females, the ovary and oviduct are distinct from the urinary tract. The ovary proper lies caudally in females. When oocytes are mature, they are moved through the coelomic cavity cranially by ciliary movement to the ostium at the head of the oviduct. As eggs pass through the oviduct, they pass through the ampulla where they obtain their egg jelly. Just before the cloaca, there is a pouch called

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the ovisac where ovulated eggs are retained until the female is ready to lay them [Sever et al, 1996; Yamahama & Onitake, 2002].

The cloaca of salamanders is a highly specialized sex organ. While caudates have no external genitalia, the cloaca functions as a sex gland that produces secretions, produces pheromones, and stores sperm in females. The Cryptobranchidae and Sirenidae orders are considered more primitive and have rudimentary specialized structures and glands that do not function as in the more evolutionarily advanced species [Sever & Brizzi, 1998; Sever et al, 1996]. Male salamanders have a proctodeal gland located at the anterior opening of the cloaca [Duellman & Trueb, 1994]. While the function of this gland is unknown in amphibians, this gland has been shown to produce secretions that increase the motility and stamina of sperm in avians [Adkins, 1999; Mohan et al, 2002; Singh et al, 2011]. Most female caudates have at least one small "pouch" off the side of the cloaca called a spermatheca. The spermatheca is responsible for storing sperm until fertilization occurs. Fertilization in most salamanders (again, the exclusions are Cryptobranchidae and Sirinidae [Sever, 1991; Duellman & Trueb, 1994; Vitt & Caldwell, 2009]) occurs internally after the female picks up what is called a spermatophore: a condensed packet of sperm deposited by the male into the environment. After a female picks up the spermatophore with her cloacal lips, the sperm is stored in the spermatheca until oviposition occurs. It is supposed that when the female is ready to lay, she pushes the eggs from the ovisac into the cloaca and they are fertilized as they pass the spermatheca. It is still unknown, however, how fertilization occurs internally in caudates once the spermatophore is inside the cloaca [Charney et al, 2014; Gonzalez-Leon & Ramirez-Pinilla, 2011; Hardy & Dent, 1987; Sever, 1991; Sever & Brizzi, 1998].

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The spermatheca allows for the storage sperm from multiple males and prior to deposition of the sperm directly upon their eggs as the eggs exit the cloaca [Adams et al, 2005; Jones et al, 2002; Osikowski, 2007; Sever, 1991; Sever, 2002; Sever et al, 1998]. This mechanism allows some amount of genetic competition, as the male which mates with the female first appears to have advantage over the other males [Adams et al, 2005; Jones et al, 2002; Tennessen & Zamudio, 2003]. The method of internal fertilization has been uniquely utilized by some species of Ambystomid salamanders that have populations which are entirely female. These populations utilize a reproductive strategy called kleptogenesis in which they steal spermatophores from a related species, using enzymes within the sperm to activate their eggs [Bogart et al, 2009; Bogart et al, 2007]. While no DNA is transferred in this fertilization mechanism, occasionally the extra genome of the male is take on by the activated egg, making the new generation polyploidic. This polyploidy is how the entirely female populations of salamander manage to maintain genetic diversity [Bi et al, 2009; Noel et al, 2011].

Salamander Sexing

In order to breed salamanders, it is crucial to know what sex the salamanders are. Although it may seem a simplistic and straightforward task to sex an animal, the sex of an amphibian is not always obtained with ease. As mentioned previously, amphibians lack external genitalia and only have a cloaca. They also are most often lacking in sexually dimorphic characteristics [Duellman & Trueb, 1994; Vitt & Caldwell, 2009]. Determining sex in an accurate, efficient, and non-invasive manner can be a true challenge in amphibians. This task can become particularly difficult in the field where

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sexing of animals requires capture and release [Ollivier & Welsh, 2003; Romano et al, 2009].

As salamanders are dioecious, it is important to ensure at least one male and one female, or gametes from both a male and a female, are present for breeding attempts. Without accurate knowledge of the sex of animals, groups may be housed inappropriately, irrelevant breeding attempts may be made, and time and effort may be put into obtaining gametes from a wrongfully sexed animal [Clulow et al, 2014; Germano et al, 2011; Kouba & Vance, 2009; Kouba et al, 2009]. For captive assurance colonies to be successful, it is critical for the success of that the true sex of animals in both breeding programs and in the remaining wild populations be known so that reproductive attempts may be successful and that genetic integrity may be upheld.

Traditionally, caudates had been sexed by visually. In many salamander families, including Salamandridae, Plethodontidae, Ambystomidae, and Proteidae, seasonal papillae are a recognizable trait in males [Sever, 1991]. These papillae are referred to as a seasonally dimorphic trait, and have frequently been used as an indicator of the sex of caudate amphibians [Ollivier & Welsh, 2003; Pauly et al, 2007; Sever, 1991]. Visual sexing of salamanders can also occur via use of sexual size dimorphism (SSD). This method has commonly been utilized as an approach to sexing caudate amphibians [Bakkegard & Guyer, 2004; Bruce 2000; Raymond & Hardy, 1990; Todd & Davis, 2007]. Most often, females are noted to be the larger of the species, which is consistent in other species of amphibians, reptiles, and fish [Parker, 1992; Shine, 1979]. There is a hypothesis that in animals with SSD, the size is the causal factor for the sex. That is, animals that are larger become female, not animals that are female become larger. This

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hypothesis applied to the lower vertebrates which have sex determination genes that are more malleable by the environment [Shine, 1979]. In salamanders, females typically have a larger girth, a longer snout vent length, and a greater mass while males have a longer tail and a longer cloaca [Alcorn et al, 2013; Bakkegard & Guyer, 2004; Bruce, 2000; Hasumi, 2007; Raymond & Hardy, 1990; Todd & Davis, 2007; Williams et al, 2009].

New, accurate, and efficient ways to sex amphibians are always under investigation. While ultrasound has become more popular for the use of tracking follicular development in female amphibians [Kouba et al, 2009; Reyer & Batting, 2004; Roth & Obringer, 2003], and can be applied to the sexing of animals [Miller & Fowler, 2012; Li et al, 2010]. The ultrasound procedure is an quick and non-invasive as collection of morphometric data. It requires minimal handling and poses little to no risk to animals. It also allows a view of the actual internal genitalia of the animals. While ultrasound is a tool that is highly available to most zoos and higher institutions of learning, it is also a tool that requires training and experience. Training beyond handling of animals would be required in order to use ultrasound as a sexing tool, unlike with collection of morphometric data.

Additional methods of sexing amphibians have potential. Collection of urine and feces for hormone analysis is a plausible sex determination method [Kouba et al, 2009; Kramer et al, 1983; Roth & Obringer, 2003]. While this is a non-invasive method and requires little to no handling of animals, it is less practical in field application than in captive application. The use of endoscopic surgery is an accurate method that has been proposed for sexing of amphibians [Chai, 2015; Kouba et al, 2009; Kramer et al, 1983; Roth & Obringer, 2003]. While this is likely an extremely accurate method of sexing, it is

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also very high risk. When considering endangered species, the risk of anesthesia may be too great for a sexing procedure and alternative methods should be considered.

Salamander reproductive hormone cycles

In almost all vertebrates, the functions of the hypothalamic pituitary gonadal axis (HPG) are conserved [Awruch, 2013; Sower et al, 2009]. The hormone luteinizing hormone releasing hormone (LHRH) stimulates the pituitary to produce and release gonadotropins, which then go on to simulate steroidogenesis, oogenesis, and spermatogenesis in the gonads. The regulation and movement of hormones, however, varies between classes of animals . In considering the endocrine system of a caudate amphibian, one must not assume that hormone cycle are the same as a mammal, an avian, or even a frog [Bentley, 1998]. Indeed, even within caudates hormone cycles will vary [Norris & Lopez, 2011; Norris et al, 1985; Norris & Jones, 1987; Ogielska, 2009]. There are animals that breed annually, biannually, and biennially. There are animals that breed in the spring and animals that breed in the fall. While, in general, the same hormones are responsible for the same actions in the reproductive system in caudates, different levels and different peaks will be occurring in different species throughout the year [Benner & Woodley, 2007; Norris & Lopez, 2011; Norris et al, 1985; Norris & Jones, 1987; Ogielska, 2009].

While there is a great deal of temporal variation within caudates, the general hormone cycle is very similar. In the caudate system, LHRH is released continuously, much like in anurans. To keep this constant secretion in check, dopamine is used as a regulator hormone. Environmental cues, including temperature and light changes, cause a release of dopamine. Dopamine appears to be responsible for partial inhibition of LHRH

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during the non-breeding season in caudates, inhibiting an increase of reproductive activity when it is inappropriate [Norris & Lopez, 2011; Norris et al, 1985; Norris & Jones, 1987; Ogielska, 2009]. However, LHRH is still secreted at a low rate during the non-breeding season and hibernation. It signals the production of luteinizing hormone (LH) and follicle stimulating hormone (FSH) in the pituitary during this time, but dopamine inhibits release of these hormones from the pituitary until hibernation is at an end [Kano et al, 2005; Norris & Lopez, 2011; Norris et al, 1985; Norris & Jones, 1987; Ogielska, 2009].

The environment signals the increase of LHRH in caudates at the beginning of the breeding season so that the secretion rate will increase and so that reproductive activities may begin [Eisthen & Krause, 2012; Norris & Lopez, 2011; Norris et al, 1985; Norris & Jones, 1987; Ogielska, 2009]. In addition to signals received from light and temperature, caudates receive chemical signals which act on a group of LHRH producing neurons located near the Terminal Nerve in the olfactory epithelium [Dellovade et al, 1998]. These neurons respond to chemosensory cues and pheromones, but research has not yet determined if the neurons respond to inhibitory cues or excitatory cues. Regardless, as newt mating relies heavily upon pheromone stimulation, the location of these cells is optimal [Dellovade et al, 1998; Rajchard, 2005].

LHRH in caudates reaches the pituitary directly through release from hypothalamic nerve endings. Once LHRH has been released into the pituitary, it stimulates additional synthesis and release of FSH and LH [Norris & Lopez, 2011; Norris et al, 1985; Norris & Jones, 1987; Ogielska, 2009]. Because the gonadotropins were increasing slowly over the non-breeding season and hibernation, the increase of LHRH at

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the beginning of the breeding season signals the release of these stored hormones and a large peak of these hormones can be noted entering the bloodstream, signaling the animal's body to prepare for reproduction [Kano et al, 2005; Norris & Lopez, 2011; Norris et al, 1985; Norris & Jones, 1987; Ogielska, 2009]. These gonadotropic hormones act upon the gonads to stimulate steroidogenesis, oogenesis, and spermatogenesis, and they upregulate the uptake of more gonadotropins. Estrogens and androgens are then produced by the gonads produce a negative feedback loop on LHRH. Estrogen also regulates vitellogenesis, or egg yolk deposition, through receptors in the liver and adds to the production of egg jelly [Guerriero et al, 2009; Polzonetti-Magni et al, 2004]. Both estrogen and androgens cause seasonal growth in the cloacal sex glands. In caudates, LH stimulates production of progesterone along with the corpus luteum as oocytes reach maturity [Norris & Lopez, 2011; Norris et al, 1985; Norris & Jones, 1987; Ogielska, 2009]. Progesterone down regulates estrogen, halting vitellogenesis and courtship behavior [Guerriero et al, 2009; Norris & Lopez, 2011; Norris et al, 1985; Norris & Jones, 1987; Ogielska, 2009]. Progesterone, secreted by both the corpora lutea and the oocytes, ensures proper maturation of the oocytes and prepares them for oviposition [Norris & Lopez, 2011; Norris et al, 1985; Norris & Jones, 1987; Ogielska, 2009]. The hormone arginine vasotocin (AVT) causes oviposition in females and spermiation in males by contraction of the oviduct and the vas deferens, respectively, but the endocrine trigger for release of this hormone is not entirely understood [Norris & Lopez, 2011; Moore et al, 1992; Toyoda et al, 2003]. AVT is an important regulator of behavior and pheromones in caudates, as well [Kim et al, 2013; Norris & Lopez, 2011; Norris et al, 1985; Norris & Jones, 1987; Ogielska, 2009; Toyoda et al, 2003]. Prostaglandin ($PGF2_a$)

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is responsible for release of stored sperm and fertilization of the eggs. As the eggs are oviposited, the stored sperm within the spermatheca is expelled by the prostaglandin release [Hardy & Dent, 1987; Norris & Lopez, 2011; Norris & Jones, 1987]. Many of the other hormones that are involved in newt reproduction are still not entirely understood. Prolactin (PRL) is involved in many behavioral responses, such as migratory behavior, tail growth, and courtship dancing, and also plays a part in egg jelly deposition [Hasumi, 1987; Polzonetti-Magni et al,2004; Toyoda et al, 2005; Toyoda et al, 1996; Yazawa et al, 2001]. Angiotensin II may play a role in fertility and osmoregulation of eggs [Toyoda et al, 2003; Norris & Lopez, 2011; Norris et al, 1985; Norris & Jones, 1987]. Thyroid hormone plays a role in vitellogenesis and regulation of estrogen receptors, but this is not well understood [Norris & Lopez, 2011; Norris et al, 1985; Norris & Jones, 1987]. Melatonin, which is primarily made in the retina of salamanders, is thought to have a role in seasonality [Chowdhury et al, 2008; Norris & Lopez, 2011; Norris et al, 1985; Norris & Jones, 1987].

Salamander oogenesis and ova structure

The oocyte is a cell within the ovary of an animal that is maintained in the diplotene stage of prophase I, awaiting ovulation and progression into the remaining stages of meiosis. During the embryonic and larval stages of amphibian life, oocytes are developed from the primordial germ cells. Primordial germ cells undergo mitosis and multiply into primary oogonia staged cells, which can be present throughout an amphibians' life. During the developmental stages of the ovary, somatic cells will migrate and proliferate through the first primary oogonia, distinguishing the centralized medulla of somatic cells and cortex of germ cells. Primary oogonia continue to undergo

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mitosis. When primary oogonia reach what will be their final mitotic division, they form cytoplasmic bridges between cells and are now called secondary oogonia. At this stage, secondary oogonia will begin to enter meiosis and form meiocyte cells. As meiocytes begin to form, primary oocytes and secondary oocytes will still be present within the ovary. Cells within the ovary are ordered, from outside of the cortex in, least mature to most mature. Therefore, primary oogonia will be near the outer cortex and meiocytes will be near the medulla. Oocytes (cells arrested in the diplotene stage of prophase I) begin to arise from the meiocytes nearest to the medulla. Follicles envelope each individual oocyte, and the oocytes begin to protrude into the ovarian lumen within the coelomic cavity, preparing for ovulation [Duellman & Trueb, 1994; Ogielska, 2009; Norris & Lopez, 2011; Norris & Jones, 1987; Sever et al, 1996; Vitt & Caldwell, 2009]. When the oocytes ovulate, they are set loose into the body cavity to make their way cranially to the ostium of the oviduct. Cilia within the wall of the body cavity actively move the oocytes to their destination [Yamahama & Onitake, 2002; Ogielska, 2009]. The ostium of the oviduct welcomes the oocytes, from which they enter the infundibulum. The first portion of the oviduct is a relatively short portion called the *pars recta*. Within the *pars recta*, oocytes are moved toward the cloaca by ciliary motion. Secretory cells within this tubule deposit the vitelline membrane onto the oocytes as they pass by. The portion of the oviduct following the *pars recta* is referred to as the *pars convoluta* and is a long, tortuous tubule. Cells are propelled forward through this tubule, again, by ciliary movement, but in a rotational manner. In the *pars convoluta*, secretory cells are responsible for deposition of the thick jelly coat of the amphibian egg. The oocyte is rotated by these secretory cells so that it may be layered with each coat of jelly

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appropriately. At the end of the pars convolute, the egg is deposited into the ovisac. The ovisac is frequently a common pouch shared by the exit of both oviducts and has been referred to as a "uterus." The ovisac, or ovisacs, depending on species, open into the cloaca. As the oocytes pass through the cloaca, they pass by the spermatheca and sperm which has been stored by the female is directly placed onto the eggs where fertilization occurs [Duellman & Trueb, 1994; Ogielska, 2009; Sever, 1991; Sever, 2002; Sever et al, 1996; Vitt & Caldwell, 2009].

Oogenesis in amphibians is generally thought to be sustained throughout life, unlike in mammals. Primary oogonia act as stem cells, rebuilding the store of germ cells before each breeding season begins. In most species, oogenesis is an annual cycle, so that oocytes are mature at the beginning of each breeding season [Gilbert, 2000; Ogielska, 2009; Vitt & Caldwell, 2009]. There is conflicting information that suggests amphibians are, in actuality, much like mammals and have a set number of ooctyes once they reach maturity. These data suggest that the oocytes are recruited each season undergo vitellogenesis, but cells cannot effectively undergo the first stages of meiosis once the animal is of a certain age [Ogieleska, 2009]. More study should be done in this area in order to fully understand oogenesis in amphibians.

If ovulation does not occur during the breeding season, mature oocytes can undergo atresia. This process is commonly referred to as resorption of the eggs. When the oocyte does not undergo ovulation, the follicular cells transform into phagocytic cells and begin to digest the oocyte [Gilbert, 2000; Ogielska, 2009]. The atresia of oocytes is widely recognized, but not well studied. It is commonly seen in animals with poor nutrition [Ogielska, 2009]. On rare occasion, oocytes may become caught in a tubular

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fold of the oviduct and unable to move forward. This phenomenon is referred to as egg retention. If the egg is not removed, it can adhere to the oviduct forming a cyst and, sometimes, resulting in sepsis. Surgery is typically required in order to remove retained oocytes [Wright & Whittaker, 2001].

There is great interest in the size of eggs and number of eggs deposited in a clutch. In it more beneficial to lay more eggs of a smaller size or fewer eggs of a large size? Is there a connection to maternal nutrition and fitness and overall egg or clutch size? While these types of questions have yet to be answered, some studies have taken place to attempt to shed light on the mystery. Older animals tend to lay larger eggs, and more evenly distributed in size, eggs than younger animals [Crespi & Lessing, 2004; Kaplan, 1985; Semlitsch & Gibbons, 1990]. Larvae from large eggs have different survivability than larvae from small eggs, dependent on environmental conditions. Larvae from large eggs develop slower and, overall, tend to survive better ideal conditions or conditions where food is limited [Kaplan, 1985; Semlitsch & Gibbons, 1990]. Conversely, more larvae from small eggs and large clutches are more likely to survive in a drought or in permanent ponds with increased predation [Semlitsch & Gibbons, 1990]. Females tend to invest more energy in large eggs and smaller clutches when laying in typical temporary ponds whereas they tend to lay large clutches with smaller eggs when they lay in a permanent pond [Woodward, 1982]. Part of the complication in these types of studies in the variability in clutch and egg size across species. It is difficult to attest that there are trends across species when the life histories of each species are so dynamic [Bernardo, 1996].

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Salamander spermatogenesis and sperm structure

The discrete testicular organ in salamanders is made up of multiple cysts. Cysts form when the primordial germ cell is enveloped by a Sertoli cell, creating the wall of a cyst [Bergmann et al, 1982; Olgielska, 2009]. Spermatogenesis takes place within the cysts of the testicle. Near the wall of the cyst, nurtured by the Sertoli cells, spermatogonia undergo mitosis within the cysts. The spermatogonia divide to form generations of secondary spermatogonia. These secondary spermatogonia are capable of entering meiosis. The process of spermatogenesis, or the transition of the spermatid to spermatozoa, occurs after the second meiotic division. During this process, the acrosome develops and the spermatids begin to elongate [Gilbert, 2000; Ogielska, 2009]. The cysts remain closed until the spermatids begin to elongate; at this point the cysts rupture and release the spermatozoa into the lumen of the vas efferens [Ogielska, 2009; Siegel et al, 2014]. The Sertoli cells remain attached (unlike in anurans, where the cells detach) and transform into hormone secreting cells [Bergmann, 1982; Ogielska, 2009]. The Sertoli cells also form the blood testes barrier of the caudate testicles. Until the later stages of spermatogenesis, this barrier remains permeable. As spermatogonia multiply and mature, the junctions between Sertoli calls become tighter and more intricate, thus making the barrier effective [Ogielska, 2009].

The process of spermatogenesis in amphibians is very similar to spermatogenesis in mammals. There are two main stages: pre-spermatogenesis and active spermatogenesis. Pre-spermatogenesis occurs in larval and juvenile stage amphibians that have not yet reached maturity. In this stage, a large supply of primary spermatogonia is formed and stored until further development can occur. Active spermatogenesis has

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multiple stages, which are based on the phase of meiotic division. The stages of sperm development are: primary spermatogonia, secondary spermatogonia, primary spermatocytes, secondary spermatocytes, spermatids, and spermatozoa [Gilbert, 2000; Ogielska, 2009].

The spermatogonia are large, spherical cells which are surrounded by the nurturing Sertoli cells. Secondary spermatogonia can by identified from primary spermatogonia by their connecting cytoplasmic "bridges." These secondary spermatogonia acquire the ability to enter meiosis as they transform into primary spermatocytes. As primary spermatocytes begin to undergo meiosis, they increase in size. However, at the end of meiosis I, primary spermatocytes divide to form small secondary spermatocytes. The secondary spermatocyte stage is very rapid. This is the phase is which meiosis II occurs and, as a result, spermatids are formed. In caudates, the spermatids are not connected by cytoplasmic bridges (contrary to in anurans). The haploid spermatids are spherical in shape early on in their development. As they undergo spermatogenesis, their shape begins to elongate and their nuclei condense. The acrosome and tail are added and the mature spermatozoa are arranged in swirl-like bundles in the center of a cyst.

The head of the mature caudate spermatozoa is long, large, and often spear or scythe-like. Many species have an acrosomal barb at the apex, which is likely utilized in penetration of the egg [Selmi et al, 1997]. Cytoplasmic "blebs" may be seen attached anywhere along the head to the connecting piece. The blebs contain vacuoles and some mitochondria. The cytoplasmic bleb is not seen consistently on all caudate spermatozoa and the role is unknown, but may play a role in maintaining the sperm during storage

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within the spermatheca [Ogielska, 2009]. The long tail of the caudate sperm is composed of three major pieces: the midpiece, the principal piece, and the endpiece. An undulating membrane travels the length of the midpiece and principal piece. Mitochondria and an axial fiber are present in the midpiece, and the short endpiece consists of only the axoneme [Selmi et al, 1997; Ogielska, 2009].

Once the spermatozoa are mature, they are ready for spermiation. A cyst ruptures and lets the spermatozoa out into the lumen of the central testicular duct. They are then moved to the vas efferentia and through the genital portion of the pelvic kidney, in most species. In some species, the vas efferentia bypass the kidneys and go straight to the Wolffian duct. The Wolffian duct then empties into collecting ducts which enter the cloaca. In many species, urine and sperm have separate collecting ducts through which they exit into the cloaca, but in some species these ducts are shared [Siegel et al 2013; Siegel et al 2012; Siegel et al, 2010]. Glands within the cloaca deposit the gel stalk and cap onto the sperm as it exits the cloaca, forming the spermatophore [Sever & Lynne, 1985].

There is evidence in many species of salamander that spermatogenesis occurs only during the breeding season. The testicles of many species appear to undergo atrophy during the non-breeding season and hypertrophy during the breeding season. Spermatogenesis is highly influenced by environmental factors, including rain, temperature, and photoperiod [Eisthen & Krause, 2011; Ogielska, 2009; Walls et al, 2013]. There are two major methods of spermatogenesis in caudates: associated and dissociated. Associated spermatogenesis occurs alongside increases in androgen levels. That is, hormones signal for the production of sperm when animals are about to breed.

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Dissociated spermatogenesis, which appears to be more common in caudates, means sperm is produced when androgens are low. In this form of spermatogenesis, hormones signal for the production of sperm in the non-breeding season so that the testicular cysts are full and ready when breeding begins [Benner & Woodley, 2007 ; Siegel et al, 2013; Woodley, 1994].

Salamander sperm motility

Very little is known about activation of salamander sperm and salamander sperm motility. In 1986, Hardy and Dent assessed the motility of sperm of the red spotted newt in solutions of different osmolalities on the assumption that caudate sperm, much like anuran sperm, will react to osmolality of the environment. They took sperm from the inside of the testicles and exposed it to hypertonic, isotonic, and hypotonic solutions. They saw the sperm was most active in the hypotonic solution, but it was also active in the other solutions. The sperm remained active the longest in the isotonic solution. They concluded that the sperm was activated, much like anuran sperm [Inoda & Morisawa, 1987], by hypotonic solutions. This conclusion has not been replicable, and on repeated experiments osmolality appears to have little effect on caudate sperm motility [Siegel et al, 2012; Marcec, Unpublished Data].

Motility of caudate sperm does appear to be affected by chemical components within the egg jelly. Motility initiating substances appear to exist in the egg jelly of caudates which activate the sperm during internal fertilization [Takahashi, et al, 2003; Watanabe et al, 2003; Watanabe et al, 2010]. In caudates, sperm from the spermatophore stored within the female spermatheca of the cloaca is deposited directly on the egg surface as the eggs exit the cloaca [Sever, 1991; Sever, 2002; Sever et al, 1996]. It is not

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necessary for sperm to swim to the egg, through the reproductive system internally or through water externally. In studies of the egg jelly factors, it appears that contact with eggs causes opening of calcium ion channels in the sperm membrane which leads to sudden, rapid activity [Takahashi, et al, 2003; Watanabe et al, 2003; Watanabe et al, 2010].

While it is clear egg jelly has much to do with activation of motility in caudate sperm, there are likely still other factors involved. Caudate sperm within spermatophores has been reported as inactive [Russell et al, 1981; Watanabe et al, 2003; Watanabe et al, 2010] and moderately active [Mansour et al, 2011; Figel, 2013]. This activity is reported as low and slow, becoming high and fast when exposed to jelly extract [Mansour et al, 2011]. Because caudate sperm is not always entirely immobile, there is likely some factor other than egg jelly involved in activation, but it is yet to be determined what this factor is.

Species Studied

One of the species to be discussed of, *Ambystoma tigrinum,* was selected as model species for development of ART in caudates. They were selected due to their physiology, which covers a broad range of physiological characteristics similar to other species, and due to their taxonomical relationships to a wide variety of endangered species. This species is consider of "least concern" by the International Union for Conservation of Nature, and are considered "common" by the Federal United States government. Common species are used for the development of protocols to ensure safety and efficiency prior to use on endangered species, and to minimize stress on endangered species. The second species mentioned in these studies, *Ambystoma laterale*, is

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considered of least concern by the IUCN but is a regionally endangered species, listed at the state level. This species was one of the target species for transfer of protocols developed in *Ambystoma tigrinum*.

Ambystoma tigrinum (Eastern tiger salamander)

The eastern tiger salamander is common throughout most of North America, ranging west to east, from Arizona to the east coast, and north to south, from Saskatchewan to Mexico City [Conant & Collins, 1998]. They are a large species, distinct by their pattern of a black background and a pattern of bright yellow or olive-green splotches. Famous for their appetites, *Ambystoma tigrinum* are ambush predators and will eat most any food item that will fit inside their large mouths. As a member of the "mole salamander" family, they burrow deep underground throughout the year and are rarely seen except for their annual breeding migrations. *Ambystoma tigrinum* are a fall breeding species, with a breeding season of October through December. Reproductive migrations to temporal pools can begin as early as September, but can occur as late as November. Egg clutch size ranges from 50 to 1,000 eggs [Conant & Collins, 1998; Duellman & Trueb, 1994; Vitt & Caldwell, 2009]. Larvae are known to be cannibalistic, with a noteworthy difference in morphology in animals which are cannibalistic and those which are not. Cannibalistic larvae have large, blunt heads and larger teeth than non-cannibalistic counterparts [Walls et al, 2013; Walls et al, 1993]. Animals reach sexual maturity at approximately three years of age. Courtship involves a male depositing a spermatophore and coaxing a female over his spermatophore with a dance [Duellman & Trueb, 1994; Vitt & Caldwell, 2009]. *Ambystoma tigrinum* are one of the species which can participate in kleptogenesis, but are less likely to do so than *Ambystoma laterale* or

Ambystoma jeffersonianum [Bogart et al, 2009; Bogart et al, 2007]. The eastern tiger salamanders used in these studies were model species on which ART protocols could be developed for endangered salamander species including the flatwoods salamander (*Ambystoma bishop* and *Ambystoma cingulatum*), the California tiger salamander (*Ambystoma californiense*), and the blue spotted salamander (*Ambystoma laterale*).

Ambystoma laterale (Blue spotted salamander)

Blue spotted salamanders range around the great lakes, through the Northeast, and up into Quebec. Like *Ambystoma tigrinum*, these salamanders are a member of the "mole salamander family." Unlike *Ambystoma tigrinum*, *Ambystoma laterale* sometimes can be found burrowed shallow underneath logs or leaf litter at certain times of year. Much like *Ambystoma tigrinum*, the best way to find a blue spotted salamander is during the annual breeding migrations. Blue spotted salamanders breed in the spring, usually in March or April during the spring rains. Egg clutch sizes range from 2 to 500 eggs [Conant & Collins, 1998; Vitt & Caldwell, 2009]. Normal mating for *Ambystoma laterale* occurs much like that of *Ambystoma tigrinum*: the male lures the female over the spermatophore with a tail dance [Duellman & Trueb, 1994; Vitt & Caldwell, 2009]. More populations of *Ambystoma laterale* are becoming entirely female populations and, therefore, more are practicing kleptogenesis. It is most common for *Ambystoma laterale* to utilize the sperm from *Ambystoma jeffersonianum* in order to reproduce via kleptogenesis [Bogart et al, 2009; Bogart et al, 2007]. The *Ambystoma laterale* utilized in this study were part of a captive assurance colony started via a partnership with the Iowa DNR and Omaha's Henry Doorly Zoo and aquarium. This species is considered regionally endangered in Iowa. There are three breeding sites for blue spotted salamanders in Iowa, one of which

has gone barren. The captive assurance population was taken from one of the sites that still has breeding animals in order to breed animals in captivity that can be released to the barren site. Protocols that were developed in model *Ambystoma tigrinum* have been used to help achieve this goal.

References

[1]   Adams EM, Jones AG, Arnold SJ. Multiple paternity in a natural population of a salamander with long-term sperm storage. Mol Ecol 2005;14:1803–10. doi:10.1111/j.1365-294X.2005.02539.x.References

[2]   Adkins, RE. Foam produced by male Coturnix quail: What is its function? Auk, 1999: 116(1), 184–193.

[3]   Alcorn, MA, Deitloff, J, Graham, SP, Timpe, EK. Sexual Dimorphism in Head Shape, Relative Head Width, and Body Size of Eurycea Aquatica and Eurycea Cirrigera. Journal of Herpetology. 2013; 47(2): 321–27. doi:10.1670/11-317.

[4]   Allen, MR., Frame DJ, Huntingford C, Jones CD, Lowe JA, Meinshausen M, and Meinshausen N. Warming Caused by Cumulative Carbon Emissions towards the Trillionth Tonne. Nature. 2009; 458 (7242): 1163–66. doi:10.1038/nature08019.

[5]   Anchordoguy TJ, Rudolph AS, Carpenter JF, Crowe JH. Modes of interaction of cryoprotectants with membrane phospholipids during freezing. Cryobiology 1987;24:324–31. doi:10.1016/0011-2240(87)90036-8.

[6]   Andersen, LW., Fog K, and Damgaard C. Habitat fragmentation causes bottlenecks and inbreeding in the European tree frog (Hyla arborea). *Proceedings of the Royal Society of London, Series B: Biological Sciences* 2004; 271.1545: 1293-1302.

[7]   Atger M, Misrahi M, Sar S, Le Flem L, Dessen P, and Milgrom E. Structure of the Human Luteinizing Hormone-choriogonadotropin Receptor Gene: Unusual Promoter and 5′ Non-coding Regions. Molecular and Cellular Endocrinology . 1995; 111.(2): 113-23.

[8]   Awruch CA. Reproductive endocrinology in chondrichthyans: The present and the future. Gen Comp Endocrinol 2013;192:60–70. doi:10.1016/j.ygcen.2013.05.021.

[9]   Bahr NI, Pryce CR, Döbeli M, Martin RD. Evidence from urinary cortisol that maternal behavior is related to stress in gorillas. Physiol Behav 1998;64:429–37. doi:10

[10]   Bakkegard KA, Guyer C. Sexual Size Dimorphism in the Red Hills Salamander, Phaeognathus hubrichti (Caudata: Plethodontidae: Desmognathinae). J Herpetol 2004;38:8–15. doi:10.1670/145-02A.

[11]   Bansal AK, Bilaspuri GS. Impacts of oxidative stress and antioxidants on semen functions. Vet Med Int 2010;2011:686137. doi:10.4061/2011/686137..1016/S0031-9384(98)00057-2.

[12] Barnosky AD, Matzke N, Tomiya S, Wogan GOU, Swartz B, Quental TB, et al. Has the Earth's sixth mass extinction already arrived? Nature 2011;471:51–7. doi:10.1038/nature09678.

[13] Bardi M, French JA, Ramirez SM, Brent L. The role of the endocrine system in baboon maternal behavior. Biol Psychiatry 2004;55:724–32. doi:10.1016/j.biopsych.2004.01.002.

[14] Beebee TJC, Griffiths RA. The amphibian decline crisis: A watershed for conservation biology? Biol Conserv 2005;125:271–85. doi:10.1016/j.biocon.2005.04.009.

[15] Benner SL, Woodley SK. The reproductive pattern of male dusky salamanders (genus Desmognathus) is neither associated nor dissociated. Horm Behav 2007;51:542–7. doi:10.1016/j.yhbeh.2007.02.004.

[16] Bentley, PJ (1998). *Comparative vertebrate endocrinology*. Cambridge University Press.

[17] Berger L, Speare L and Hyatt A. Chytrid fungi and amphibian declines: overview, implications and future directions. *Declines and disappearances of Australian frogs. Environment Australia, Canberra.* 1999: 23-33.

[18] Bergmann M, Schindelmeiser J, Greven H. The zone of mature spermatozoa in the testis of *Salamandra salamandra*. Z. Mikrosk Anat, Forsch. 1982; 96: 221-234.

[19] Bernardo J. The Particular Maternal Effect of Propagule Size, Especially Egg Size: Patterns, Models, Quality of Evidence and Interpretations. Integr Comp Biol 1996;36:216–36. doi:10.1093/icb/36.2.216.

[20] Bi K, Bogart JP, Fu J. An examination of intergenomic exchanges in A. laterale-dependent unisexual salamanders in the genus Ambystoma. Cytogenet Genome Res 2009;124:44-50

[21] Blaustein AR, Belden LK, Olson DH, Green DM, Root TL, Kiesecker JM. Amphibian breeding and climate. Conserv Biol 2001;15:1804–9. doi:10.1038/374219a0.

[22] Bogart JP, Bartoszek J, Noble DW a, Bi K. Sex in unisexual salamanders: discovery of a new sperm donor with ancient affinities. Heredity (Edinb) 2009;103:483–93. doi:10.1038/hdy.2009.83.

[23] Bogart JP, Bi K, Fu J, Noble DW a, Niedzwiecki J. Unisexual salamanders (genus Ambystoma) present a new reproductive mode for eukaryotes. Genome 2007;50:119–36. doi:10.1139/G06-152.

[24] Bogerd J, Granneman JCM, Schulz RW, Vischer HF. Fish FSH Receptors Bind LH: How to Make the Human FSH Receptor to Be More Fishy? General and Comparative Endocrinology. 2005; 142 (1-2): 34-43.

[25] Boone MD, Cowman D, Davidson C, Hayes T, Hopkins W, Relyea R, et al. Evaluating the Role of Environmental Contamination in Amphibian Population Declines. In: Gascon C.; Collins J. P.; Moore, R. D; Church, D. R.; McKay, J. E.; Mendelson III, J. R. 2005. Amphibian Conservation Action Plan. 2005.

[26] Bruce RC. Sexual Size Dimorphism in the Plethodontidae. In: Bruce RC, Jaeger RG, Houck LD, editors., Springer US; 2000, p. 243–60.

[27] Brucker RM, Baylor CM, Walters RL, Lauer A, Harris RN, Minbiole KPC. The identification of 2,4-diacetylphloroglucinol as an antifungal metabolite produced by cutaneous bacteria of the salamander plethodon cinereus. J Chem Ecol 2008;34:39–43. doi:10.1007/s10886-007-9352-8.

[28] Browne RK, Seratt J, Vance C, Kouba A. Hormonal priming, induction of ovulation and in-vitro fertilization of the endangered Wyoming toad (Bufo baxteri). Reprod Biol Endocrinol 2006;4:34. doi:10.1186/1477-7827-4-34.

[29] Browne, R K Zippel K. Reproduction and Larval Rearing of Amphibians. ILAR J 2007;8:214–34.

[30] Burton TM, Likens GE. Salamander populations and biomass in the Hubbard Brook experimental forest, New Hampshire. Copeia. 1975:541-46.

[31] Cabrita E, Sarasquete C, Martínez-Páramo S, Robles V, Beirão J, Pérez-Cerezales S, et al. Cryopreservation of fish sperm: Applications and perspectives. J Appl Ichthyol 2010;26:623–35. doi:10.1111/j.1439-0426.2010.01556.x.

[32] Calatayud NE, Langhorne CJ, Mullen AC, Williams CL, Smith T, Bullock L, et al. A hormone priming regimen and hibernation affect oviposition in the boreal toad (Anaxyrus boreas boreas). Theriogenology 2015;84:600–7. doi:10.1016/j.theriogenology.2015.04.017.

[33] Calatayud, NE, Shier, DM, Swaisgood, RR, & Durrant, BS. Hormone-assited reproduction in a captive mountain yellow-legged frog population. Reproduction, Fertility and Development, 2016; 28(2), 189-190.

[34] Caldwell I, Vitt LJ, Gibbons JW. Declining amphibian populations: the problem of separating human impacts from natural fluctuations. Science 1991;253:892–5. doi:10.1126/science.253.5022.892.

[35] Calisi RM, Bentley GE. Lab and field experiments: Are they the same animal? Horm Behav 2009;56:1–10. doi:10.1016/j.yhbeh.2009.02.010

[36] Carey C, Cohen N, Rollins-Smith L. Amphibian declines: An immunological perspective. Dev Comp Immunol 1999;23:459–72. doi:10.1016/S0145-305X(99)00028-2.

[37] Carlstead K, Brown JL. Relationships between patterns of fecal corticoid excretion and behavior, reproduction, and environmental factors in captive black (Diceros bicornis) and white (Ceratotherium simum) rhinoceros. Zoo Biol 2005;24:215–32. doi:10.1002/zoo.20050.

[38] Caro, T. *Behavioral Ecology and Conservation Biology*. Oxford University Press, 1998.

[39] Ceballos G, Ehrlich PR, Barnosky AD, García A, Pringle RM, Palmer TM. Accelerated modern human – induced species losses : Entering the sixth mass extinction. Sci Adv 2015;1:1–5. doi:10.1126/sciadv.1400253.

[40] Chai, N. Endoscopy in amphibians. Veterinary Clinics of North America: Exotic Animal Practice. 2015; 18(3): 479-491.

[41] Charney, ND Castorino, JJ Dobro, MJ Steely, SL. Embryo development inside female salamander (Ambystoma jeffersonianum-laterale) rrior to egg laying. PloS One. 2014: 9(3), e91919. http://doi.org/10.1371/journal.pone.0091919

[42] Chowdhury, VS., Yamamoto K, Saeki I, Hasunuma I, Shimura T, and Tsutsui K. Melatonin Stimulates the Release of Growth Hormone and Prolactin by a Possible Induction of the Expression of Frog Growth Hormone-Releasing Peptide and Its Related Peptide-2 in the Amphibian Hypothalamus. Endocrinology. 2008;149(3): 962-70.

[43]   Clare, John. Personal Communication. April 2014.

[44] Clulow J, Trudeau VL, Kouba AJ. Amphibian declines in the twenty-first century: why we need assisted reproductive technologies. Adv Exp Med Biol 2014;753:275–316. doi:10.1007/978-1-4939-0820-2_12.

[45] Collins JP, Storfer A. Global amphibian declines : sorting the hypotheses. Divers Distrib 2003;9:89–98.

[46] Conant R., & Collins JT. 1998. *A field guide to reptiles & amphibians: eastern and central North America* (Vol. 12). Houghton Mifflin Harcourt.

[47] Corn PS. Climate change and amphibians P. S. Corn. Anim Biodivers Conserv 2005;1:59–67.

[48] Crespi EJ, Lessig H. Mothers influence offspring body size through post-oviposition maternal effects in the redbacked salamander, Plethodon cinereus. Oecologia 2004;138:306–11. doi:10.1007/s00442-003-1410-5.

50

[49]   Cushman SA. Effects of habitat loss and fragmentation on amphibians: A review and prospectus. Biol Conserv 2006;128:231–40. doi:10.1016/j.biocon.2005.09.031.

[50]   Daszak P, Cunningham AA, Hyatt AD. Infectious disease and amphibian population declines. Divers Distrib 2003;9:141–50. doi:10.1046/j.1472-4642.2003.00016.x.

[51]   Davic D, Welsh H. Roles on the Ecological of Salamanders. Annu Rev Ecol Evol Syst 2014;35:405–34. doi:10.2307/annurev.ecolsys.35.112202.30000016.

[52]   Debyser IWJ. Platyrrhine juvenile mortality in captivity and in the wild. Int J Primatol 1995;16:909–33. doi:10.1007/BF02696110.

[53]   Dellovade T, Schwanzel-Fukuda M, Gordan J, Pfaff D. Aspects of GnRH neurobiology conserved across vertebrate forms. Gen Comp Endocrinol 1998;112:276–82. doi:10.1006/gcen.1998.7157.

[54]   Diffendorfer JE, Gaines ME, and Holt RD. Habitat Fragmentation and Movements of Three Small Mammals (Sigmodon, Microtus, and Peromyscus). Ecology 1995; 76(3): 827–39. doi:10.2307/1939348.

[55]   Dixo M, Metzger JP, Morgante JS, and Zamudio KR. Habitat fragmentation reduces genetic diversity and connectivity among toad populations in the Brazilian Atlantic Coastal Forest. Biol Conserv 2009;142:1560–9. doi:10.1016/j.biocon.2008.11.016.

[56]   Dresser, BL. Cryobiology, Embryo Transfer, and Artificial Insemination in Ex Situ Animal Conservation programs. Wilson, E. O., and Frances M. Peter, eds. *Biodiversity*. Washington (DC): National Academies Press (US), 1988.

[57]   Duellman DE, and Trueb L. Biology of Amphibians. Baltimore USA: John Hopkins University Press; 1994.

[58]   DuRant SE, Hopkins WA. Amphibian predation on larval mosquitoes. Can J Zool 2008;86:1159–64. doi:10.1139/Z08-097.

[59]   Duvall D, and Norris DO. Prolactin and substrate stimulation of locomotor activity in adult tiger salamanders (*Ambystoma tigrinum*). J. Exp. Zool. 1977; 200:103–106.

[60]   Eisthen HL, and Krause BC. Ambiguities in the relationship between gonadal steroids and reproduction in axolotls (Ambystoma mexicanum). Gen Comp Endocrinol 2012;176:472–80. doi:10.1016/j.ygcen.2011.12.034.

[61]   Fauth JE., William J. Resetarits. Interactions Between the Salamander Siren Intermedia and the Keystone Predator Notophthalmus Viridescens. Ecology 1991; 72(3): 827.

[62]   Fickel J, Wagener A, and Ludwig A. Semen cryopreservation and the conservation of endangered species. Eur J Wildl Res 2007;53:81–9. doi:10.1007/s10344-007-0089-z.

[63]   Figel C. Cryopreservation of s perm from the axolotl Ambystom mexicanum : Implications for conservation. Herpetol Conserv Biol 2013;8:748–855.

[64]   Fisher MC, Garner TWJ, and Walker SF. Global Emergence of Batrachochytrium Dendrobatidis and Amphibian Chytridiomycosis in Space, Time, and Host. Annual Review of Microbiology. 2009; 63(1): 291–310. doi:10.1146/annurev.micro.091208.073435.

[65]   Foote RH. The history of artificial insemination : Selected notes and notables The online version of this article , along with updated information and services , is located on the World Wide Web at : The history of artificial insemination : Selected notes and notab. J Anim Sci 2002;80:1–10.

[62]   Forson D, and Storfer A. Effects of atrazine and iridovirus infection on survival and life-history traits of the long-toed salamander (Ambystoma macrodactylum). Environ Toxicol Chem 2006;25:168–73. doi:10.1897/05-260R.1.Ambystoma Tigrinum. Ecol Appl 2006;16:2325–32.

[63]   Fuller BJ. Cryoprotectants : the Essential Antifreezes To Protect Life in the Frozen State Cryoletters. 2004;25:375–88.

[64]   Gascon, C Collins, JP Moore, RD Church, DR McKay, JE, and Mendelson J. Amphibian Conservation Action Plan. IUCN/SCC Amphib. Conserv. Summit 2005, 2005.

[65]   Germano J, Cree A, and Bishop P. Ruling out the boys from the girls: Can subtle morphological differences identify sex of the apparently monomorphic frog, Leiopelma pakeka? New Zealand Journal of Zoology. 2011; 38:161-71.

[66]   Gilbert SF. Developmental Biology. 6th edition. Sunderland (MA): Sinauer Associates; 2000.

[67]   Godwin, J. The Promise of Perfect Adult Tissue Repair and Regeneration in Mammals: Learning from Regenerative Amphibians and Fish. BioEssays: News and Reviews in Molecular, Cellular and Developmental Biology. 2014:36(9): 861–71. doi:10.1002/bies.201300144.

[68] González-León, E., & Ramírez-Pinilla, M. P. Cloacal Morphology in Bolitoglossa nicefori (Caudata: Plethodontidae): Variation During the Reproductive Cycle. Anatomical Record. 2011; 294:349–362. http://doi.org/10.1002/ar.21306

[69] Grace, MS. Timing of reproductive immigration in salamanders: roles of environmental cues and endogenous biological clocks. Herpetol. Rev. 2003; 34:17–20.

[70] Gray MJ, Miller DL, and Hoverman JT. Ecology and pathology of amphibian ranaviruses. Dis Aquat Organ 2009;87:243–66. doi:10.3354/dao02138.

[71] Gualandris-Parisot, L., D. Husson, F. Foulquier, P. Kan, J. Davet, C. Aimar, C. Dournon, and A.m. Duprat. Pleurodeles Waltl, Amphibian, Urodele, Is a Suitable Biological Model for Embryological and Physiological Space Experiments on a Vertebrate. Advances in Space Research. 2011; 28(4): 569-78.

[72] Guerriero G, Roselli CE, and Ciarcia G. The Amphibian (Rana Esculenta) Brain Progesterone Receptor: Relationship to Plasma Steroids and Vitellogenic Cycle during the Gonadal Recovery Phase. Annals of the New York Academy of Sciences. 2009; 1163(1): 407-09.

[73] Gurtovenko AA, Anwar J. Modulating the structure and properties of cell membranes: the molecular mechanism of action of dimethyl sulfoxide. J Phys Chem B. 2007;111:10453–60. doi:10.1021/jp073113e.

[74] Hahn, Thomas P. Integration of Photoperiodic and Food Cues to Time Changes in Reproductive Physiology by an Opportunistic Breeder, the Red Crossbill, Loxia Curvirostra (Aves: Carduelinae). Journal of Experimental Zoology 1995; 272(3): 213–26. doi:10.1002/jez.1402720306.

[75] Harada Y, Kawazoe M, Eto Y, Ueno S, Iwao Y. The Ca2+ increase by the sperm factor in physiologically polyspermic newt fertilization: its signaling mechanism in egg cytoplasm and the species-specificity. Dev Biol 2011;351:266–76. doi:10.1016/j.ydbio.2011.01.003.

[76] Hardy, MP & Dent, JN. Hormonal facilitation in the release of sperm from the spermatheca of the red-spotted newt. Experientia, 1987:43(3), 302–304. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/3470194

[77] Hasumi, M. Prolactin-Based 'Water-Drive Theory' in Migratory Salamanders: Some Disprovable Data. Current Herpetology 2007; 26(2): 107–16. doi:10.3105/1881-1019(2007)26[107:PWTIMS]2.0.CO;2.

[78] Hau M, Wikelski M, Wingïfield JC. Visual and nutritional food cues fine-tune timing of reproduction in a neotropical ranforest bird. J Exp Zool 2000;286:494–504. doi:10.1002/(SICI)1097-010X(20000401)286.

53

[79] Hayes TB, Falso P, Gallipeau S, Stice M. The cause of global amphibian declines: a developmental endocrinologist's perspective. J Exp Zool 2010;213:921–33. doi:10.1242/jeb.040865.

[80] Hitchings SP, Beebee TJ. Genetic substructuring as a result of barriers to gene flow in urban Rana temporaria (common frog) populations: implications for biodiversity conservation. Heredity (Edinb) 1997;79 (Pt 2):117–27. doi:10.1038/sj.hdy.6882010.

[81] Hoffmann M, Hilton-taylor C, Angulo A, Böhm M, Brooks TM, Butchart SHM, et al. The Impact of Conservation on the Status of the World 's Vertebrates. Science 2010;330:1503–9. doi:10.1126/science.1194442.

[82] Hollinger, TG, and GL Corton. Artificial Fertilization of Gametes from the South African Clawed Frog, Xenopus Laevis. Gamete Research. 1980; 3(1): 45–57. doi:10.1002/mrd.1120030106.

[83] Holt WV. Basic aspects of frozen storage of semen. Anim Reprod Sci 2000;62:3–22. doi:10.1016/S0378-4320(00)00152-4.

[84] Holt WV. Fundamental aspects of sperm cryobiology: The importance of species and individual differences. Theriogenology 2000;53:47–58. doi:10.1016/S0093-691X(99)00239-3.

[85] Horne MT, and Dunson WA. Behavioral and Physiological Responses of the Terrestrial Life Stages of the Jefferson Salamander, Ambystoma Jeffersonianum, to Low Soil pH. Archives of Environmental Contamination and Toxicology 1994: 2(2): 232–38. doi:10.1007/BF00214267.

[86] Horne MT, and Dunson WA. Exclusion of the Jefferson Salamander, Ambystoma Jeffersonianum, from Some Potential Breeding Ponds in Pennsylvania: Effects of pH, Temperature, and Metals on Embryonic Development. Archives of Environmental Contamination and Toxicology. 1994; 27(3): 323–30. doi:10.1007/BF00213167.

[87] Inoda T, Morisawa M. Effect of osmolality on the initiation of sperm motility in Xenopus laevis. Comp Biochem Physiol -- Part A Physiol 1987;88:539–42. doi:10.1016/0300-9629(87)90077-6.

[88] Islam MD, Nakamura RK, Casco-Robles MM, Kunahong A, Inami A, Toyama F, Maruo F, and Chiba C. 2014. "The Newt Reprograms Mature RPE Cells into a Unique Multipotent State for Retinal Regeneration." *Scientific Reports Sci. Rep.* 4.

[89] Iwao Y. Egg activation in physiological polyspermy. Reproduction 2012;144:11–22. doi:10.1530/REP-12-0104.

54

[90]   Jones AG, Adams EM, Arnold SJ. Topping off: a mechanism of first-male sperm precedence in a vertebrate. Proc Natl Acad Sci U S A 2002;99:2078–81. doi:10.1073/ pnas.042510199.

[91]   Kano Y, Nakano T, Kumakura M, Wasa T, Suzuki M, Yamauchi K, et al. Seasonal expression of LHβ and FSHβ in the male newt pituitary gonadotrophs. Gen Comp Endocrinol 2005;141:248–58. doi:10.1016/j.ygcen.2005.01.003.

[92]   Kaplan RH. Maternal Influences on Offspring Development in the California Newt, Taricha torosa. Copeia 1985;1985:1028–35. doi:10.2307/1445258.

[93]   Keramitsoglou T, Grispou E, Margaritis LH, Koussoulakos S. Implantation of MNNG crystals into a Triturus intact limb affects mitotic and labeling indices, regeneration rate, and morphogenesis in the contralateral, regenerating limb. Teratog Carcinog Mutagen 2002;22:473–83. doi:10.1002/tcm.10043.

[94]   Khan MZ, Law FCP. Adverse Effects of Pesticides and Related Chemicals on Enzyme and Hormone Systems of Fish , Amphibians and Reptiles : a Review 2005;42:315–23.

[95]   Kiesecker JM. Synergism between trematode infection and pesticide exposure: a link to amphibian limb deformities in nature? Proc Natl Acad Sci U S A 2002;99:9900–4. doi:10.1073/pnas.152098899.

[96]   Kikuyama S, Nakada T, Toyoda F, Iwata T, Yamamoto K, and Conlon JM. Amphibian Pheromones and Endocrine Control of Their Secretion. Annals of the New York Academy of Sciences. 2005; 1040 (1): 123-30.

[97]   Kim JW, Im WB, Choi HH, Ishii S, Kwon HB. Seasonal Fluctuations in Pituitary Gland and Plasma Levels of Gonadotropic Hormones In Rana. General and Comparative Endocrinology. 1998; 109 (1): 13-23.

[98]   Kim JK, Kim IH, Heo J-H, Lee J-H, Ra N-Y, Eom J, et al. Arginine vasotocin (AVT) triggers courtship behavior without exposure to external stimuli and modulates the olfactory response of male Hynobius leechii salamanders. Zoolog Sci 2013;30:929–37. doi:10.2108/zsj.30.929.

[99]   Kolbert, E. The Sixth Extinction: An Unnatural History. New York, USA: Bloomsbury Publishing: 2014.

[100]   Kouba AJ, and Vance CK. Applied reproductive technologies and genetic resource banking for amphibian conservation. Reprod Fertil Dev 2009;21:719–37. doi:10.1071/RD09038.

[101]   Kouba AJ, Vance CK, Willis EL. Artificial fertilization for amphibian conservation: Current knowledge and future considerations. Theriogenology 2009;71:214–27. doi:10.1016/j.theriogenology.2008.09.055.

55

[102] Koussoulakos S. Evidence that chemical carcinogens interfere with specific regeneration-controlling mechanisms of urodeles. Tumour Biol J Int Soc Oncodevelopmental Biol Med 1992;13:189–94.

[103] Kramer L, Dresser B, Maruska E. Sexing aquatic salamanders by laparoscopy. In.; 1983; 193-4. Proceedings Am Assoc Zoo Vet Ann Meeting San Diego, CA.

[104] Kutka FJ., and Bachmann MD. Acid Sensitivity and Water Chemistry Correlates of Amphibian Breeding Ponds in Northern Wisconsin, USA. Hydrobiologia. 1990; 208 (3): 153–60. doi:10.1007/BF00007781.

[105] Kwok, HF. Zebrafish Gonadotropins and Their Receptors: I. Cloning and Characterization of Zebrafish Follicle-Stimulating Hormone and Luteinizing Hormone Receptors-- Evidence for Their Distinct Functions in Follicle Development. Biology of Reproduction 2005; 72(6): 1370-381.

[106] Lam BA, Walton DB, Harris RN. Motile zoospores of batrachochytrium dendrobatidis move away from antifungal metabolites produced by amphibian skin bacteria. Ecohealth 2011;8:36–45. doi:10.1007/s10393-011-0689-7.

[107] Langhorne, Cecilia. 2015. Assisted Reproductive Technologies for Endangered North American Amphibians. (Doctoral Dissertation)

[108] Langwig KE, Voyles J, Wilber MQ, Frick WF, Murray KA, Bolker BM, et al. Context-dependent conservation responses to emerging wildlife diseases. Front Ecol Environ 2015;13:195–202. doi:10.1890/140241.

[109] Li PQ, Zhu BC, Wang YF, Xiang XJ. Sex identification of Chinese giant salamander (Andrias davidianus) by Doppler B-ultrasound method. Journal of Biology. 2010; 1.

[110] Linklater S. The Effects of Carcinogens and Irradiation on Cells and Tissues of the Eastern Red Spotted Newt (Notophthalmus viridescens). University of Ottawa, 2011.

[111] Lips KR, Diffendorfer J, Mendelson JR, Sears MW. Riding the Wave: Reconciling the Roles of Disease and Climate Change in Amphibian Declines. PLoS Biol 2008;6:e72. doi:10.1371/journal.pbio.0060072.

[112] Macneil JE., and Williams RN. Effects of Timber Harvests and Silvicultural Edges on Terrestrial Salamanders. PLoS ONE. 2014:12.

[113] Mansour N, Lahnsteiner F, Patzner R a. Collection of gametes from live axolotl, Ambystoma mexicanum, and standardization of in vitro fertilization. Theriogenology 2011;75:354–61. doi:10.1016/j.theriogenology.2010.09.006.

[114] Marcec, R. Motility of *Ambystoma tigrinum* sperm before and after contact with egg jelly. Unpublished raw data.

[115] Marcec R., Langhorne C, Vance C, Kouba A, and Willard S. Cryopreservation of Spermic Milt in the Model Species Ambystoma Tigrinum (Tiger Salamander) for Application in Endangered Salamanders. Cryobiology. 2014; 69(3): 515. doi:10.1016/j.cryobiol.2014.09.342.

[116] Marco, A Ortiz-Santaliestra M. Pollution: Impact of Reactive Nitrogen on Amphibians. In: Heatwole H, editor. Amphib. Biol. Conserv. Amphib. 8th ed., 2009, p. 3145–85.

[117] Martel A, Spitzen-van der Sluijs A, Blooi M, Bert W, Ducatelle R, Fisher MC, et al. *Batrachochytrium salamandrivorans* sp. nov. causes lethal chytridiomycosis in amphibians. Proc Natl Acad Sci U S A 2013;110:15325–9. doi:10.1073/pnas.1307356110.

[118] Mason GJ. Species differences in responses to captivity: Stress, welfare and the comparative method. Trends Ecol Evol 2010;25:713–21. doi:10.1016/j.tree.2010.08.011.

[119] Means DB, J.G. P, Baggett M. Effects of Slash Pine Silviculture on a Florida Population of Flatwoods Salamanders. Conserv Biol 1996;10:426.

[120] Medeiros CMO, Forell F, Oliveira ATD, Rodrigues JL. Current status of sperm cryopreservation: Why isn't it better? Theriogenology 2002;57:327–44. doi:10.1016/S0093-691X(01)00674-4.

[121] Mellen, J.D. Factors influencing reproductive suc cess in small captive exotic felids (Felis spp.): A multiple regression analysis. Zoo Bio. 1991; 10; 95-110.

[122] Merola M. A Reassessment of Homozygosity and the Case for Inbreeding Depression in the Cheetah, Acinonyx jubatus: Implications for Conservation. Conserv Biol 1994;8:961–71. doi:10.1046/j.1523-1739.1994.08040961.

[123] Miller RE, Fowler ME. Fowler's Zoo and Wild Animal Medicine. St. Louis, MO: Saunders-Elsevier; 2012.

[124] Mohan, J Moudgal, RP Sastry, KVH Tyagi, J Singh,R. Effects of hemicastration and castration on foam production and its relationship with fertility in male Japanese quail. Theriogenology, 2002: 58(1), 29–39. http://doi.org/10.1016/S0093-691X(02)00863-4

[125] Moore FL, Wood RE, Boyd SK. Sex steroids and vasotocin interact in a female amphibian (Taricha granulosa) to elicit female-like egg-laying behavior or male-like courtship. Horm Behav 1992;26:156–66. doi:10.1016/0018-506X(92)90039.

[126] Moyle WR, Campbell RK, Rao SNV, Ayad NG, Bernard NP, Han Y, and Wang Y. Model of Human Chorionic Gonadotropin and Lutropin Receptor Interaction That Explains Signal Transduction of the Glycoprotein Hormones. Journal of Biological Chemistry 1995; 270 (34): 20020-0031.

[127] Muchlisin Z. Current Status of Extenders and Cryoprotectants on Fish Spermatozoa Cryopreservation. Biodiversitas 2005;6:66–9. doi:10.13057/biodiv/d060114.

[128] Noël S, Labonté P, Lapointe F-J. Genomotype Frequencies and Genetic Diversity in Urban and Protected Populations of Blue-spotted Salamanders (Ambystoma laterale) and Related Unisexuals. J Herpetol 2011;45:294–9. doi:10.1670/09-151.1.

[129] Noël S, Ouellet M, Galois P, Lapointe FJ. Impact of urban fragmentation on the genetic structure of the eastern red-backed salamander. Conserv Genet 2007;8:599–606. doi:10.1007/s10592-006-9202-1.

[130] Norris, DO, and Lopez KH. *Amphibians*. Amsterdam: Elsevier, 2011.

[131] Norris DO, Norman MF, Pancak MA, Duvall D. Seasonal variations in spermatogenesis, testicular weights, vasa deferentia, and androgen levels in neotenic male tiger salamanders, Ambystoma tigrinum. Gen Comp Endocrinol 1985;60:51–7.

[132] Norris, DO, and Jones RE. *Hormones and Reproduction in Fishes, Amphibians, and Reptiles*. New York: Plenum, 1987.

[133] Ogielska, Maria. *Reproduction of Amphibians*. Enfield, NH: Science, 2009

[134] Ollivier L, Welsh Jr. HH. Determining sex and life stage of Del Norte salamanders from external cues. Northwest Nat 2003;84:129–34.

[135] Osikowski A. Sperm transport after insemination in the Alpine newt (Triturus alpestris, Caudata, salamandridae). Folia Biol (Praha) 2007;55:109–14. doi:10.3409/173491607781492641.

[136] Palis, J.G.; Aresco, M.J.; Kilpatrick, S. Breeding biology of a Florida population of Ambystoma cingulatum (Flatwoods salamander) during a drought. Southeast. Nat. 2006; 5: 1-8.

[137] Parker, GA. The Evolution of Sexual Size Dimorphism in Fish. Journal of Fish Biology. 1992;41: 1–20. doi:10.1111/j.1095-8649.1992.tb03864.

[138] Parks JE, Graham JK. Effects of cryopreservation procedures on sperm membranes. Theriogenology 1992;38:209–22. doi:10.1016/0093-691X(92)90231-F.

[139] Pauly GB, Piskurek O, Shaffer HB. Phylogeographic concordance in the southeastern United States: The flatwoods salamander, Ambystoma cingulatum, as a test case. Mol Ecol 2007;16:415–29. doi:10.1111/j.1365-294X.2006.03149.

[140] Pedrono M. Wasted efforts: why captivity is not the best way to conserve species. Madagascar Conserv Dev 2011;6:57–9. doi:10.4314/mcd.v6i2.3.

[141] Peng LY, Xiao YM., Liu Y, Kouba AJ, Vance CK, Zippel K, Shishova NV. Effect of cryopreservation and short-term storage of Chinese giant salamander sperm. Reproduction, Fertility and Development. 2013; 25, 1134.

[142] Petranka JW, Harp EM, Holbrook CT, Hamel JA. Long-term persistence of amphibian populations in a restored wetland complex. Biol Conserv 2007;138:371–80. doi:10.1016/j.biocon.2007.05.002.

[143] Piatkowski T, and Braun T. Long-Term Organ Cultures of Newt Hearts. Methods in Molecular Biology Salamanders in Regeneration Research. 2014: 241-51.

[144] Polzonetti-Magni AM, Mosconi G, Soverchia L, Kikuyama S, and Carnevali O. Multihormonal Control of Vitellogenesis in Lower Vertebrates. International Review of Cytology. 2004; 1-46.

[145] Polzonetti-Magni A, Carnevali O, Yamamoto K, Kikuyama S. Growth hormone and prolactin in amphibian reproduction. Zoolog Sci 1995;12:683–94.

[146] Propper CR, Dixon TB. Differential effects of arginine vasotocin and gonadotropin-releasing hormone on sexual behaviors in an anuran amphibian. Horm Behav 1997;32:99–104. doi:10.1006/hbeh.1997.1408.

[147] Qu M, Tong C, Kong L, Yan X, Chernikov O V., Lukyanov PA, et al. Purification of a secreted lectin from Andrias davidianus skin and its antibacterial activity. Comp Biochem Physiol Part - C Toxicol Pharmacol 2015;167:140–6. doi:10.1016/j.cbpc.2014.08.008.

[148] Rajchard J. Sex pheromones in amphibians : a review. Vet Med (Praha) 2005;50:385–9.

[149] Rannap, R., A. Lõhmus, and L. Briggs. Restoring Ponds for Amphibians: A Success Story. In *Pond Conservation in Europe*, edited by Beat Oertli, Régis Céréghino, Jeremy Biggs, Steven Declerck, Andrew Hull, and Maria Rosa Miracle, 243–51. Developments in Hydrobiology. Springer Netherlands, 2009. http://link.springer.com/chapter/10.1007/978-90-481-9088-1_20.

[150] Raymond LR, Hardy LM. Demography of a Population of Ambystoma talpoideum (Caudata: Ambystomatidae) in Northwestern Louisiana. Herpetologica 1990;46:371–82.

[151] Reyer HU, Bättig I. Identification of reproductive status in female frogs-A quantitative comparison of nine methods. Herpetologica. 2004; 60:349-57.

[152] Roe JH, Hopkins WA, DuRant SE, Unrine JM. Effects of competition and coal-combustion wastes on recruitment and life history characteristics of salamanders in temporary wetlands. Aquat Toxicol 2006;79:176–84. doi:10.1016/j.aquatox.2006.06.007.

[153] Rohr JR, Schotthoefer AM, Raffel TR, Carrick HJ, Halstead N, Hoverman JT, et al. Agrochemicals increase trematode infections in a declining amphibian species. Nature 2008;455:1235–9. doi:10.1038/nature07281.

[154] Romano A, Bruni G, Paoletti C. Sexual dimorphism in the Italian endemic species Salamandrina perspicillata (Savi, 1821) and testing of a field method for sexing salamanders. Amphibia-Reptilia 2009;30:425–34. doi:10.1163/156853809788795128.

[155] Roth, TL, Obringer AR. Reproductive research and the worldwide amphibian extinction crisis. In *Reproductive Science and Integrated Conservation.* Eds WV Pickard, AR Pickard, JC Rodger and DE Wildt. 2003 pp. 359–374.

[156] Roth TL, Szymanski DC, Keyster ED. Effects of age, weight, hormones, and hibernation on breeding success in boreal toads (Bufo boreas boreas). Theriogenology 2010;73:501–11. doi:10.1016/j.theriogenology.2009.09.033.

[157] Rowe CL, Hopkins WA, and Coffman VR. Failed Recruitment of Southern Toads (Bufo Terrestris) in a Trace Element-Contaminated Breeding Habitat: Direct and Indirect Effects That May Lead to a Local Population Sink. Archives of Environmental Contamination and Toxicology. 2001; 40(3): 399–405. doi:10.1007/s002440010189.

[158] Rowe CL, and Dunson WA. Relationships among Abiotic Parameters and Breeding Effort by Three Amphibians in Temporary Wetlands of Central Pennsylvania. Wetlands. 1993;13(4): 237–46. doi:10.1007/BF03161290.

[159] Rugh R. Induced ovulation and artivicial fertilization in the frog. Biol Bull 1934;66: 22–9.

[160] Russell LD, Brandon RA, Zalisko EJ, Martan J. Spermatophores of the salamander Ambystoma texanum. Tissue Cell 1981;13:609–21.

[161] Sargent MG, Mohun TJ. Cryopreservation of sperm of Xenopus laevis and Xenopus tropicalis. Genesis 2005;41:41–6. doi:10.1002/gene.20092.

[162] Selmi MG, Brizzi R, Bigliardi E. Sperm morphology of salamandrids (Amphibia, Urodela): implications for phylogeny and fertilization biology. Tissue Cell 1997;29:651–64. doi:10.1016/S0040-8166(97)80041-9.

[163] Semlitsch RD. Differentiating Migration and Dispersal Processes for Pond-Breeding Amphibians. J Wildl Manage 2008;72:260–7. doi:10.2193/2007-082.

[164] Semlitsch, RD, Gibbons, JW. Effects of Egg Size on Success of Larval Salamanders in Complex Aquatic Environments. Ecology. 1990;71(5): 1789–1795. http://doi.org/10.2307/1937586

[165] Semlitsch RD, O'Donnell KM, Thompson FR. Abundance, Biomass Production, Nutrient Content, and the Possible Role of Terrestrial Salamanders in Missouri Ozark Forest Ecosystems. Can. J. Zool. Canadian Journal of Zoology. 2014: 92(12): 997-1004.

[166] Senger, P. L. *Pathways to Pregnancy and Parturition*. Pullman, WA: Current Conceptions, 2005.

[167] Sever, DM. Comparative anatomy and phylogeny of the cloacae of salamanders (Amphibia: Caudata). 1. Evolution at the family level. Herpetologica, 1991:47(September), 165–193. http://doi.org/10.1002/jmor.1052070306

[168] Sever DM. Female sperm storage in amphibians. J Exp Zool 2002;292:165–79. doi:10.1002/jez.1152.

[169] Sever, DM & Brizzi, R. Comparative biology of sperm storage in female salamanders. The Journal of Experimental Zoology, 1998: 282(4-5), 460–476.

[170] Sever, DM Rania, LC Krenz, JD. Reproduction of the Salamander Siren intermedia Le Conte With Especial Reference to Oviducal Anatomy and Mode of Fertilization. Journal of Morphology, 1996:227, 335–348. http://doi.org/10.1002/(SICI)1097-4687(199603)227:3<335::AID-JMOR5>3.3.CO;2-E

[171] Sever, David M Lynne DH. Spermatophore Formation in Desmognathus ochrophaeus (Amphibia : Plethodontidae). Copeia 1985;1985:394–402.

[172] Siegel DS, Aldridge RD, Rheubert JL, Gribbins KM, Sever DM, Trauth SE. The testicular sperm ducts and genital kidney of male Ambystoma maculatum (Amphibia, Urodela, Ambystomatidae). J Morphol 2013;274:344–60. doi:10.1002/jmor.20100.

[173] Siegel DS, Alvino S, Trauth SE, Sever DM, Gribbins KM. Spermatogenic cycle of a plethodontid salamander, *Eurycea longicauda* (Amphibia, Urodela). Spermatogenesis 2014;4:e982420. doi:10.4161/21565562.2014.982420.

[174] Siegel DS, Murray CM, Wilson ER, Puleo ER, True K a., Aldridge RD. Sexual Kidneys in the Red-spotted Newt (Notophthalmus viridescens). J Herpetol 2012;46:136–44. doi:10.1670/11-013.

[175] Siegel DS, Nicholson AE, Rabe B, Beran B, Trauth SE. The evolution of the sperm transport complex in male plethodontid salamanders (Amphibia, Urodela, Plethodontidae). Copeia 2014;2014:489–502. doi:10.1643/CG-14-026.

[176] Siegel, DS Sever, DM Aldridge, RD. The pelvic kidney of male Ambystoma maculatum (Amphibia, urodela, ambystomatidae) with special reference to the sexual collecting ducts. Journal of Morphology. 2010:271(12), 1422–39. http://doi.org/10.1002/jmor.10883

[177] Silk JB, Clark-Wheatley CB, Rodman PS, Samuels A. Differential reproductive success and facultative adjustment of sex ratios among captive female bonnet macaques (Macaca radiata). Anim Behav 1981;29:1106–20. doi:10.1016/S0003-3472(81)80063-2.

[178] Singh, BN Weaver, C Koyano-Nakagawa, N Garry, D. Visualizing Vascular Structure in the Adult Newt (Notophtalmus Viridescens) Heart. J. Mech. Des. Journal of Mechanical Design. 2015: 9(2). doi:10.115/1.4030125

[179] Singh, RP Sastry, KVH Shit, N Pandey, NK Singh, KB Mohan, J Moudgal, RP. Cloacal gland foam enhances motility and disaggregation of spermatozoa in Japanese quail (Coturnix japonica). Theriogenology, 2011: *75*(3), 563–9. http://doi.org/10.1016/j.theriogenology.2010.09.028

[180] Sherman, C. D. H., et al. Males with high genetic similarity to females sire more offspring in sperm competition in Peron's tree frog Litoria peronii. *Proceedings of the Royal Society of London B: Biological Sciences*.2008; 275.1637: 971-978.

[181] Shine, R. Sexual Selection and Sexual Dimorphism in the Amphibia. Copeia 1979;2: 297–306. doi:10.2307/1443418.

[182] Smith, KG. Keystone Predators (eastern Newts, Notophthalmus Viridescens) Reduce the Impacts of an Aquatic Invasive Species. Oecologia 2006:148(2): 342-49.

[183] Smith, AU and Polge C. Survival of spermatozoa at low temperatures. Nature. 1950; 166:668-669.

[184] Snyder NFR, Derrickson SR, Beissinger SR, Wiley JW, Smith TB, Toone WD, et al. Limitations of Captive Breeding in Endangered Species Recovery. Conserv Biol 1996;10:338–48. doi:10. 1046/j.1523-1739.1996.10020338.

[185] Sower SA, Freamat M, Kavanaugh SI. The origins of the vertebrate hypothalamic-pituitary-gonadal (HPG) and hypothalamic-pituitary-thyroid (HPT) endocrine systems: New insights from lampreys. Gen Comp Endocrinol 2009;161:20–9. doi:10.1016/j.ygcen.2008.11.023.

[186] Stoops MA, Campbell MK, Dechant CJ. Successful captive breeding of Necturus beyeri through manipulation of environmental cues and exogenous hormone administration: a model for endangered Necturus. Herpetol Rev 2014;45:251–6.

[187] Storey BT, Noiles EE, Thompson K a. Comparison of glycerol, other polyols, trehalose, and raffinose to provide a defined cryoprotectant medium for mouse sperm cryopreservation. Cryobiology 1998;37:46–58. doi:10.1006/cryo.1998.2097.

[188] Stuart SN, Chanson JS, Cox NA, Young BE, Rodrigues ASL, Fischman DL, et al. Status and trends of amphibian declines and extinctions worldwide. Science 2004;306:1783–6. doi:10.1126/science.1103538.

[189] Suquet M, Dreanno C, Fauvel C, Cosson J, Billard R. Cryopreservation of sperm in marine fish. Aquac Res 2000;31:231–43. doi:10.1046/j.1365-2109.2000.00445.x.

[190] Swanson WF, Brown JL, and Wildt DE. Influence of Seasonality on Reproductive Traits of the Male Pallas' Cat (Felis Manul) and Implications for Captive Management. Journal of Zoo and Wildlife Medicine 1996; 27(2): 234–40.

[191] Sztein JM, Noble K, Farley JS, Mobraaten LE. Comp arison of permeating and nonpermeating cryoprotectants for mouse sperm cryopreservation. Cryobiology 2001;42:28–39. doi:10.1006/cryo.2001.2300.

[192] Takahashi T, Kutsuzawa M, Shiba K, Takayama-Watanabe E, Inaba K, Watanabe A. Distinct Ca2+ channels maintain a high motility state of the sperm that may be needed for penetration of egg jelly of the newt, Cynops pyrrhogaster. Dev Growth Differ 2013;55:657–67. doi:10.1111/dgd.12073.

[193] Taylor, RR. In Vitro Preparation of Newt Inner Ear Sensory Epithelia as a Model for Repair and Regeneration. *Salamanders in Regeneration Research.* Anoop Kumar and András Simon, ed. pp 253–65. Methods in Molecular Biology. Springer New York, 2015. http://dx.doi.org/10.1007/978-1-4939-2495-0_20.

[194] Taylor VJ, Poole TB. Captive breeding and infant mortality in Asian elephants: A comparison between twenty western zoos and three eastern elephant centers. Zoo Biol 1998;17:311–32. doi:10.1002/(SICI)1098-2361(1998)17:4<311::AID-ZOO5>3.0.

[195] Tennessen JA, Zamudio KR. Early male reproductive advantage, multiple paternity and sperm storage in an amphibian aggregate breeder. Mol Ecol 2003;12:1567–76. doi:10.1046/j.1365-294X.2003.01830.x.CO;2-C.

[196] Todd, BD & Davis AK. Sexual Dichromatism in the Marbled Salamander, Ambystoma Opacum. Canadian Journal of Zoology. 2007; 85(9): 1008–13. doi:10.1139/Z07-082.

[197] Toro E, Michael SF. In vitro fertilization and artificial activation of eggs of the direct-developing anuran Eleutherodactylus coqui. Reprod Biol Endocrinol 2004;2:60. doi:10.1186/1477-7827-2-60.

[198] Toyoda F, Hasunuma I, Yamamoto K. Prolactin acts centrally to enhance newt courtship behavior. Gen Comp Endocrinol 2005;141:172–7. doi:10.1016/j.ygcen.2004.12.015.

[199] Toyoda F, Matsuda K, Yamamoto K, Kikuyama S. Involvement of endogenous prolactin in the expression of courtship behavior in the newt, Cynops pyrrhogaster. Gen Comp Endocrinol 1996;102:191–6. doi:10.1006/gcen.1996.0060.

[200] Toyoda F, Yamamoto K, Ito Y, Tanaka S, Yamashita M, Kikuyama S. Involvement of arginine vasotocin in reproductive events in the male newt Cynops pyrrhogaster. Horm Behav 2003;44:346–53. doi:10.1016/j.yhbeh.2003.06.001.

[201] Trottier TM, Armstrong JB. Hormonal stimulation as an aid to artificial insemination in Ambystoma mexicanum. Can J Zool 1975;53:171–3.

[202] Trudeau VL, Schueler FW, Navarro-Martin L, Hamilton CK, Bulaeva E, Bennett A, et al. Efficient induction of spawning of northern leopard frogs (Lithobates pipiens) during and outside the natural breeding season. Reprod Biol Endocrinol 2013;11:14. doi:10.1186/1477-7827-11-14.

[203] Trudeau VL, Somoza GM, Natale GS, Pauli B, Wignall J, Jackman P, et al. Hormonal induction of spawning in 4 species of frogs by coinjection with a gonadotropin-releasing hormone agonist and a dopamine antagonist. Reprod Biol Endocrinol 2010;8:1–9. doi:10.1186/1477-7827-8-36.

[204] Tsonis PA. Effects of carcinogens on regenerating and non-regenerating limbs in amphibia (review). Anticancer Res 1983;3:195–202.

[205] Unger S, Mathis A, Wilkinson R. A comparison of sperm health in declining and stable populations of hellbenders (Cryptobranchus alleganiensis alleganiensis and C . a . bishopi). Am Midl Nat 2013;170:382–92.

[206] Uteshev VK, Kaurova S, Shishova N, Stolyarov S, Browne R, and Gakhova E. In Vitro Fertilization with Hormonally Induced Sperm and Eggs from Sharp-Ribbed Newts Pleurodeles Waltl. Russian Journal of Herpetology. 2015; 22:1:35–40.

[207] Uysal O, Bucak MN. Effects of oxidized glutathione, bovine serum albumin, cysteine and lycopene on the quality of frozen-thawed ram semen. Acta Vet Brno 2007;76:383–90. doi:10.2754/avb200776030383.

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[208]   Vanderberg MR, Adams MB, Wiseman MS. Evaluating Forest Biomass Utilization in the Appalachians : A Review of Potential Impacts and Guidelines for Management. Gen Tech Rep NRS-106 2012.

[209]   Vitt LJ, Caldwell JP. Herpetology. 3rd ed. Singapore China: Elsevier; 2009.

[210]   Waggener WL, Carroll EJ. A method for hormonal induction of sperm release in anurans (eight species) and in vitro fertilization in lepidobatrachus species. Dev Growth Differ 1998;40:19–25.

[211]   Wake DB, Vredenburg VT. Are we in the midst of the sixth mass extinction? A view from the world of amphibians. Proc Natl Acad Sci U S A 2008;105:11466–73. doi:10.1073/pnas.0801921105.

[212]   Walke, JB Becker, MH Loftus, SC House, LL Cormier, G Jensen, RV Belden, KL. Amphibian Skin May Select for Rare Environmental Microbes. ISME J. 2014: 8(11): 2207-217.

[213]   Walls S, Barichivich W, Brown M. Drought, Deluge and Declines: The Impact of Precipitation Extremes on Amphibians in a Changing Climate. Biology (Basel) 2013;2:399–418. doi:10.3390/biology2010399.

[214]   Walls SC, Beatty JJ, Tissot BN, Hokit DG, Blaustein a R. Morphological Variation and Cannibalism in a Larval Salamander (Ambystoma-Macrodactylum-Columbianum). Can J Zool Can Zool 1993;71:1543–51. doi:Doi 10.1139/Z93-218.

[215]   Walls SC, Belanger SS, Blaustein AR. Morphological variation in a larval salamander: dietary induction of plasticity in head shape. Oecologia 1993;96:162–8. doi:10.1007/BF00317728.

[216]   Walton BM, Steckler S. Contrasting effects of salamanders on forest-floor macro- and mesofauna in laboratory microcosms. Pedobiologia (Jena) 2005;49:51–60. doi:10.1016/j.pedobi.2004.07.012.

[217]   Watanabe T, Itoh T, Watanabe A, Onitake K. Characteristics of sperm motility induced on the egg-jelly in the internal fertilization of the newt, Cynops pyrrhogaster. Zool Sci 2003;20:345–52. doi:10.2108/zsj.20.345.

[218]   Watanabe T, Kubo H, Takeshima S, Nakagawa M, Ohta M, Kamimura S, et al. Identification of the sperm motility-initiating substance in the newt, Cynops pyrrhogaster, and its possible relationship with the acrosome reaction during internal fertilization. Int J Dev Biol 2010;54:591–7. doi:10.1387/ijdb.092894tw.

[219]   Watson, PF and Holt WV, Ed. *Cryobanking the genetic resource: Wildlife conservation for the future?* London, Taylor and Francis, 2001.

[220] Wijer P, Watt PJ, and Oldham RS. Amphibian Decline and Aquatic Pollution: Effects of Nitrogenous Fertiliser on Survival and Development of Larvae of the Frog *Rana Temporaria*. Applied Herpetology 2003; 1(1): 3–12. doi:10.1163/157075403766451180.

[221] Wildt, DE. Fertilization in cats. Pp. 299-328 in *A Comparative Overview of Mammalian Fertilization*. B.S. Dunbar; M.G. O'Rand, eds. New York, Plenum Press, 1991.

[222] Wildt D, Pukazhenthi B, Brown J, Monfort S, Howard J, Roth T. Spermatology for understanding, managing and conserving rare species. Reprod Fertil Dev 1995;7:811–24.

[223] Wildt DE, and Wemmer C. Sex and wildlife: The role of reproductive science in conservation. Biodivers Conserv 1999;8:965–76. doi:10.1023/A:1008813532763.

[224] Williams RN, Gopurenko D, Kemp KR, Williams B, DeWoody JA. Breeding Chronology, Sexual Dimorphism, and Genetic Diversity of Congeneric Ambystomatid Salamanders. Journal of Herpetology. 2009; 43(3): 438–449. http://doi.org/10.1670/08-034R2.1

[225] Witman, N Murtuza, B Davis, B Arner, A Morrison, JI. Recapitulation of developmental cardiogenesis governs the morphological and functional regeneration of adult newt hearts following injury. Developmental Biology. 2011: 354(1), 67–76. http://doi.org/10.1016/j.ydbio.2011.03.021

[226] Wolf DP, Hedrick JL. A molecular approach to fertilization. II. Viability and artificial fertilization of Xenopus laevis gametes. Dev Biol 1971;25:348–59. doi:10.1016/0012-1606(71)90036-4.

[227] Wolff JO, Schauber EM, Edge WD. Effects of Habitat Loss and Fragmentation on the Behavior and Demography of Gray-Tailed Voles. Efectos de la Pérdida y Fragmentación de Hábitat Sobre el Comportamiento y la Demografía de Ratones de Campo de Cola Gris. Conserv Biol 1997;11:945–56. doi:10.1046/j.1523-1739.1997.96136.

[228] Woodley SK. Plasma Androgen Levels, Spermatogenesis, and Secondary Sexual Characteristics in Two Species of Plethodontid Salamanders with Dissociated Reproductive Patterns. Gen Comp Endocrinol 1994;96:206–14. doi:10.1006/gcen.1994.1175.

[229] Woolley SC, Sakata JT, Crews D. Evolutionary insights into the regulation of courtship behavior in male amphibians and reptiles. Physiol Behav 2004;83:347–60. doi:10.1016/j.physbeh.2004.08.021.

[230]   Woodward, BD. Local Intraspecific Variation in Clutch Parameters in the Spotted Salamander (Ambystoma maculatum). Copeia. 1982; 1982 (1): 157–160. http://doi.org/10.2307/1444279

[231]   Wright, KM, & Whitaker, BR. 2001. *Amphibian medicine and captive husbandry*. Krieger Publishing Company.

[232]   Wyman, RL. Experimental Assessment of Salamanders as Predators of Detrital Food Webs: Effects on Invertebrates, Decomposition and the Carbon Cycle. Biodiversity and Conservation. 1998;7: 641-50.

[233]   Yamahama, Y & Onitake, K. Egg Transport in the Coelom of the Newt Cynops pyrrhogaster. I. The Ovulated Egg is Transported to the Ostium by the Ciliary Movement of Coelomic Epithelial Cells (Reproductive Biology). Zoological Science, 2002:19(8), 899–906. http://doi.org/10.2108/zsj.19.899

[234]   Yamashita, Masamichi, Akemi Izumi-Kurotani, Mari Imamizo, Hajime Koike, Makoto Okuno, Carl J. Pfeiffer, Shinji Komazaki, Fumie Sasaki, Yoshinobu Ohira, Isamu Kashima, Sakae Kikuyama, Takeo Ohnishi, Yoshihiro Mogami, and Makoto Asashima. Japanese Red-Bellied Newts in Space - AstroNewt Experiment on Space Shuttle IML-2 and Space Flyer Unit. Biol. Sci. Space Biological Sciences in Space 15.Suppl (2001)

[235]   Yazawa T, Yamamoto T, Kubokawa K, Nakayama Y, Fujimoto K, Ito R, et al. Cold suppression of follicle-stimulating hormone activity on proliferation and survival of newt spermatogonia. GenComp Endocrinol 2001;122:296–303. doi:10.1006/gcen.2001.7631.

[236]   Zhang, Manna, Guoyu Tong, Yanling Liu, Yiming Mu, Jianping Weng, Yaoming Xue, Zuojie Luo, Yuanming Xue, Lixin Shi, Xueyan Wu, Shouyue Sun, Yanhua Zhu, Ying Cao, Jie Zhang, Hong Huang, Ben Niu, Hong Li, Qinghua Guo, Yan Gao, Zhibin Li, Guang Ning, Dalong Zhu, and Xiaoying Li. 2015. "Sequential versus Continual Purified Urinary FSH / HCG in Men with Idiopathic Hypogonadotropic Hypogonadism." *The Journal of Clinical Endocrinology & Metabolism*

[237]   Zukor, KA Kent, DT Odelberg, SJ. Meningeal cells and glia establish a permissive environment for axon regeneration after spinal cord injury in newts. Neural Development. 2011:6(1), 1. http://doi.org/10.1186/1749-8104-6-1

CHAPTER II

DEVELOPMENT OF HORMONE PROTOCOLS FOR THE INDUCTION OF

SPERMIATION IN MALE TIGER SALAMANDERS

(*AMBYSTOMA TIGRINUM*)

Introduction

Amphibians are essential to maintaining the health of an ecosystem, and they are key indicator species which are important sentinels for the status of the global environment [Beebee & Griffiths, 2005; Collins & Storfer, 2003; Davic & Welsh, 2014; Kouba & Vance, 2009]. Amphibians are experiencing extensive population declines and extinction rates worldwide; these collective experiences are being called the "Amphibian Extinction Crisis" [Beebee & Griffiths, 2005; Collins & Storfer, 2003; Gascon et al., 2005; Kouba & Vance, 2009]. Over one-third (32%) of known amphibian species are threatened with extinction; an additional 25% of amphibians do not have enough documented data in order to ascertain the status of their populations [Stuart et al., 2004]. As a result of the Amphibian Extinction Crisis, it is imperative that captive breeding facilities are successfully established for the future survival of amphibian species. Over the last decade, attempts by governmental and zoological conservation institutions have been put forth to establish captive assurance colonies for critically endangered species of amphibians [Browne et al., 2006; Griffiths & Pavajeau, 2008; Kouba & Vance, 2009; Obringer et al., 2000]. Unfortunately, due to the specific environmental and physiological

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cues needed in order to induce breeding in these animals, amphibian captive assurance colonies face frequent failure [Griffiths & Pavajeau, 2008; Kouba & Vance, 2009; Obringer et al., 200; Stoops et al., 2014]. Some of these very specific cues include changes in barometric pressure, humidity, temperature, and light cycles. Many species rely in specific types of habitat for breeding, such as vernal pools, presence of specific types of vegetation, soil pH, and presence of other fauna in the ecosystem [Duellman & Trueb, 1994; Means et al., 1996; Vitt & Caldwell, 2009]. Without these specific cues, amphibian species will not breed. This has been one of many complications seen in wild amphibian populations as a result of global climate change [Blaustein et al., 2001; Corn, 2005; Lips et al., 2008; Walls et al., 2013].

Until recently, the majority of amphibian conservation efforts have focused on anurans (frogs/toads) despite the fact that caudates (salamanders/newts) face a larger threat of extinction (49.8% of salamanders are threatened or endangered compared to 31.6% of anurans) [Stuart et al., 2004]. As the number of caudates in both captivity and the wild decreases rapidly, populations become unsustainable. It is in the best interest of species preservation that assisted reproductive technologies (ART) be developed before population recovery is no longer possible. While research efforts have been devoted to anuran ART [Griffiths & Pavajeau, 2008; Kouba & Vance, 2009; Kouba et al., 2012; McDonough et al., 2015; Obringer et al., 2000; Uteshev et al., 2012; Uteshev et. al. 2015; Waggener & Carroll, 1998], reliable technologies have yet to be developed for caudates. The lack of ART for caudates is in part due to the complicated reproductive physiology of the salamander and newt. Caudates display a wide variety of reproductive strategies, many of which the mechanisms are still not entirely understood [Adams et al., 2005;

Duellman & Trueb, 1994; Jones et al., 2002; Selmi et al., 1997; Sever, 1991; Tennessen & Zamudio, 2003; Vitt & Caldwell, 2009; Woolley et al., 2004]. While most species within this taxa undergo internal fertilization, there are some genre which are known to undergo external fertilization, similar to anurans [Duellman & Trueb, 1994; Sever, 1991; Vitt & Caldwell, 2009]. Most salamanders transfer sperm by encasing it within a dense, gelatinous packet called a spermatophore. The spermatophore can be stored within the cloaca of a female salamander for many months, dependent on species [Sever, 1991; Zalisko et al., 1984]. The variations in caudate reproductive methods present challenges to the development of ART. These challenges increase the importance of developing ART protocols sooner, rather than later, in order that such obstacles may be surmounted before populations of animals become beyond recovery.

Hormone protocols designed for the induction of spermiation and ovulation are the foundation of ART development. Much of the failure of amphibian captive breeding is due to two causes: 1) failure to release gametes; or 2) failure of gametes to successfully undergo fertilization [Kouba & Vance, 2009; Kouba et al. 2012]. The use of hormones helps avoid the first cause of failure by inducing gamete release. Refinement of hormone protocols, altering the amount and timing of doses, allows correction of the second cause of failure by increasing gamete quality and/or number. The ability to induce production of gametes on demand via hormone stimulation can circumvent environmental breeding cues in caudates.. Induction of gamete production facilitates collection of gametes for *in vitro* fertilization or cryopreservation [Kouba et al. 2012].

Very little information is available regarding the use of hormone stimulation in caudates, The little information that is known comes from wild caught animals during

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breeding season [Hardy & Dent, 1986; Stoops et al., 2014; Uteshev et al., 2015; Watanabe et al., 2003; Watanabe et al., 2010], or animals that are highly inbred laboratory specimens [Figel, 2013; Mansour et al., 2011; Trottier & Armstrong, 1975]. Animals captured during breeding season will have an increased amount of gonadotropins and steroid sex hormones cycling in the blood [Norris & Jones, 2011; Norris & Lopez, 2011; Norris et al., 1985; Ogielska, 2009; Woodley, 1994] and are more likely susceptible to artificial stimulation [Calisi & Bentley, 2009; St. Aubin et al., 1996]. Inbred lab specimens are only models for wild species, and their responses are not completely analogous to wild or endangered species [Calisi & Bentley, 2009]. Thus anurans, laboratory model species and wild caught animals in the breeding season are limited in their utility for ART development in captive breeding, and an optimal physiological model should be designed and tested for best results in future transfer of protocols to endangered species of caudates.

In these experiments, an array of hormone protocols and a control protocol are developed using the model species *Ambystoma tigrinum* (the eastern tiger salamander). This species was chosen due to physiological similarities and its taxonomic relation to threatened and endangered target species: *Ambystoma laterale* (the blue spotted salamander); *Ambystoma cingulatum* (the flatwoods salamander); and *Ambystoma californiense* (the California tiger salamander).

Hormone protocols were selected by extrapolating from previous available data in caudates [Hardy & Dent, 1986; Mansour et al., 2011; Trottier & Armstrong, 1975; Uteshev et al., 2015; Watanabe et al., 2003; Watanabe et al., 2010] and anurans [Browne et al., 2006; Kouba & Vance, 2009; Kouba et al., 2012; Obringer et al., 2000; Wright &

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Whitaker, 2001]. The hormones utilized for the treatments are human chorionic gonadotropin (hCG) and luteinizing hormone releasing hormone (LHRH). The neuropeptide LHRH is a highly conserved hormone, even sharing its genetic sequence across vertebrate and multiple invertebrate species [Kim et al., 2011; Roch et al., 2011]. The functions of LHRH also appear to be conserved across species, including the less understood functions involving regulation of sexual behaviors [Dellovade et al., 1998; Pawson & McNeilly, 2005]. The peptide hormone hCG is naturally produced by a developing primate embryo and fetus for support of the corpus luteum and maintenance of pregnancy [Cole, 2010]. While the hormone is naturally specific to humans and primates, it is an analog for luteinizing hormone (LH). The two hormones, LH and hCG are structurally similar and share a common receptor, the luteinizing hormone-chorionic gonadotropin receptor (LH/CGR). Of these two hormones, hCG has been shown to have a higher affinity for the LH/CGR receptor [Atger et al., 1995; Moyle et al., 1995] than LH. The LH/CGR receptor is highly conserved across species, therefore use of hCG is a reasonable way to produce effects of LH in non-primate species [Moyle et al., 1995; Swanson et al., 2003].

Materials and Methods

All protocols for animal care and use were reviewed and approved by the IACUC at Mississippi State University.

Experimental Animals

Fifteen sexually mature, terrestrial male eastern tiger salamanders were obtained from private breeders. The salamanders were allowed a five month acclimation period

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prior to any experimentation. Animals were housed in tanks, (66 cm x 45.7 x 30.5 cm) , in groups of three to six animals. Tanks were furnished with hiding spaces in the form of a substrate of moist sheet moss (Mosser Lee, Black River Falls, WI, USA) with a burrowing depth of at least six cm. UVB lighting sources were provided for each tank, and were set to timers (Intermatic, Spring Grove, IL, USA) on a natural light cycle.

Salamanders were fed on a rotating diet of earthworms and mealworms three times a week, and each animal was hand fed to monitor intake carefully and minimize aggression between animals within the tanks.

Induction of spermiation using exogenous hormones

Hormone treatments were scheduled over a recovery period of four weeks apart or greater; this recovery period was selected based on previously reported recovery periods in amphibians [McDonough et al., 2015] to ensure appropriate time between treatments for elimination of hormones.

Preliminary trials of 26 hormone protocols in six categories were run on treatment groups of n = 5 male eastern tiger salamanders (See *Appendix A*) from which 13 hormone protocols were selected (see Table 2.1) for further analysis against a control of sterile phosphate buffered saline ([PBS], Sigma Aldrich, St. Louis MO, USA) without any hormone. The control and treatment groups consisted of randomly selected male eastern tiger salamanders (n = 15). Lyophilized hCG and synthetic LHRH ([des Gly ^{10} D-Ala^6] LHRH ethylamide acetate salt hydrate; Sigma Aldrich, St. Louis MO, USA). were either used as an individual dose, as a combination treatment, or as a primed treatment in which a small amount of hormone was administered as a "prime" 24 hours prior to a larger "spermiation dose." (See Table 2.1). All doses were selected based on safe levels of

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hormone administered in other amphibian species [Bogaerts et al., 2012; Browne et al., 2006; Kouba et al., 2009; Lips et al., 2008; Mansour et al. 201; Silla, 2011; Stoops et al., 2014; Trottier & Armstrong, 1975; Uteshev et al. 2015; Uteshev et al., 2012; Waggener & Carroll, 1998] and on preliminary trials [Marcec, Unpublished data]. For protocols on reconstitution and administration of hormones, see *Appendix B*. Due to the large number of treatments, the treatments will be referred to by coded names in order to simplify text and tables (See Table 2.2).

Collection of sperm

Sperm collection and animal observation occurred over 72 hours, starting 1 hour after hormone treatment. Sperm was collected hourly during the first 12 hours, and every other hour in the period between 12-24 hours, and then every 6 hours for the remaining 48 hours. During the final 48 hour period, if sperm was obtained then the collection interval would return to every other hour until sperm was no longer produced, at which point 6 hour collection intervals were reinstated.

During the 72 hour collection period, animals placed in individually in plastic containers (36 cm x 24 cm x 18 cm), placed on a gentle slope with water covering half the accessible area. Plastic plants were placed in the water in order to allow for spermatophore deposition. Water was changed every 24 hours, or as needed. Containers were equipped with locking lids to prevent animal escape and covered with dark towels during the 72 hour observation period, so as to reduce animal stress.

If an animal produced milt (a thick, milky semen-like substance) upon collection, and/or passively produced spermatophores, it was classified as a general responder and designated specific sperm type was noted. Quality of the two forms of sperm was

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assessed separately (see *2.2.4 Evaluation and Analysis of Milt* and *2.2.5 Evaluation and Analysis of Spermatophores*).

Table 2.1   Male hormone administration protocols and their respective codes by which they will be referred to in text and tables in Chapter II

Code	Treatment
H1	100 IU hCG
H2	200 IU hCG
H3	300 IU hCG
L1	0.025 µg/g LHRH
L2	0.05µg/g LHRH
L3	0.10 µg/g LHRH
L4	0.25 µg/g LHRH
L5	0.50 µg/g LHRH
PL1	Prime* 0.025 µg/g LHRH; Spermiation dose** 0.1 µg/g LHRH
PL2	Prime* 0.05 µg/g LHRH; Spermiation dose** 0.25 µg/g LHRH
C1	100 IU hCG + 0.05 µg/g LHRH
C2	200 IU hCG + 0.025 µg/g LHRH
PC	Prime* 100 IU hCG; Spermiation dose** 0.05 µg/g LHRH

*Prime (priming dose) was administered 24 hours prior to the spermiation dose
** Spermiation dose was administered 24 hours following the priming dose (prime)

At each collection time point, any spermatophores observed in the water were gently removed from the container via transfer pipette and immediately placed on a glass slide for further analysis. The milt collection procedure was performed by picking up the animal and holding it such that the ventral side of the animal faced the collector. The animal was observed for sexual behaviors and cloacal puckering at this time (see *Appendix C: Physical and Behavioral Analysis*). After cloacal stimulation, the sides of the animal were firmly massaged for the stimulation of milt production (See *Appendix B*

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for more detailed procedures). Milt would be pipetted directly into a microcentrifuge tube in 10 µL increments. If the animal did not produce milt via massage, a small piece flexible 0.86 mm x 1.32 mm micro medical tube (Scientific Commodities Inc., Lake Harvest City AZ) was used as a catheter in order to stimulate milt production. Milt was allowed to fill the catheter tube a maximum of three times before discontinuing catheterization.

Evaluation and analysis of milt

Two µL of the freshly collected milt was diluted 1:100 in a 10% Holtfreter's solution (5.9mM NaCl, 0.67mM KCl, 0.76mM CaCl$_2$, 2.4mM NaHCO$_3$), and sperm was analyzed for concentration, percent total motility, percent progressive motility, percent non-progressive motility, and percent abnormal spermatozoa.

In order to analyze milt for concentration, 10 µL of the 1:100 diluted milt solution was placed on a hemocytometer, and spermatozoa within the four 1 mm^2 corner squares of the hemocytometer were counted. In order to obtain the concentration of spermatozoa/mL, the total count of the spermatozoa in these four squares was divided by 0.4 (to account for volume within the squares), multiplied by 100 (the dilution factor), and then multiplied by 1,000 (for 1 mL):

$$\frac{spermatozoa}{mL} = \left(\frac{sperm\ count}{0.4}\right) \times dilution\ factor\ \times 1{,}000 \qquad (2.1)$$

In order to determine percent motility, 5 µL of the 1:100 diluted milt solution was placed on a glass slide that had been rinsed with 1% BSA (bovine serum albumin, Sigma Aldrich, St. Louis MO, USA), without a coverslip. . The number of spermatozoa that were non-motile (unmoving), progressively motile (moving forward across the slide or

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actively moving in a circle), and non-progressively motile (tail-twitching or immobile with an active undulating membrane) were counted until a count of 100 cells was reached The progressive motile cell count and the non-progressive motile cell count were added together to obtain the percent total motility.

Cells that were normal (normal structure and complete cell with an intact undulating membrane) and cells that were abnormal (broken or malformed, including detached undulating membranes) were counted until a count of 100 cells was reached (See Figure 2.1).

Evaluation and analysis of spermatophores

Any spermatophores observed in the container at a collection time point were immediately removed and placed on a glass slide without a cover slip. They were inspected for gel cap completion at 10X magnification. Each spermatophore was then placed in a microcentrifuge tube in 20 µL of 10% Holtfreter's solution, and gently pipetted into a fine paste. A measure of 2µL of the paste was diluted 1:100 in 10% Holtfreter's solution, and the sperm within was analyzed for concentration, percent total motility, progressive motility, non-progressive motility, and abnormal spermatozoa, as above.

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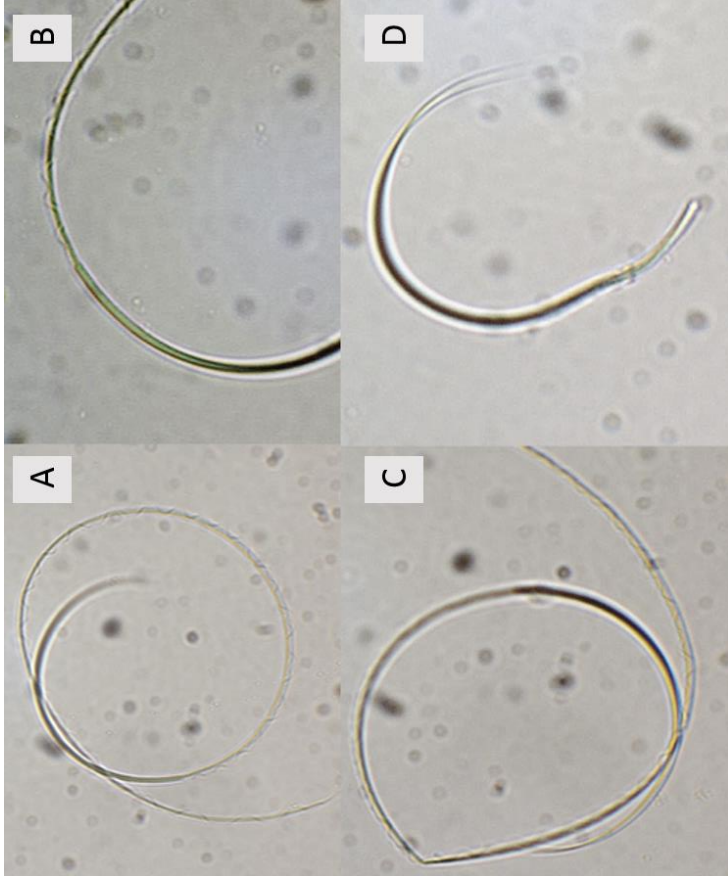
Figure 2.1   Examples of normal and some abnormal sperm from the eastern tiger salamander (*Ambystoma tigrinum*)

Figure 2.1 A-B: Normal spermatozoa, seen on 40 X magnification. Measures approximately 500 microns. Note the undulating membrane that starts along the tail at the base of the head. Note the large, thin, hook shaped head. head/tail junction of a normal spermatozoa. Note there is no mitochondrial vesicle.
Figure 2.1 C: Abnormal spermatozoa: "Bent tail." Note the abnormal angle about 1/3 of the way down the tail, proximate to the head.
Figure 2.1 D: Abnormal spermatozoa: "Broken head." Note the abnormal bend at the base of the head and the missing tail.

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Based on the formation of the gel cap and the concentration of sperm, the spermatophores were given a grade on a scale of one (G1) to five (G5) (see Table 2).

Table 2.2   Grading of spermatophore quality

Grade	Sperm Density Description	Gel	Concentration
G1	No sperm present	Gel cap only	0
G2	Sperm present in clumps or spread thin	+/- Gel cap	$< 1 \times 10^5$
G3	Sperm present in dense clumps or patches	+/- Gel cap	$> 1 \times 10^5$ $< 1 \times 10^6$
G4	Sperm dense throughout	Little to no gel cap	$\geq 1 \times 10^6$
G5	Sperm dense throughout	Complete gel cap	$\geq 1 \times 10^6$

Statistical Methods

Normality and equal variances of the data were assessed using the Shapiro Wilk test and Bartlett's test and data was found to be not normal. Data that was not normal was transformed via logarithmic transformation for non-percentage data and arcsine transformation for percentage data. For all statistical analysis, significance was determined by a p-value of < 0.05. A multivariate analysis of variance (MANOVA) was used in order to assess the differences between treatment groups. Post hoc testing was done via Tukey HSD and the Least Squares Means analysis. For the binary variable of response to treatment, a Fisher's exact test was used to assess the differences between treatments and the control.

A generalized linear mixed model (GLIMMIX) procedure with a gamma distribution and a correlated error structure was used to assess the data over time. For the

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binary variable of response to treatment over time, the model was given a logarithmic link function with a binary distribution.

Results

Control animals received PBS injections and produced no sperm. All other responding animals were evaluated verses control (see table 2.3). Parameters for milt were assessed only from animals that produced milt (see table 2.4) and parameters for spermatophores were assessed only from animals that produced spermatophores (see table 2.5).

Multivariate Analysis of Variance

Except for treatment H2 ($p=0.05$; $F=4.3$), all treatments resulted in animals responding significantly more frequently than animals treated with the control (see Table 2.3). There was no significant difference in the amount of milt produced (μL), the concentration of milt produced (spermatozoa/mL), the total motility of milt, the non-progressive motility of milt, the percent abnormal spermatozoa in milt, the number of spermatophores produced, the grade of spermatophores produced, or the total motility of spermatophores (see Table 2.4 for milt and Table 2.5 for spermatophores).

While total motility was not significantly different between treatments, a significant difference was seen in progressive motility ($p=0.007$; $F=2.5$). Animals treated with H1 or with C1 had significantly higher progressive motilities than animals treated with three of the other treatments (L2, L5, H3). Animals treated with PL2 had significantly higher progressive motilities than animals treated with L2 alone (Table 2.4).

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A significant difference was seen between treatments in the time to milt production (hr) post spermiation dose (p=0.02; F=2.1). Animals treated with PL1 or PL2 produced milt significantly sooner following the spermiation dose than in two of the other treatments (L2 and L3). Similarly, the time to production (hr) of spermatophores post spermiation dose was significantly different between treatments (p=0.04; F=1.5). Animals treated with a PL1 produced spermatophores significantly sooner following the time of the spermiation dose than in one of the other treatments (L3) (See Table 2.5).

There was a significant difference between treatments in numbers of Grade 4 spermatophores produced (p<0.0001; F=4.04) as well as Grade 5 spermatophores produced (p=0.001; F=3.2). Animals treated with the combination treatments of C1, C2, and PC produced significantly more grade 4 spermatophores than four of the other treatments (H2, L3, L4, PL1). Animals treated with the PC or with H2 produced significantly more grade 5 spermatophores than any other treatments (Table 2.5). While all hormone protocols produced more spermatophores overall than the control, only a few produced significantly more spermatophores of each grade than the control. G1 spermatophores, also called "empty" spermatophores, consist of only the gel component of the spermatophore (Table 2.1), and it is possible that these "empty" spermatophores may be produced when an animal is not hormonally stimulated. While none of these G1 spermatophores were seen from control animals, the amount of G1 spermatophores that was seen from hormone treated animals was insignificant. A number of the hormone protocols failed to produce a significant amount of G2, G3, G4, or G5 spermatophores, the grades of spermatophores that contained sperm, when compared to the control. The inverse relationship that was seen between milt production and spermatophore production

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likely accounted for the low number of spermatophores in each grade, and because the numbers of spermatophores were so low they could not be assessed for significance, given the amount of error.

The concentration of spermatophores was significantly different between treatments ($p < 0.0001$; $F = 4.9$). The treatment of L1 produced spermatophores with significantly higher concentrations than all other treatments. Percent abnormal sperm within spermatophores was also significantly different ($p < 0.0002$; $F = 3.8$). Animals treated with H2 or H3 had significantly more abnormal spermatozoa in their spermatophores than animals treated with nine of the other treatments (H1, L1, L2, L5, C1, C2 PL1, PL2, and PC). Animals treated with L3 or with L4 had significantly more abnormal spermatozoa in their spermatophores than animals treated with two of the other treatments (L1 and PL1) (Table 2.5).

All of the thirteen treatments were effective in eliciting a sperm response from the male salamanders. Two of the treatments, the L5 treatment and the PL1 treatment, were more likely to induce spermiation. The ability to induce spermiation is a key factor in determining which treatment is the most successful when there is not a significant difference in concentration or total motility across treatments. There was significant difference in progressive motility across treatments, but only one treatment (L2) had exceptionally low progressive motility. There is also evidence that in the natural state, caudate sperm must be activated prior to becoming functional [Onitake, Wantanabe[1-2]], and thus motility may not be an ideal basis for judging quality of caudate sperm. Although the L5 and PL1 treatment were effective at inducing spermiation in eastern tiger salamanders, they may not be ideal protocols for inducing spermiation in other

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caudate species. Eleven of the thirteen treatment protocols (excluding H2 and L2, which were not significantly different at inducing sperm production from the control) were at effective at inducing spermiation. Having a list of multiple working protocols may be of benefit when approaching application of hormone protocols in new species, as protocols may need to be adapted to individual species' needs. Hormone protocols for use in amphibians have frequently needed species specific modification [Kouba[1-2], Stoops]. If the protocol that is most effective in one species fails when applied to a new species, it may be advantageous to have a list of effective protocols to draw from rather than needing to improvise protocol adaptations. Because the other eleven protocols are functional, they may be used or integrated as adaptations to spermiation protocols when approaching assisted reproductive technologies in new species.

Analysis was also performed looking at differences in the dependent variables across the different months individuals were treated in order to account for seasonal variation. No significant difference was noted in any of the dependent variables when compared across the months in which treatments were administered.

Additionally, analysis was performed in order to determine differences in individual variation. There were significant differences in individual responses to treatment ($p=0.0013$; $F=2.5$), in individual responses by production of milt ($p=0.0001$; $F=3.0$), and in percent of abnormal sperm produced by each individual ($p=0.0003$,; $F=3.4$). There were no significant differences between individuals in any of the other dependent variables. More animals responded to LHRH treatments than hCG treatments ($p=0001$). Animals that did not have as frequent responses overall were also more likely to respond to an LHRH treatment than an hCG treatment ($p=0.001$). There was no

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difference in the likelihood of animals responding to Combination treatments and LHRH treatments, but animals responded to Combination treatments significantly more often than hCG treatments (p=0.04).

Generalized Linear Mixed Model Analysis

Models were assessed via residual plots, with good scatter determining a good model fit. Milt quality and response varied considerably over the 72 hour observation period for each treatment.

For amount of milt, a main effect of both the fixed effects treatment (p=0.008; F=2.59) and time (p<0.0001; F=8.35) was seen, as well as an interaction effect of treatment and time (p=0.0006; F=2.24). For concentration of milt, only the fixed effect of time had a main effect (p=0.005; F=1.42), although an interaction between treatment and time was noted (p<0.0001; F=3.21). In assessing total motility of milt, both fixed effects of treatment (p=0.005; F=2.8) and time (p=0.04; F=2.0) were seen to have main effects. Additionally, an interaction effect was seen between the two fixed effects (p=0.007; F=2.0). The assessment of the number of animals responding to milt showed that there was a main effect of both treatment (p<0.0001; F=18.2) and time (p<0.0001; F=40.3). For number of animals responding to treatment, there was also an interaction effect between the fixed effects of treatment and time.

Discussion

While all treatments were effective in producing milt and spermatophores, the PL1 treatment (a priming dose of 0.025 µg/g of LHRH followed by a spermiation dose of 0.1 µg/g of LHRH 24 hours later) proved to have the most satisfactory and consistent

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results. It had the highest total sperm, the fastest time to first milt, and was one of the two treatments with the highest number of animals responding to treatment. Over the 72 hour observation period, the PL1 treatment was fairly consistent, compared to other treatments, in maintaining sperm production and continual response to treatment. Because of these qualities, the PL1 treatment would be recommended in zoological and conservation institution for use in hormonally induced breeding attempts or in *in vitro* fertilization attempts.

Table 2.3   Percent animals responding to respective hormone treatments via milt, spermatophores, or both

Treatment	% Animals responding to treatment (n=15)	% Animals producing milt*	% Animals producing spermatophores**	% Animals producing milt and spermatophores
H1	46.7 [+]	71.4	42.8	14.3
H2	33.3	100.0	20.0	20.0
H3	53.3 [+]	60.0	60.0	40.0
L1	53.3 [+]	62.5	50.0	12.5
L2	40.0	50.0	50.0	0.0
L3	60.0 [+]	66.7	44.4	11.1
L4	60.0 [+]	77.8	44.4	22.2
L5	73.3 [+]	90.9	81.8	72.7
PL1	73.3 [+]	90.9	26.4	9.1
PL2	66.7 [+]	90.0	50.0	40.0
C1	66.7 [+]	60.0	80.0	40.0
C2	53.3 [+]	62.5	62.5	25.0
PC	53.3 [+]	87.5	37.5	25.0
PBS	0	0	0	0

For treatment code identification, see Table 2.1

+: Indicates a significant difference from the control (within the first column)

*Percent of animals producing milt is taken from the number of animals that responded to treatment by producing milt, spermatophores or both

** Percent of animals producing spermatophores is taken from the number of animals that responded to treatment by producing milt, spermatophores or both

Results from analysis of milt parameters (shown only from animals that produced milt)

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Table 2.4   Mean sperm parameters from analysis of milt

Treatment	Volume (µL)	Conc. (sperm/mL)	Total Sperm	% Abnormal sperm	% Motility Total	% Motility Progressive
H1	290.4 ± 113.5 [a]	$8.0 \times 10^7 \pm 7.5 \times 10^7$	197.7 ± 147.8	1.5 ± 0.6 [b]	46.8 ± 7.0	30.0 ± 7.0 [a]
H2	188.9 ± 105.4 [ab]	$4.3 \times 10^7 \pm 2.3 \times 10^7$	322.0 ± 226.8	4.9 ± 3.9 [b]	46.4 ± 6.6	21.6 ± 3.9 [ab]
H3	143.9 ± 50.1 [ab]	$2.7 \times 10^7 \pm 2.0 \times 10^7$	2871.5 ± 3248.7	4.5 ± 2.0 [b]	33.1 ± 9.2	12.0 ± 5.2 [b]
L1	50.2 ± 13.7 [b]	$1.3 \times 10^7 \pm 5.4 \times 10^6$	712.2 ± 361.2	0.7 ± 0.3 [b]	41.7 ± 3.4	17.7 ± 2.1 [ab]
L2	137.5 ± 58.9 [ab]	$1.2 \times 10^7 \pm 9.1 \times 10^6$	387.3 ± 428.3	6.0 ± 5.0 [b]	19.0 ± 10.0	5.7 ± 3.5 [b]
L3	199.7 ± 69.6 [ab]	$1.2 \times 10^7 \pm 3.9 \times 10^5$	349.9 ± 254.4	20.2 ± 3.2 [a]	43.9 ± 5.6	20.2 ± 3.2 [ab]
L4	194.4 ± 49.6 [ab]	$1.4 \times 10^7 \pm 5.6 \times 10^6$	350.1 ± 257.7	1.2 ± 0.5 [b]	43.8 ± 5.1	27.6 ± 4.8 [ab]
L5	58.0 ± 18.3 [b]	$9.5 \times 10^6 \pm 8.6 \times 10^5$	471.8 ± 42.9	3.9 ± 3.0 [b]	32.3 ± 5.7	12.3 ± 2.9 [b]
PL1	138.8 ± 40.6 [ab]	$5.8 \times 10^7 \pm 1.5 \times 10^7$	4438.3 ± 2110.6	1.5 ± 0.4 [b]	43.1 ± 4.1	20.1 ± 2.1 [ab]
PL2	307.4 ± 57.4 [a]	$3.4 \times 10^7 \pm 1.2 \times 10^7$	745.8 ± 837.6	2.8 ± 0.7 [b]	48.7 ± 3.3	23.9 ± 3.3 [ab]
C1	73.2 ± 43.1 [ab]	$6.9 \times 10^6 \pm 5.5 \times 10^6$	545.1 ± 241.9	4.5 ± 2.0 [b]	38.1 ± 9.0	23.3 ± 8.6 [ab]
C2	111.0 ± 43.2 [ab]	$1.8 \times 10^7 \pm 6.6 \times 10^6$	304.0 ± 263.6	1.8 ± 0.6 [b]	47.3 ± 5.9	19.4 ± 3.9 [ab]
PC	182.1 ± 90.7 [ab]	$8.4 \times 10^6 \pm 3.1 \times 10^6$	442.7 ± 269.7	2.6 ± 1.0 [b]	41.7 ± 5.0	19.1 ± 4.6 [ab]
PBS	-	-	-	-	-	-

For treatment code identification, see Table 2.1
[abc]: Values with different superscripts within the same column are significantly different from one another ($p < 0.05$)

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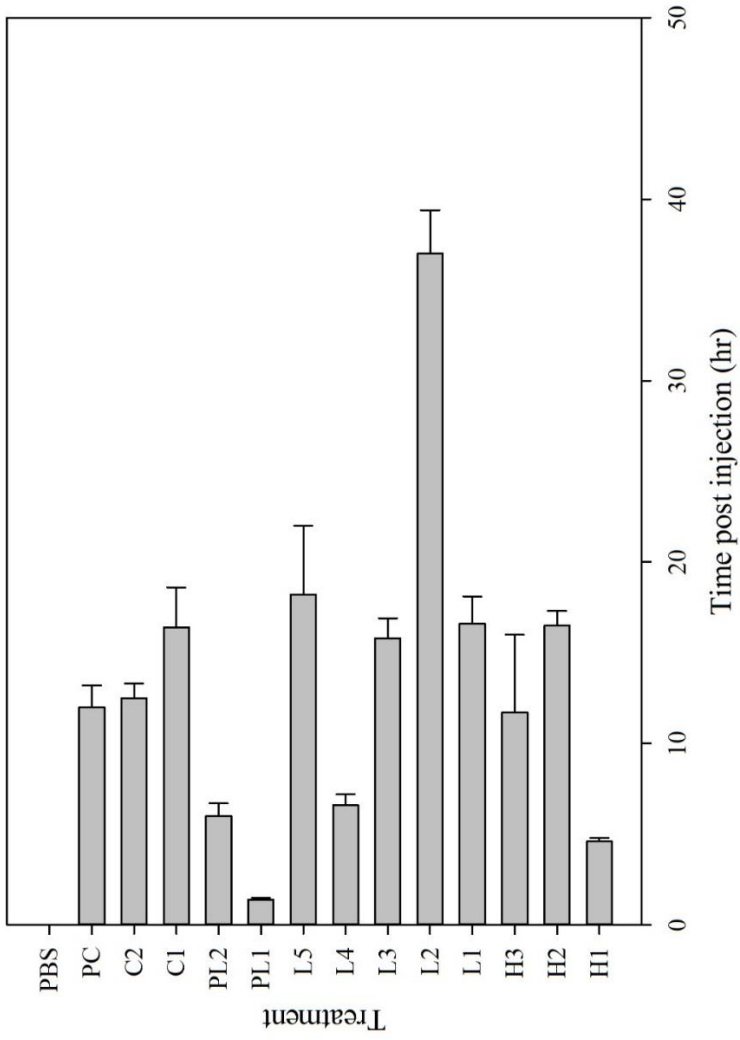
Figure 2.2   Time (hr) post injection of hormone to the first production of milt

Table 2.5   Mean sperm parameters from analysis of spermatophores

Treatment	Number of spermatophores	Concentration (sperm/mL)	Total Sperm	% Abnormal sperm	% Total motility[+]
H1	1.9 ± 0.3	$8.9\text{x}10^5 \pm 3.1\text{x}10^5$ [b]	48.3 ± 9.2	9.2 ± 1.5 [ab]	1.6 ± 1.0 [bc]
H2*	2.0	$1.2\text{x}10^6$ [ab]	60.0	16.0 [a]	0.0 [c]
H3	1.3 ± 0.2	$1.5\text{x}10^6 \pm 6.1\text{x}10^5$ [ab]	145.8 ± 61.5	7.5 ± 2.4 [ab]	4.7 ± 3.1 [bc]
L1	2.0 ± 0.4	$9.7\text{x}10^6 \pm 8.8\text{x}10^6$ [a]	966.0 ± 885.5	8.0 ± 1.9 [ab]	2.0 ± 2.0 [bc]
L2	2.6 ± 0.9	$5.6\text{x}10^5 \pm 1.8\text{x}10^5$ [b]	48.9 ± 20.2	3.2 ± 1.0 [b]	1.7 ± 1.5 [bc]
L3	1.3 ± 0.2	$1.2\text{x}10^5 \pm 8.1\text{x}10^4$ [b]	7.7 ± 6.9	9.5 ± 3.2 [ab]	3.8 ± 3.1 [bc]
L4	1.6 ± 0.2	$1.3\text{x}10^4 \pm 3\text{x}10^3$ [b]	0.4 ± 0.2	15.3 ± 5.0 [ab]	3.3 ± 2.6 [bc]
L5	1.6 ± 0.2	$8.6\text{x}10^5 \pm 2.6\text{x}10^5$ [b]	65.4 ± 26.0	7.1 ± 1.8 [ab]	3.4 ± 1.4 [bc]
PL1	1.6 ± 0.4	$5.2\text{x}10^5 \pm 3.3\text{x}10^5$ [b]	46.7 ± 33.9	3.8 ± 1.2 [b]	1.3 ± 1.0 [bc]
PL2	1.7 ± 0.4	$1.4\text{x}10^6 \pm 4.3\text{x}10^5$ [ab]	52.5 ± 12.2	6.3 ± 0.5 [ab]	2.0 ± 0.7 [b]
C1	2.1 ± 0.4	$9.9\text{x}10^5 \pm 3.4\text{x}10^5$ [b]	76.6 ± 18.1	7.3 ± 1.3 [ab]	6.5 ± 1.8 [b]
C2	3.2 ± 0.7	$9.6\text{x}10^5 \pm 2.1\text{x}10^5$ [b]	88.9 ± 30.4	5.1 ± 0.6 [b]	5.5 ± 1.1 [b]
PC	1.5 ± 0.3	$1.6\text{x}10^6 \pm 5.0\text{x}10^6$ [b]	120.0 ± 36.6	5.0 ± 1.9 [ab]	15.2 ± 4.9 [a]
PBS	-	-	-	-	-

For treatment code identification, see Table 2.1

[abc]. Values with different superscripts within the same column are significantly different from one another ($p < 0.05$)

*Values shown for treatment H2 represent only one animal

+ Only total motility is shown as no spermatophores were observed to have sperm with progressive motility

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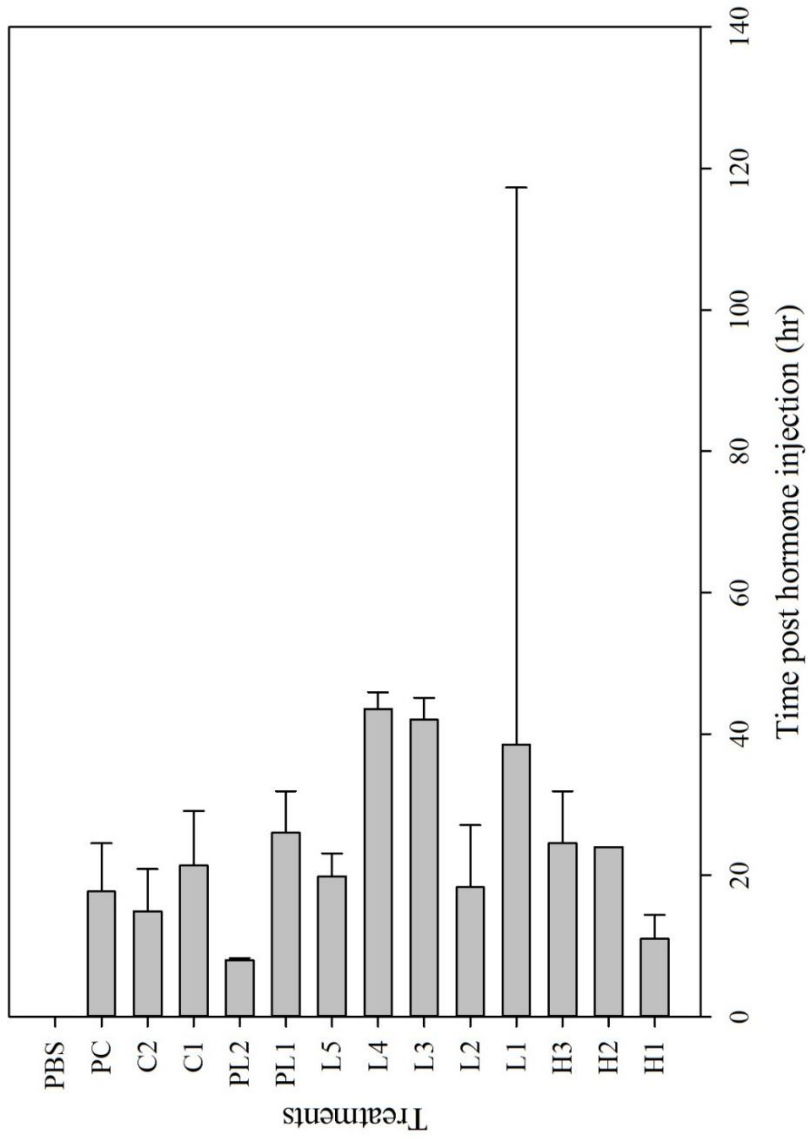
Figure 2.3   Time (hr) post injection of hormone to the first production of spermatophores

90
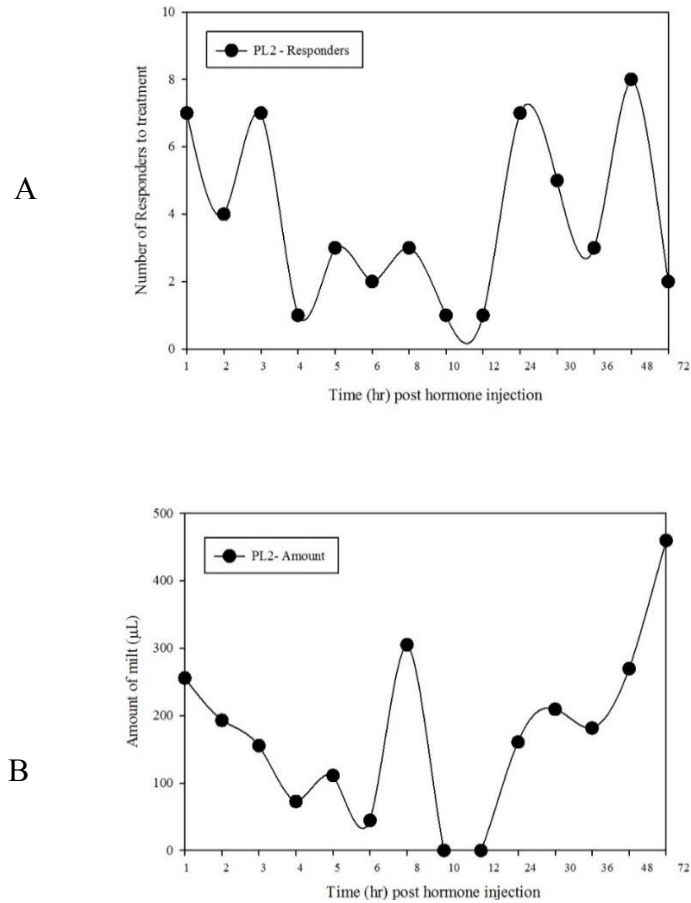
Figure 2.4   Observation at collection time points (time in hours post hormone injection) of number of animals responding to the PL2 treatment and amount of milt produced (μL) by animals treated with the PL2 treatment

Figure 2.4 A: The number of animals responding at each collection point is designated as animals that responded by production of either milt or spermatophores, with a total of n=15 animals treated.
Figure 2.4 B: The amount of milt only (no spermatophores) produced at each collection time point for the PL2 treatment is shown.
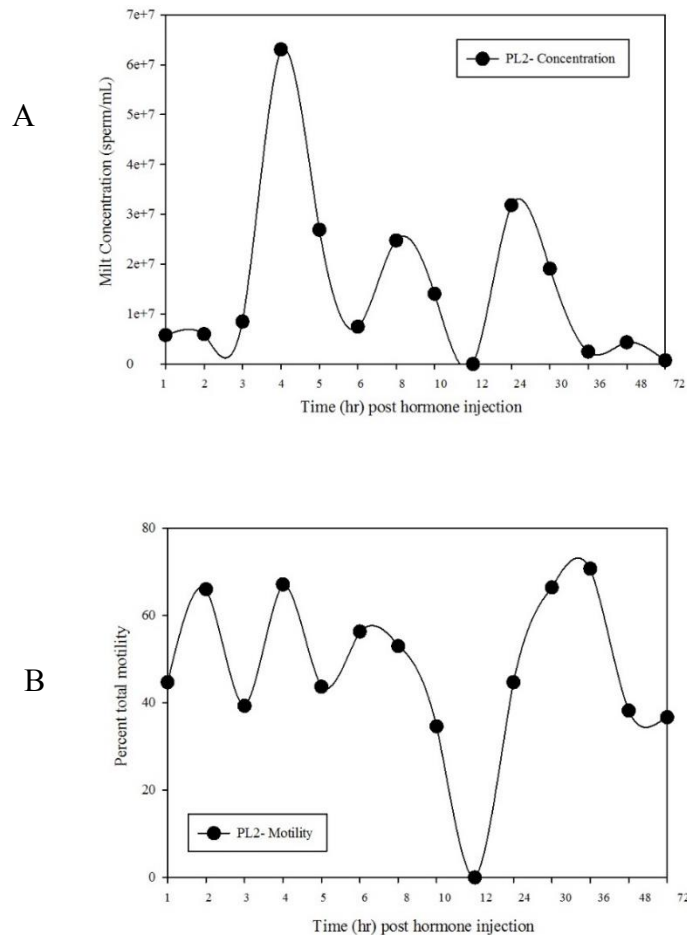
Figure 2.5   Observation at collection time points (time in hours post hormone
              injection) of sperm concentration (sperm/mL) and percent total motility for
              animals treated with the PL2 treatment

Figure 2.5 A: Observation of concentration (sperm/mL) from animals treated with PL2
Figure 2.5 B: Observation of percent total motility from animals treated with PL2

Hormone doses for hCG were not adjusted to the weight of the animals, based off

findings in previous studies [Browne et al, 2006; Kouba et al, 2012; Kouba & Vance,

2009; Mansour et al, 2011; Trottier & Armstrong, 1975]. These findings suggested that

adjustment to weight may result in inappropriate, and potentially refractory, doses.

Consideration was taken that weight might be an indication of overall fitness of the

animals, thus causing a discrepancy in ability of individuals to produce sperm. There was

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no significant difference between individuals' production or quality of sperm based on weight, neither was weight a predicting variable in the production of quality of sperm. Each individual animal, when assessed by his or her identity and not by weight, showed both a significant difference in response and in quality, in regards to the percent of abnormal sperm produced. The variation among individuals can be attributed to many factors, involving overall fitness of the animals, individual animal body chemistry, or difference in age of animals. As these animals had not been raised in captivity, there was no indication as to their age. They were known to be sexually mature, but they may have been many years beyond sexual maturity. Seasonality was not a factor as variation in response and sperm quality was assessed for each month in which animals were treated, and no significant differences were noted. While *Ambystoma tigrinum* breed once in the year and have a seasonal sexual hormone change [Norris et al, 1985], their sex hormones may be altered by environmental factors, including stress [Norris & Jones, 1987; Ogielska & Lopez, 2011; Rose et al, 1995]. While each animal was handled in an identical manner which was designed to minimize stress, individuals perceive stress differently [Koolhaas et al, 2010]. This, and natural individual variation in circulating testosterone and estrogens, may have accounted for the significant variation in individual response. In the future, utilizing assays to determine the endogenous hormone levels of an animal may help to idealize both which animals are likely to respond best to treatments and which treatments will be best to for a given animal. This variation in individuals re-emphasizes the benefit of having multiple protocols which are effective to fall back upon when attempting application of hormones in a new group of animals.

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In looking at which animals responded to what treatments, overall any treatment containing LHRH, including combination treatments, resulted in significantly more responses than hCG alone. This suggests that *Ambystoma tigrinum* are more sensitive to LHRH than hCG, or that LHRH acts faster in LHRH that hCG. The latter hypothesis may be more likely, as one animal that was injected with a preliminary primed dose of hCG did not produce milt within 72 hours but did produce milt one week later. Looking at the response time and the length of response of both LHRH and hCG in salamanders is a critical component of future study. Additionally, studying the long term effects of these hormones on the salamander physiology is crucial to understanding the safety and worth of their use in conservation. In comparison to what was seen in this study, in previous studies with anurans, animals were more likely to respond to hCG than to LHRH [Koub & Vance, 2009; Kouba et al, 2012], although little to no effect was seen by hCG in some species [Mann et al, 2009].

Although the PL1 treatment was effective at inducing spermiation in*Ambystoma tigrinum*, it may not be an ideal protocol for inducing spermiation in other caudate species. Twelve of the thirteen treatment protocols (excluding H2, which was not significantly different at inducing sperm production from the control) were at effective at inducing spermiation. Having a list of multiple working protocols may be of benefit when approaching application of hormone protocols in new species, as protocols may need to be adapted to individual species' needs. Hormone protocols for use in amphibians have frequently needed species specific modification [Kouba & Vance, 2009; Stoops et al, 2014]. If the protocol that is most effective in one species fails when applied to a new species, it may be advantageous to have a list of effective protocols to draw from rather

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than needing to improvise protocol adaptations. Because the other twelve protocols are functional, they may be used or integrated as adaptations to spermiation protocols when approaching assisted reproductive technologies in new species. Some of these protocols were attempted in the regionally endangered blue spotted salamander (*Ambystoma laterale*) and successful spermiation was induced in 100% of males treated. Results of these trials can be found in *Appendix F.* While the sperm quality from *A. laterale* was not ideal, each animal produced sperm. This may suggest that, with some adjustment, protocols developed in *Ambystoma tigrinum* may be successfully transferred to endangered target species.

    Ambystoma tigrinum naturally produce spermatophores [Duellman & Trueb, 1994; Ogielska, 2009; Siegel et al, 2014; Vitt & Caldwell, 2009], unlike the few salamanders that naturally produce milt, like those in the family Cryptobranchidae [Duellman & Trueb, 1994; Sever, 1991; Vitt & Caldwell, 2009]. In general, spermatophore production was fairly low in this study (1.8 ± 0.1; see Table 2.5 for individual treatments). In previous studies where hormones were used to induce spermatophore production in salamanders, spermatophore production was fairly high [Toyoda et al, 1996; Toyoda et al, 2005; Trottier & Armstrong, 1975], as was the case in studies where spermatophore production was allowed to occur naturally [Doyle et al, 2011; Figel, 2013]. The unnatural process of obtaining milt from *Ambystoma tigrinum* likely lead to a decrease in production of spermatophores, because milt was obtained by massaging the animals' internal testicles. Spermatophores are produced as they exit the collecting ducts and the cloaca [Sever, 1991]. By massaging the testicles, sperm was mobilized prematurely and the biological process of spermatophore production was likely

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disrupted. The sperm that would have later moved to the collecting ducts and been packaged into spermatophores was expressed as milt instead.

Unlike *Ambystoma tigrinum*, salamanders in the families Cryptobranchidae, Sirenidae, and Hynobiidae undergo external fertilization, much like anurans. The Ambystomid family, along with other salamander families excluding the aforementioned three, undergo internal fertilization. Internal fertilization in salamanders occurs through use of the spermatophore. The female salamander picks up the condensed packet of sperm into her cloaca and stores it in a specialized compartment called a spermatheca until time of fertilization. As the eggs pass by the spermatheca, sperm is released and fertilization occurs. In animals that produce spermatophores, it is not natural to produce milt. The milt obtained in this study had concentrations similar to those reported in the natural milt of eastern hellbenders (*Cryptobranchus allegiansis allegiansis*). Average sperm concentration collected from milt in wild eastern hellbenders has been reported as having average concentrations of $3.1 \times 10^7 \pm 7.6 \times 10^5$ [Unger et al, 2013]. Average concentration found in the eastern tiger salamander milt milked from the internal testicles in this study was $2.4 \times 10^7 \pm 6.4 \times 10^6$. This concentration is similar to findings in a study where milt was artificially milked from the internal testicles of the Mexican axolotl (*Ambystoma tigrinum*), in which concentrations ranged from $1.4 \times 10^7 \pm 5.1 \times 10^6$ to $1.6 \times 10^7 \pm 3.9 \times 10^6$ [Mansour et al, 2011]. Total motility, however, was found to be lower in the milt of obtained in this study compared to that reported in hellbenders. Average total motility reported in eastern hellbenders from the same study mentioned previously was $85.3 \pm 21.3\%$, while average total motility of the tiger salamanders in this study was $40.4 \pm 2.2\%$. In some species, spermatophores have been noted to have 0% motility

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[Watanabe et al, 2003; Watanabe et al, 2010], and in this study it was noted that spermatophores had very low motility and had no progressive motility, only non-progressive motility. The motility of sperm in *Ambystoma tigrinum* is unusual in itself. The progressive movement is a circular motion and appears to be propelled mostly by the undulation of the tail membrane, not by the motion of the tail. Once the sperm contacts the egg or egg jelly, the tail may begin to beat rapidly; however, before that time, it appears either immobile, vibrating in place, or spinning based on the movement of the undulating membrane. In theory, the sperm of internally fertilizing salamanders does not need to have much forward propulsion. Within the body of the female, a spermatophore is deposited almost directly onto the eggs as they pass out of the cloaca [Norris & Jones, 1987; Norris & Lopez, 2011; Norris et al, 1985; Ogielska, 2009; Sever, 1991]. Therefore, energy of the sperm is likely conserved for penetration rather than swimming. Indeed, experiments have shown that when salamander sperm is exposed to salamander egg jelly, there is a component of the egg jelly which activates the sperm and up to 100% of the sperm becomes motile [Marcec, Unpublished data; Watanabe et al, 2003; Watanabe et al, 2010]. The motility of this egg jelly-activated sperm is rapid, although in *Ambystoma tigrinum*, much of the sperm still moves in a circular pattern [Mansour et al, 2009; Marcec, Unpublished data]. In addition to activation by egg jelly, the anatomy of the sperm supports the supposition that salamander sperm is idealized for penetration, having long (approximately 200 μm), hook-shaped heads containing small barbs on the tips [Ogielska, 2009; Selmi et al, 1997]. It is supposed that these barbs are used for hooking on to the egg in a harpoon-like manner so that the sperm may penetrate easily through the thick membrane [Selmi et al, 1997]. While the basic structure of most salamander sperm

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is the same, the lack of necessity for sperm movement in spermatophore producing salamanders is contrary to motion in externally fertilizing salamanders. In externally fertilizing salamanders, the sperm is required to swim a distance outside the body to reach the eggs [Babcock et al, 1994; Dzminski et al, 2009; Stoltz & Neff, 2006]. These differences in physiology of fertilization may explain the differences in the motility of the hellbender milt compared to the tiger salamander milt.

The curve of sperm concentration and amount over time in anurans is typically a smooth bell curve [Kouba et al, 2012; Langhorne, 2015]. In *Ambystoma tigrinum*, the time curves for amount, concentration, and motility were seen to make many rises and falls. It is possible that because these animals normally package their sperm into spermatophores, these peaks are artifacts of the packing process. The sperm of the salamanders may be in the process of packaging while it is being collected as milt, and what is seen in the curves is the high density of the spermatophores and the low density between packaging.

Overall, the treatment found to be the most efficient in *Ambystoma tigrinum* for production of good quality sperm was a prime of 0.025 µg/g of LHRH followed 24 hours later by a spermiation dose of 0.1 µg/g of LHRH. While this performed greater than 12 other treatments and a PBS control, 11 of the hormone treatments tested were successful at producing sperm in *Ambystoma tigrinum*. Two of the 13 hormone treatments were chosen and utilized in *Ambystoma laterale* in order to attempt spermiation in this regionally endangered species, and 100% of these males produced sperm (see *Appendix E* for *Ambystoma laterale* data). This suggests that these protocols may be successfully transferred to endangered species related to *Ambystoma tigrinum*.

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References

[1] Adams EM, Jones AG, Arnold SJ. Multiple paternity in a natural population of a salamander with long-term sperm storage. Mol Ecol 2005;14:1803–10. doi:10.1111/j.1365-294X.2005.02539.

[2] Atger M, Misrahi M, Sar S, Le Flem L, Dessen P, Milgrom E. Structure of the human luteinizing hormone-choriogonadotropin receptor gene: unusual promoter and 5′ non-coding regions. Mol Cell Endocrinol 1995;111:113–23. doi:10.1016/0303-7207(95)03557-N.

[3] Babcock RC, Mundy CN, Whitehead D. Sperm diffusion models and in situ confirmation of long-distance fertilization in the free-spawning asteroid Acanthaster planci. Biol Bull 1994;186:17–28. doi:10.2307/1542033.

[4] Beebee TJC, Griffiths RA. The amphibian decline crisis: A watershed for conservation biology? Biol Conserv 2005;125:271–85. doi:10.1016/j.biocon.2005.04.009.

[5] Blaustein AR, Belden LK, Olson DH, Green DM, Root TL, Kiesecker JM. Amphibian breeding and climate. Conserv Biol 2001;15:1804–9. doi:10.1038/374219a0.

[6] Bogaerts S, Janssen H, Macke J, Schultschik G, Ernst K, Maillet F, et al. Conservation biology, husbandry, and captive breeding of the endemic Anatolia newt, Neurergus strauchii Steindachner (1887) (Amphibia: Caudata: Salamandridae). Amphib Reptil Conserv 2012;6:9–29.

[7] Browne RK, Seratt J, Vance C, Kouba A. Hormonal priming, induction of ovulation and in-vitro fertilization of the endangered Wyoming toad (Bufo baxteri). Reprod Biol Endocrinol 2006;4:34. doi:10.1186/1477-7827-4-34.

[8] Calisi RM, Bentley GE. Lab and field experiments: Are they the same animal? Horm Behav 2009;56:1–10. doi:10.1016/j.yhbeh.2009.02.010.

[9] Cole L a. Biological functions of hCG and hCG-related molecules. Reprod Biol Endocrinol 2010;8:102. doi:10.1186/1477-7827-8-102.

[10] Collins JP, Storfer A. Global amphibian declines : sorting the hypotheses 2003:89–98.

[11] Corn PS. Climate change and amphibians. Anim Biodivers Conserv 2005;1:59–67.

[12] Davic D, Welsh H. Roles on the Ecological of Salamanders. Annu Rev Ecol Evol Syst 2014;35:405–34. doi:10.2307/annurev.ecolsys.35.112202.30000016.

[13] Dellovade T, Schwanzel-Fukuda M, Gordan J, Pfaff D. Aspects of GnRH neurobiology conserved across vertebrate forms. Gen Comp Endocrinol 1998;112:276–82. doi:10.1006/gcen.1998.7157.

[14] Doyle JM, McCormick CR, DeWoody JA. The quantification of spermatozoa by real-time quantitative PCR, spectrophotometry, and spermatophore cap size. Mol Ecol Resour 2011;11:101–6. doi:10.1111/j.1755-0998.2010.02892.

[15] Duellman DE, Trueb L. Biology of Amphibians. Baltimore USA: John Hopkins University Press; 1994.

[16] Dziminski MA, Roberts JD, Beveridge M, Simmons LW. Sperm competitiveness in frogs: slow and steady wins the race. Proc R Soc B Biol Sci 2009;276:3955–61. doi:10.1098/rspb.2009.1334.

[17] Figel C. Cryopreservation of s perm from the axolotl Ambystom mexicanum : Implications for conservation. Herpetol Conserv Biol 2013;8:748–855.

[18] Gascon, C Collins, JP Moore, RD Church, DR McKay, JE Mendelson J. Amphibian Conservation Action Plan. IUCN/SCC Amphib. Conserv. Summit 2005, 2005.

[19] Griffiths RA, Pavajeau L. Captive Breeding, Reintroduction, and the Conservation of Amphibians. Conserv Biol 2008;22:852–61. doi:10.1111/j.1523-1739.2008.00967.

[20] Hardy, MP Dent J. Hardy and Baronet.pdf. J Exp Zool 1986;240:385–96.

[21] Jones AG, Adams EM, Arnold SJ. Topping off: a mechanism of first-male sperm precedence in a vertebrate. Proc Natl Acad Sci U S A 2002;99:2078–81. doi:10.1073/pnas.042510199.

[22] Kim D-K, Cho EB, Moon MJ, Park S, Hwang J-I, Kah O, et al. Revisiting the evolution of gonadotropin-releasing hormones and their receptors in vertebrates: Secrets hidden in genomes. Gen Comp Endocrinol 2011;170:68–78. doi:10.1016/j.ygcen.2010.10.018.

[23] Kim J-K, Kim I-H, Heo J-H, Lee J-H, Ra N-Y, Eom J, et al. Arginine vasotocin (AVT) triggers courtship behavior without exposure to external stimuli and modulates the olfactory response of male Hynobius leechii salamanders. Zoolog Sci 2013;30:929–37. doi:10.2108/zsj.30.929.

[24] Koolhaas JM, de Boer SF, Coppens CM, Buwalda B. Neuroendocrinology of coping styles: Towards understanding the biology of individual variation. Front Neuroendocrinol 2010;31:307–21. doi:10.1016/j.yfrne.2010.04.001.

[25] Kouba AJ, DelBarco-Trillo J, Vance CK, Milam C, Carr M. A comparison of human chorionic gonadotropin and luteinizing hormone releasing hormone on the induction of spermiation and amplexus in the American toad (Anaxyrus americanus). Reprod Biol Endocrinol 2012;10:59. doi:10.1186/1477-7827-10-59.

[26] Kouba AJ, Vance CK. Applied reproductive technologies and genetic resource banking for amphibian conservation. Reprod Fertil Dev 2009;21:719–37. doi:10.1071/RD09038.

[27] Langhorne, Cecilia. 2015. Assisted Reproductive Technologies for Endangered North American Amphibians. (Doctoral Dissertation)

[28] Lips KR, Diffendorfer J, Mendelson JR, Sears MW. Riding the Wave: Reconciling the Roles of Disease and Climate Change in Amphibian Declines. PLoS Biol 2008;6:e72. doi:10.1371/journal.pbio.0060072.

[29] Mann Reinier Ross M, Hyne V, and Choung CB. Hormonal Induction of Spermiation, Courting Behavior and Spawning in the Southern Bell Frog, Litoria Raniformis. Zoo Biology. 2009; 29 (6): 774–82. doi:10.1002/zoo.20331.

[30] Mansour N, Lahnsteiner F, Patzner R a. Collection of gametes from live axolotl, Ambystoma mexicanum, and standardization of in vitro fertilization. Theriogenology 2011;75:354–61. doi:10.1016/j.theriogenology.2010.09.006.

[31] Marcec, Ruth. Motility of *Ambystoma tigrinum* sperm before and after contact with egg jelly. Unpublished raw data.

[32] Mcdonough CE, Martin MW, Vance CK, Kouba JACAJ. Frequency of exogenous hormone therapy impacts spermiation in male Fowler ' s toad (Bufo fowleri). Reprod Fertil Dev 2015.

[33] Means DB, J.G. P, Baggett M. Effects of Slash Pine Silviculture on a Florida Population of Flatwoods Salamanders. Conserv Biol 1996;10:426.

[34] Mohan J, Moudgal RP. Biochemical characteristics of transparent fluid and its effect on in vitro storage of cock spermatozoa. Indian J Poult Sci 1996;31:96–100.

[35] Mohan J, Moudgal RP, Sastry KVH, Tyagi J, Singh R. Effects of hemicastration andcastration on foam production and its relationship with fertility in male Japanese quail. Theriogenology 2002;58:29–39. doi:10.1016/S0093-691X(02)00863-4.

[36] Moore FL, Boyd SK, Kelley DB. Historical perspective: Hormonal regulation of behaviors in amphibians. Horm Behav 2005;48:373–83. doi:10.1016/j.yhbeh.2005.05.011.

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[37]   Moore FL, Wood RE, Boyd SK. Sex steroids and vasotocin interact in a female amphibian (Taricha granulosa) to elicit female-like egg-laying behavior or male-like courtship. Horm Behav 1992;26:156–66. doi:10.1016/0018-506X(92)90039.

[38]   Moyle, WR Campbell, RK Venkateswara Rao, SN Ayad, NG Bernard, MP Han, Y Wang Y. Model of Human Chorionic Gonadotropin and Lutropin Receptor Interaction That Explains Signal Transduction of the Glycoprotein Hormones. J Biol Chem 1995;270:20020–31.

[39]   Norris DO, Jones RE. Hormones and Reproduction in Fishes, Amphibians, and Reptiles. Verlag USA: Springer; 1987.

[40]   Norris DO, Lopez KH. Hormones and Reproduction of Vertebrates: Volume 2: Amphibians. San Diego USA: Elsevier; 2011.

[41]   Norris DO, Norman MF, Pancak MA, Duvall D. Seasonal variations in spermatogenesis, testicular weights, vasa deferentia, and androgen levels in neotenic male tiger salamanders, Ambystoma tigrinum. Gen Comp Endocrinol 1985;60:51–7.

[42]   Obringer AR, O'Brien JK, Saunders RL, Yamamoto K, Kikuyama S, Roth TL. Characterization of the spermiation response, luteinizing hormone release and sperm quality in the American toad (Bufo americanus) and the endangered Wyoming toad (Bufo baxteri). Reprod Fertil Dev 2000;12:51–8.

[43]   Ogielska, M. Reproduction of Amphibians. Enfield USA: Science Publishers; 2009.

[44]   Onitake K, Takai H, Ukita M, Mizuno JI, Sasaki T, Watanabe A. Significance of egg-jelly substances in the internal fertilization of the newt, Cynops pyrrhogaster. Comp Biochem Physiol - B Biochem Mol Biol 2000;126:121–8. doi:10.1016/S0305-0491(00)00191-7.

[45]   Pawson AJ, McNeilly AS. The pituitary effects of GnRH. Anim Reprod Sci 2005;88:75–94. doi:10.1016/j.anireprosci.2005.05.010.

[46]   Roch GJ, Busby ER, Sherwood NM. Evolution of GnRH: diving deeper. Gen Comp Endocrinol 2011;171:1–16. doi:10.1016/j.ygcen.2010.12.014.

[47]   Rose, JD., Kinnaird JR, Moore FL. Neurophysiological Effects of Vasotocin and Corticosterone on Medullary Neurons: Implications for Hormonal Control of Amphibian Courtship Behavior. Neuroendocrinology 1995:62;4: 406–17. doi:10.1159/000127030.

[48] Rowson AD, Obringer AR, Roth TL. Non-invasive treatments of luteinizing hormone-releasing hormone for inducing spermiation in American (Bufo americanus) and Gulf Coast (Bufo valliceps) toads. Zoo Biol 2001;20:63–74. doi:10.1002/zoo.1007.

[49] Seiwart, CM Adkins-Regan E. The foam production system of the male japanese quail: Characterization of structure and function. Brain Behav Evol 1998;52:61–80.

[50] Selmi MG, Brizzi R, Bigliardi E. Sperm morphology of salamandrids (Amphibia, Urodela): implications for phylogeny and fertilization biology. Tissue Cell 1997;29:651–64. doi:10.1016/S0040-8166(97)80041-9.

[51] Sever DM. Comparative anatomy and phylogeny of the cloacae of salamanders (Amphibia: Caudata). 1. Evolution at the family level. Herpetologica 1991;47:165–93. doi:10.1002/jmor.1052070306.

[52] Siegel DS, Alvino S, Trauth SE, Sever DM, Gribbins KM. Spermatogenic cycle of a plethodontid salamander, *Eurycea longicauda* (Amphibia, Urodela). Spermatogenesis 2014;4:e982420. doi:10.4161/21565562.2014.982420.

[53] Silla AJ. Effect of priming injections of luteinizing hormone-releasing hormone on spermiation and ovulation in Gunther's Toadlet, Pseudophryne guentheri. Reprod Biol Endocrinol 2011;9:68. doi:1477-7827-9-68 [pii]\n10.1186/1477-7827-9-68.

[54] Singh RP, Sastry KVH, Shit N, Pandey NK, Singh KB, Mohan J, et al. Cloacal gland foam enhances motility and disaggregation of spermatozoa in Japanese quail (Coturnix japonica). Theriogenology 2011;75:563–9. doi:10.1016/j.theriogenology.2010.09.028.

[55] St. Aubin DJ, Ridgway SH, Wells RS, Rhinehart H. Dolphin Thyroid and Adrenal Hormones: Circulating Levels in Wild and Semidomesticated Tursiops Truncatus, and Influence of Sex, Age, and Season. Marine Mammal Science,1996: 12; 1; 1–13. doi:10.1111/j.1748-7692.1996.tb00301.

[56] Stoltz JA, Neff BD. Sperm competition in a fish with external fertilization: The contribution of sperm number, speed and length. J Evol Biol 2006;19:1873–81. doi:10.1111/j.1420-9101.2006.01165.x.

[57] Stoops MA, Campbell MK, Dechant CJ. Successful captive breeding of Necturus beyeri through manipulation of environmental cues and exogenous hormone administration: a model for endangered Necturus. Herpetol Rev 2014;45:251–6.

[58] Stuart SN, Chanson JS, Cox NA, Young BE, Rodrigues ASL, Fischman DL, et al. Status and trends of amphibian declines and extinctions worldwide. Science 2004;306:1783–6. doi:10.1126/science.1103538.

[59]   Swanson P, Dickey JT, Campbell B. Biochemistry and physiology of fish gonadotropins. Fish Physiol Biochem 2003;28:53–9. doi:10.1023/B:FISH.0000030476.73360.07.

[60]   Tennessen JA., Zamudio KR. Early male reproductive advantage, multiple paternity and sperm storage in an amphibian aggregate breeder. Mol Ecol 2003;12:1567–76. doi:10.1046/j.1365-294X.2003.01830.

[61]   Toyoda F, Hasunuma I, Yamamoto K. Prolactin acts centrally to enhance newt courtship behavior. Gen Comp Endocrinol 2005;141:172–7. doi:10.1016/j.ygcen.2004.12.015.

[62]   Toyoda F, Matsuda K, Yamamoto K, Kikuyama S. Involvement of endogenous prolactin in the expression of courtship behavior in the newt, Cynops pyrrhogaster. Gen Comp Endocrinol 1996;102:191–6. doi:10.1006/gcen.1996.0060.

[63]   Trottier TM, Armstrong JB. Hormonal stimulation as an aid to artificial insemination in Ambystoma mexicanum. Can J Zool 1975;53:171–3.

[64]   Unger S, Mathis A, Wilkinson R. A comparison of sperm health in declining and stable populations of hellbenders (Cryptobranchus alleganiensis alleganiensis and C . a . bishopi). Am Midl Nat 2013;170:382–92.

[65]   Uteshev VK, Kaurova S, Shishova N, Stolyarov S, Browne R, and Gakhova E. In Vitro Fertilization with Hormonally Induced Sperm and Eggs from Sharp-Ribbed Newts Pleurodeles Waltl. Russian Journal of Herpetology. 2015; 22:1:35–40.

[66]   Uteshev VK, Shishova N V, Kaurova SA, Browne RK, Gakhova EN. Hormonal induction of spermatozoa from amphibians with Rana temporaria and Bufo bufo as anuran models. Reprod Fertil Dev 2012;24:599–607. doi:10.1071/RD10324.

[67]   Vitt LJ, Caldwell JP. Herpetology. 3rd ed. Singapore China: Elsevier; 2009.

[68]   Waggener WL, Carroll EJ. A method for hormonal induction of sperm release in anurans (eight species) and in vitro fertilization in lepidobatrachus species. Dev Growth Differ. 1998; 40 (1):19-25.

[69]   Walls S, Barichivich W, Brown M. Drought, Deluge and Declines: The Impact of Precipitation Extremes on Amphibians in a Changing Climate. Biology (Basel) 2013;2:399–418. doi:10.3390/biology2010399.

[70]   Watanabe T, Itoh T, Watanabe A, Onitake K. Characteristics of sperm motility induced on the egg-jelly in the internal fertilization of the newt, Cynops pyrrhogaster. Zool Sci 2003;20:345–52. doi:10.2108/zsj.20.345.

[71]   Watanabe T, Kubo H, Takeshima S, Nakagawa M, Ohta M, Kamimura S, et al. Identification of the sperm motility-initiating substance in the newt, Cynops pyrrhogaster, and its possible relationship with the acrosome reaction during internal fertilization. Int J Dev Biol 2010;54:591–7. doi:10.1387/ijdb.092894tw.

[72]   Woodley SK. Plasma Androgen Levels, Spermatogenesis, and Secondary Sexual Characteristics in Two Species of Plethodontid Salamanders with Dissociated Reproductive Patterns. Gen Comp Endocrinol 1994;96:206–14. doi:10.1006/gcen.1994.1175.

[73]   Woolley SC, Sakata JT, Crews D. Evolutionary insights into the regulation of courtship behavior in male amphibians and reptiles. Physiol Behav 2004;83:347–60. doi:10.1016/j.physbeh.2004.08.021.

[74]   Wright KM, Whitaker BR. Amphibian Medicine and Captive Husbandry. Malabar USA: Krieger Publishing Company; 2001.

[75]   Zalisko, Edward J Brandon, Ronald A Martan J. Zalisko 1984 Spermatophores. Copeia 1984;1984:739–47.

CHAPTER III

DEVELOPMENT OF PROTOCOLS FOR CRYOPRESERVATION OF SPERM IN

THE TIGER SALAMANDER (*AMBYSTOMA TIGRINUM*)

Introduction

As amphibian populations dwindle, this last decade has seen an increased focus
on protocol development to cryopreserve anuran (frog/toad) of sperm [Clulow et al.,
2014; Kouba et al., 2009]. Cryopreserved sperm is maintained in genetic resource banks
to manage genetic diversity such that gametes may be maintained long after valuable
animals have become senescent or have died. Additionally, genetic resource banks
maintain readily available sperm for artificial insemination or *in vitro* fertilization when
natural mating fails or when an animal is out of season. A reduction of animals housed at
a breeding facility can also be beneficial if the genetic diversity and genetic pairings can
be maintained through gamete cryostorage. Thus, genetic resource banks decrease the
cost and space required for housing animals yet help maintain genetic diversity within a
species. Using cryopreserved sperm and IVF in breeding events also allows animal
pairings to occur over long distances without the risks associated with shipping animals
[Clulow et al., 2014; Kouba et al., 2009; Watson & Holt, 2001; Wildt, 2000].

Most cryopreservation practices for anurans are based on protocols for freshwater
fish, as fish sperm and anuran have similar osmolality-related properties. Effectively fish
and anuran spermatozoa are activated and motile in a low osmotic environment [Browne

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et al, 2015; Byrne et al, 2015]. Yet the knowledge base for anuran sperm cryopreservation is more advanced than that of caudates, for which nearly nothing is known. In fact, only four reports are available on cryopreservation of salamander sperm: hellbenders [Unger et al., 2013], Chinese giant salamanders [Peng et al., 2013], axolotls [Figel, 2013], and tiger salamanders [Marcec et al., 2014].

The cryoprotectant is critical in the success of a protocol; however, the needs of sperm are species specific [Holt, 2000; Holt2, 2000; Kouba et al., 2009; Wildt, 2000]. During cooling and thawing, cryoprotectants interact with the phospholipid membrane of the sperm in order to prevent damage from water crystals and from dehydration. During cooling, ice crystals can form both inside and outside the cell and the membrane hardens making it more likely to shatter. Additionally, ice crystals form outside of the cells and into hyperosmolar "pockets" and any cells trapped in these "pockets" may undergo severe dehydration. During thawing, the ice crystals melt and the membrane becomes fluid again, a process in which additional structural damage is likely to occur [Holt, 2000; Holt2, 2000; Parks & Graham, 1992; Sztein et al. 2001] .

The goal of the addition of cryoprotectants is to protect cells from the stress on the cell caused by freezing and thawing. Cryoprotectants can be classified a number of ways, but for the purposes of this study there are two major classes of cryoprotectants: permeating and non-permeating. Permeating cryoprotectants (i.e. dimethyl sulfoxide) cross the cell membrane easily, while non-permeating cryoprotectants (i.e. sucrose, trehalose or bovine serum albumin) do not cross the cell membrane [Holt, 2000; Holt2, 2000; Sztein et al., 2001]. There are a number of ways in which cryoprotectants assist in decreasing osmotic stress and damage during cooling and thawing. The first goal of

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cryoprotectants is to prevent dramatic changes in osmolality during freezing and thawing. While the addition of cryoprotectants should help to reduce osmotic stress on a cell, if an inappropriate amount or type of cryoprotectant is used, the cryoprotectants themselves can cause severe cell stress or lysis [Gurtovenko & Anwar, 2007; Holt, 2000; Holt2, 2000; Parks & Graham, 1992; Sztein, 2001]. The second goal is the prevention or protection from ice crystal formation. The third major goal of cryoprotectants is to increase fluidity of the phospholipid membrane so that it becomes less fragile and less susceptible to rupture during cryopreservation [Holt, 2000; Holt2, 2000; Parks & Graham, 1992]. Finally, many cryoprotectants act as antioxidants, reducing the number of reactive oxygen species which are produced during the freezing and thawing process and protecting the cell membrane from lipid peroxidation [Bansal & Bilaspuri, 2010; Uysal & Bucak, 2007].

Four cryoprotectants were selected for use in this study: dimethyl sulfoxide (DMSO), sucrose, trehalose, and bovine serum albumin (BSA). DMSO is a low molecular weight permeating cryoprotectant, and while not all of its mechanisms of action are entirely understood, it is well known that DMSO increases cell permeability, especially for hydrophobic particulates. DMSO causes this increase in permeability by producing holes in the phospholipid membrane which allow small molecules to pass through the membrane freely. Another known cryoprotective function of DMSO is that it disrupts the formation of ice crystals, which form jagged edges that rupture the cell membrane [Anchordoguy et al., 1987; Gurtovenko & Anwar, 2007; Holt, 2000; Holt2, 2000].

Sucrose and trehalose are sugars that have been recognized as natural cryoprotectants in some species of animals, including arthropods, many reptiles and amphibians, and polar fish, where they help prevent freezing in the blood and cell damage during the winter months [Fuller, 2004; Storey, 1990]. Both of these sugars are glucose disaccharides and non-permeating cryoprotectants which maintain cell membrane integrity and protein structures [Anchordoguy et al., 1987; Fuller, 2004; Storey, 1990]. Bovine serum albumin (BSA) functions as an antioxidant, and this is its main role as a cryoprotectant. The powerful blocking of the formation of reactive oxygen species and protection of the lipid membrane from lipid peroxidation is what makes BSA a useful additive in cryopreservation [Bansal & Bilasupuri, 2010; Uysal & Bucak, 2007].

While cryoprotectants are crucial to successful cryopreservation, appropriate freezing and thawing rates are what are also key to success. A freezing rate must be finely balanced so that localized variations in osmolality do not arise causing the cells to dehydrate. Likewise, thawing must be regulated so that cells do not lyse [Holt, 2000; Holt2, 2000; Watson & Holt, 2001]. In this study, frozen salamander sperm was thawed at 40ºC and at 20ºC to test rapid and slow thawing rates, respectively. These temperatures were selected from highly successful thawing temperatures utilized in anuran sperm studies [Langhorne, 2015].

In this study, the recovered motility and percent abnormal sperm of *Ambystoma tigrinum* sperm is assessed upon thawing from cryopreservation. Sperm is collected in the form of milt and assessed when sperm is treated with one of five different cryoprotective solutions and thawed at one of two thawing temperatures.

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Materials and Methods

Experimental animals

All protocols for animal care and use were reviewed and approved by the IACUC at Mississippi State University.

Ten male eastern tiger salamanders (*Ambystoma tigrinum*) were used in these experiments and were maintained in conditions identical to section 2.2.1. A total of five straws of frozen sperm were collected and tested for each treatment per 10 males (50 straws total).

Collection and Evaluation of Fresh Sperm

Hormone induced spermiation consisted of a priming dose of 0.025 µg/g LHRH and a spermiation dose of 0.1 µg/g LHRH for all animals. Administrations and methods for use of this hormone treatment are described in *Chapter II, Section 2.2.2*.

Sperm was collected as described in *Chapter II, Section 2.2.3*.

Only spermic milt was used for cryopreservation studies, and fresh spermic milt was evaluated as described in *Chapter II, Section 2.2.4*.

See *Appendix B* for additional methods on hormone administration and sperm collection.

Cryopreservation of Milt

Milt was diluted in 10% Holtfreter's solution (see *Chapter II* for solution components) to a concentration of 2×10^6 spermatozoa/mL. The diluted sperm was cooled at 4ºC for 30 minutes, after which, the sperm was diluted 1:1 with one of seven stock cryoprotectant solutions, listed in Table 3.1.

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Table 3.1   Cryoprotective Treatments

Cryoprotectant Stock Solution	Final Cryoprotectant Concentration
1% BSA	0.5% BSA
10% Trehalose	5% Trehalose
10% Sucrose	5% Sucrose
10% DMSO	5% DMSO
10% DMSO + 1% BSA	5% DMSO + 0.5% BSA
10% DMSO + 10% Trehalose	5% DMSO + 5% Trehalose
10% DMSO + 10% Sucrose	5% DMSO + 5% Sucrose

Sperm was diluted 1:1 in the initial cryoprotectant

After dilution of the sperm in the cryoprotectant, the solution was drawn up into a 0.25cc cryo-straw and allowed to sit at 4ºC for an additional five minutes. The straws were then moved to liquid nitrogen vapor, 20cm above the liquid. The straws were allowed to sit in the vapor for 15 minutes to ensure they reached a temperature of -80ºC (Figure 3.1). After 15 minutes, the straws were plunged into the liquid nitrogen to reach a temperature of -180ºC (Figure 3.1) and were then stored in liquid nitrogen dewars for at least 12 hours prior to thawing and analysis.

Thawing of cryopreserved milt

Two thawing methods were tested for efficacy in recovery of motility in salamander milt. The first method was thawing at 40ºC in a warm water bath. For this procedure, straws were removed from storage in liquid nitrogen with tongs and immediately plunged into the warm water bath. Straws were held in the bath for a total of five seconds. Upon thawing, straws were emptied into a microcentrifuge tube for analysis of sperm.

The second method of thawing was "bench top thawing." In this method, straws were removed from storage in liquid nitrogen with tongs and placed at 20ºC (room

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temperature) on the lab bench to thaw for five minutes (see hobo logger data in figure 6.3). After five minutes, straws were emptied into microcentrifuge tubes for further analysis.

Analysis of thawed sperm

Sperm was assessed for percent motility and abnormalities upon thawing. In order to determine percent motility, 5 μL of the thawed milt solution was placed on a glass slide that had been rinsed with 1% BSA (bovine serum albumin, Sigma Aldrich, St. Louis MO, USA). As in *Section 2.2.4*, motility was assessed without a coverslip and cell counts for motility were performed at a 20 X magnification. The number of spermatozoa that were non-motile (unmoving), progressively motile (moving forward across the slide or actively moving in a circle), and non-progressively motile (tail-twitching or immobile with an active undulating membrane) were counted until a count of 100 cells was reached. The progressive motile cell count and the non-progressive motile cell count were added together to obtain the percent total motility. Once these counts were obtained, relative motility was calculated for each total motility, progressive motility, and non-progressive motility by taking the motility count upon thawing and dividing it by the initial motility count:

$$Relative \text{ \%} Motility = \frac{Thawed \text{ } Motility}{Initial \text{ } Motility} \tag{3.1}$$

The same slide used to obtain percent motility was used to obtain percent abnormal spermatozoa. The cell count for abnormality was also performed at 20 X magnification. Cells that were normal (normal structure and complete cell with an intact undulating membrane) and cells that were abnormal (broken or malformed, including

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detached undulating membranes; see Figure 2.1) were counted until a count of 100 cells was reached.

Statistical Analysis

Data was assessed for normality using histogram plots and the Shapiro-Wilk test. An arcsine transformation was performed on non-normal data.

Randomization was achieved using a random number generator applied to the cryoprotective treatment. Each of the five straws collected for cryoprotective treatment were collected at the 24 hour collection time point.

A multivariate analysis of variance (MANOVA) was used to assess the effects of the cryoprotective treatments on the sperm quality. Variables found significant were assessed post hoc by the Least Square Means procedure.
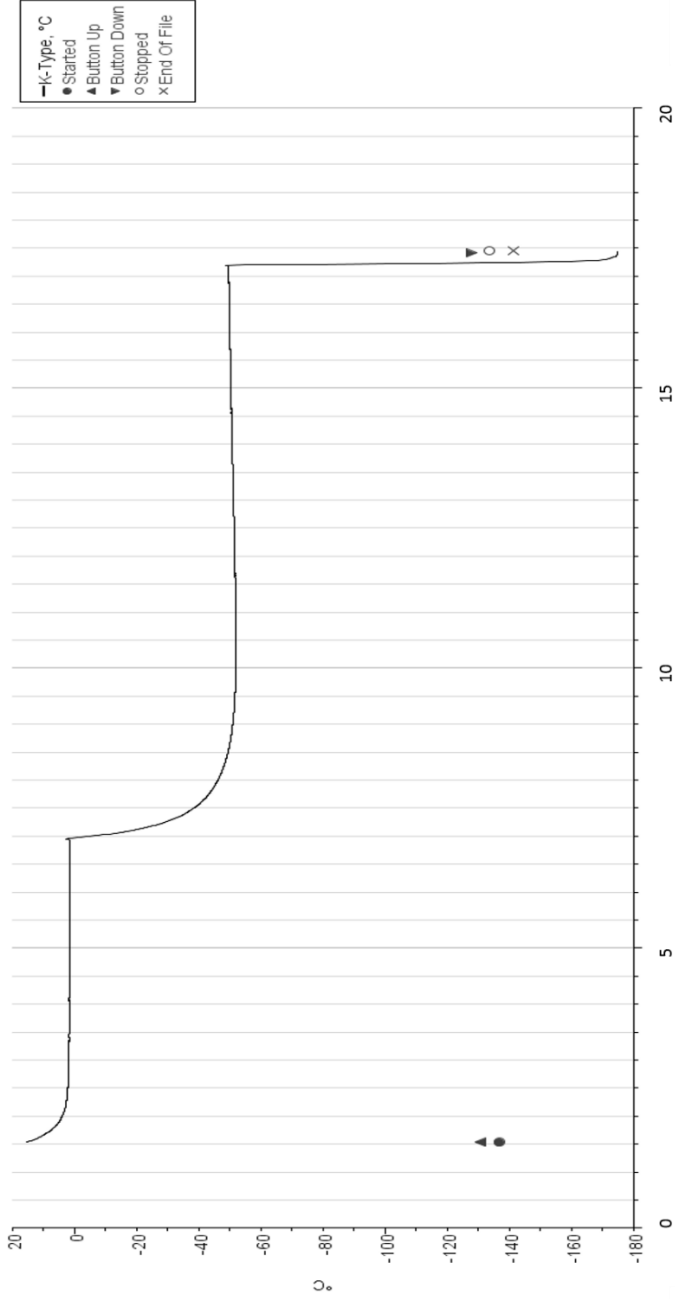
Figure 3.1   HOBO logger temperature data for freezing process

Figure 3.1 shows the temperature change (°C) over time (in minutes) starting at room temperature to placement in a refrigerator at 4°C. The straw is kept at 4°C prior to movement to the liquid nitrogen vapor, where it is kept for 15 minutes. After 15 minutes, the straw is plunged into the liquid nitrogen A "dummy straw" was used to log this data and was only kept at 4°C for 5 minutes prior to moving to the liquid nitrogen vapors.

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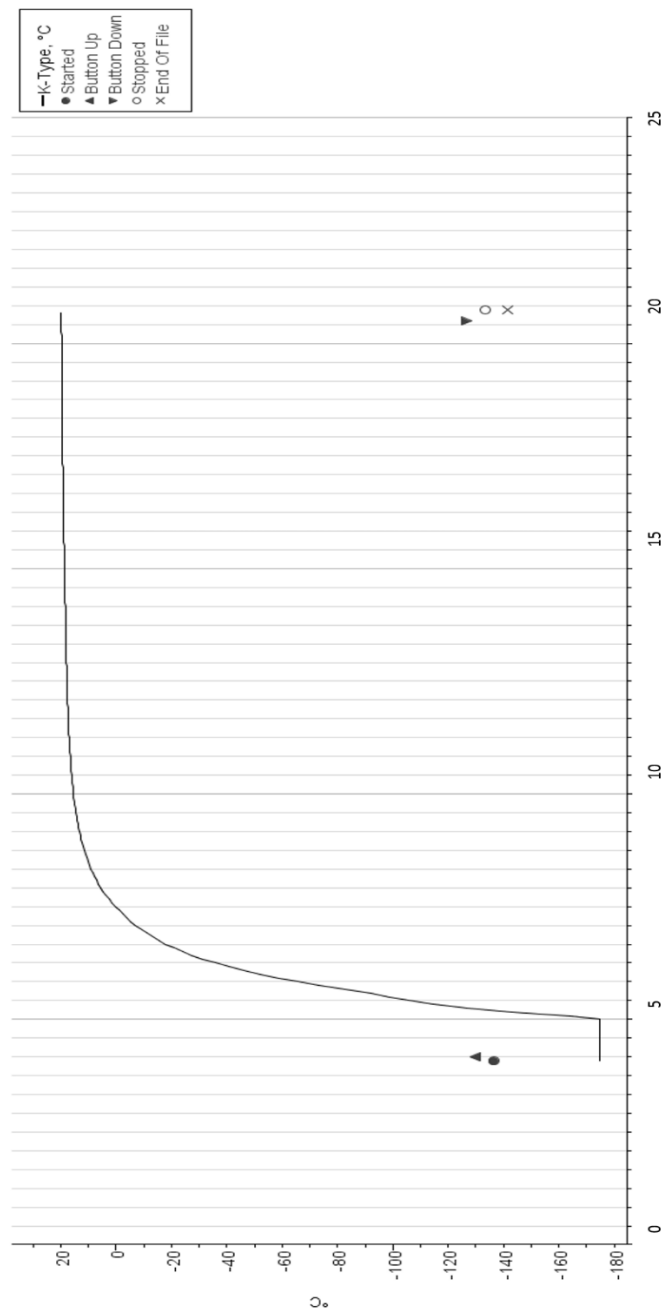
Figure 3.2   HOBO logger temperature data for "bench top" thawing process at room temperature

Figure 3.2 shows the temperature change (°C) over time (in minutes) starting at -80°C with a frozen straw brought out of liquid nitrogen and thawed to room temperature. The straw is removed from liquid nitrogen using tongs and placed directly on the bench top, where it is allowed to cool for 3 minutes until it has reached room temperature (20°C)

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Results

The overall model of both cryoprotectant and thawing temperature affecting motility parameters showed significant differences between treatments (total motility: $p<0.001$; $F=17.4$). The overall model of cryopreservation and thawing temperature affecting percent abnormal sperm was also showed significant differences between treatments (total motility: $p<0.0001$; $F=33.02$).

The overall effect of differing thawing rates on recovered motility and abnormalities was significant with a Wilk's Λ significance of < 0.0001, $F = 34.9$. There was no effect of thawing temperature on motility parameters, however there was an effect of thawing temperature on the percent of abnormal sperm generated through the thawing process ($p < 0.0001$; $F = 136.3$). Least square means also showed significantly more abnormal sperm were formed by rapidly thawing at 40ºC compared to slow thawing at 20ºC.

Treatment with different cryoprotectants had an effect on percent motility ($p < 0.0001$; $F = 16.7$) and on percent abnormal sperm ($p < 0.0001$; $F = 46.1$). Main effects of the cryoprotectant were significant overall with a Wilk's Λ of $p < 0.0001$, $F=21.3$. Least square means analysis showed recovered motility was highest in the 5% DMSO with 5% Trehalose and lowest in the 0.5% BSA or the 5% Trehalose. While significant differences showed across the treatments in motility, these were due to the main effect of the cryoprotectant and were not resultant of an interaction effect. The most abnormal sperm ($p < 0.05$) were found in the 0.5% BSA treatment, while the 5% DMSO and 0.5% BSA, the 5% DMSO and 0.5% Sucrose, the 5% Trehalose, and the 5% Sucrose treatments.

There was a significant interaction of cryoprotectant and thawing temperature on the percent of abnormal sperm ($p = 0.03$; $F = 2.4$). The interaction of the cryoprotectant and thawing temperature were significantly different across treatments ($p < 0.0001$, $F = 21.28$). The only variable affected by the interaction of cryoprotectant and thawing rate was the percent of abnormal sperm ($p = 0.03$; $F = 2.4$), with the greatest amount of abnormal sperm formed when frozen with 0.5% BSA and thawed rapidly at 40ºC, see table 3.3. Otherwise, the cryoprotective treatments were similar ($p > 0.05$) in preserving sperm morphology. however, these treatments had significantly fewer abnormalities when thawed at 20ºC than when thawed at 40ºC. The 5% sucrose treatment, when thawed at 20ºC, had the fewest percentage of broken heads while the 0.5% BSA and the 5% DMSO thawed at 40ºC had the most (see table 3.3). The 5% sucrose and the 5% DMSO with 0.5% BSA treatments had the fewest bent tails of all cryoprotective treatments when thawed at 20ºC (see table 3.3).

Table 3.2   Effects of cryoprotective treatments and thawing temperature on percent initial, thawed, and relative sperm motilities

Thaw Temp.	Cryoprotectants	% Initial Motility		% Thawed Motility		% Relative Motility	
		Total	Progressive	Total	Progressive	Total	Progressive
20°C	0.5% BSA	59.9 ± 6.1	34.8 ± 8.0	0.2 ± 0.1 d	0.0 e	0.3 ± 0.2 c	0.0 d
	5% Trehalose	59.0 ± 6.8	38.8 ± 9.1	0.0 d	0.0 e	0.0 c	0.0 d
	5% Sucrose	49.2 ± 8.3	28.4 ± 8.1	5.9 ± 3.3 bc	1.5 ± 1.0 cde	12.4 ± 7.6 b	8.5 ± 6.8 bc
	5% DMSO	51.9 ± 7.3	28.2 ± 6.5	5.5 ± 1.0 c	1.3 ± 0.4 cd	12.1 ± 2.0 b	14.8 ± 10.7 abc
	5% DMSO + 0.5% BSA	56.9 ± 6.4	37.7 ± 7.1	9.3 ± 2.5 abc	1.3 ± 0.4 cd	18.2 ± 0.6 b	5.8 ± 2.8 c
	5% DMSO + 5% Trehalose	55.4 ± 7.2	36.5 ± 8.6	13.1 ± 3.9 a	3.9 ± 1.6 bc	28.7 ± 7.6 ab	16.6 ± 5.5 b
	5% DMSO + 5% Sucrose	45.9 ± 5.1	19.7 ± 3.0	6.5 ± 1.9 bc	0.8 ± 0.2 de	14.5 ± 4.2 b	4.0 ± 1.3 c
40°C	0.5% BSA	54.9 ± 6.0	32.7 ± 6.2	0.0 d	0.0 e	0.0 d	0.0 d
	5% Trehalose	59.0 ± 6.8	38.8 ± 9.1	0.0 d	0.0 e	0.0 d	0.0 d
	5% Sucrose	51.2 ± 7.8	30.0 ± 7.9	7.2 ± 3.3 bc	1.6 ± 0.8 cd	14.5 ± 7.1 b	8.0 ± 5.0 bc
	5% DMSO	50.3 ± 4.4	30.4 ± 3.4	5.6 ± 1.1 c	1.0 ± 0.3 cd	12.2 ± 0.03 b	0.05 ± 0.02 cd
	5% DMSO + 0.5% BSA	55.7 ± 4.6	30.0 ± 2.7	10.4 ± 2.1 ab	7.9 ± 1.6 a	19.6 ± 5.0 ab	26.7 ± 2.4 a
	5% DMSO + 5% Trehalose	53.8 ± 6.9	35.1 ± 8.1	13.8 ± 5.1 a	3.5 ± 1.9 bc	30.9 ± 9.9 a	10.6 ± 3.9 bc
	5% DMSO + 5% Sucrose	45.9 ± 5.1	26.0 ± 5.0	6.2 ± 1.5 bc	1.1 ± 0.4 cd	16.1 ± 4.7 b	7.2 ± 3.9 c

abc Values with different letter superscripts within the same column are significantly different from one another (p<0.05)
Relative motility is expressed as the percentage of motile sperm upon thawing relative to the initial percentage of motile sperm
Non-progressive motility not shown

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Table 3.3   Effects of cryoprotectants and thawing temperatures on percent abnormal sperm 1.0±0.06

Thawing Temperature	Cryoprotectants	% Broken Heads	% Bent Tails	% Supercoiled Tails	% Total Abnormal Sperm
20°C	0.5% BSA	7.8 ± 0.7 [b]	3.5 ± 0.5 [a]	6.7 ± 0.5 [b]	15.6 ± 4.1 [bc]
	5% Trehalose	2.4 ± 0.9 [c]	1.6 ± 0.2 [b]	1.7 ± 0.8 [c]	4.8 ± 0.9 [e]
	5% Sucrose	0.6 ± 0.5 [d]	0.5 ± 0.4 [c]	3.2 ± 0.5 [bc]	4.5 ± 0.7 [e]
	5% DMSO	3.7 ± 0.6 [c]	3.4 ± 0.5 [a]	1.4 ± 0.2 [c]	7.1 ± 1.2 [de]
	5% DMSO + 0.5% BSA	1.3 ± 0.2 [c]	0.5 ± 0.2 [c]	2.4 ± 0.4 [c]	3.9 ± 1.3 [e]
	5% DMSO + 5% Trehalose	2.6 ± 0.3 [c]	3.1 ± 0.6 [a]	2.4 ± 0.3 [c]	7.6 ± 4.5 [de]
	5% DMSO + 5% Sucrose	1.9 ± 0.9 [c]	1.6 ± 0.8 [b]	4.4 ± 1.7 [bc]	5.8 ± 2.5 [de]
40°C	0.5% BSA	17.5 ± 3.5 [a]	3.3 ± 0.6 [a]	13.6 ± 4.3 [a]	34.7 ± 4.8 [a]
	5% Trehalose	4.6 ± 1.7 [bc]	2.3 ± 0.9 [ab]	8.9 ± 1.2 [ab]	11.3 ± 1.5 [cd]
	5% Sucrose	2.6 ± 0.7 [c]	2.1 ± 0.4 [ab]	6.1 ± 0.8 [b]	10.9 ± 2.5 [cd]
	5% DMSO	14.0 ± 3.3 [a]	3.2 ± 0.7 [a]	3.4 ± 1.9 [bc]	17.8 ± 2.9 [bc]
	5% DMSO + 0.5% BSA	3.0 ± 0.6 [c]	3.5 ± 1.0 [a]	4.6 ± 1.0 [bc]	9.9 ± 1.6 [cd]
	5% DMSO + 5% Trehalose	2.4 ± 0.7 [c]	3.1 ± 0.6 [a]	13.7 ± 5.2 [a]	15.6 ± 4.6 [bc]
	5% DMSO + 5% Sucrose	5.2 ± 4.3 [bc]	3.3 ± 0.9 [a]	10.8 ± 3.1 [a]	14.0 ± 2.6 [bc]

abc. Values with different letter superscripts within the same column are significantly different from one another ($p < 0.05$)

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Discussion

Overall, thawing *Ambystoma tigrinum* sperm at 20ºC showed to be less damaging to the sperm than thawing at 40ºC. There was not, however, and advantage to using wither thawing rate in regards to recovery of sperm motility. As for cryoprotectants, relative recovered total motility was highest in the 5% DMSO with 5% trehalose and in the 5% DMSO with 0.5% BSA. The 5% DMSO with 0.5% BSA thawed at 40ºC had the highest relative progressive motility of all treatments (see table 3.2). In the 5% trehalose treatment and the 0.5% BSA treatment, when no DMSO was added, there was little to no recovered motility, but when these additives were combined with DMSO, the relative recovered motility appeared to increase significantly. This increase was significant as compared to the 5% trehalose, 5% BSA, as well as the 5% DMSO when each of these agents were applied alone. This suggests there is an additive effect of when BSA or trehalose is combined with DMSO in a cryoprotective solution. Both BSA and trehalose are typically used as additives, and are not commonly used as cryoprotective agents on their own. When used as additives, their antioxidant effects and effects on water add to the protection of the tissue undergoing cryopreservation and help prevent further damage. They protect the cells in ways that the base cryoprotective agent cannot, and so their addition may increase the amount of recovery upon thawing [Watson & Holt, 2001].

The treatments which achieved the best recovery overall were the 5% DMSO with 5% trehalose and the 5%DMSO with 0.5% BSA. Of the treatments used for this study, these two would be recommended for use in cryopreservation of sperm in species of Ambystomids and, potentially, other caudate species. This study used very low strength cryoprotectant solutions, in order to ensure the least amount of toxicity. It is

recommended that further testing be performed with increased concentrations of cryoprotective agents and additives in order to determine if increased recovery can be achieved with stronger solutions. This study also used a set concentration of one million sperm/mL for all trials. It is possible a higher concentration of *Ambystoma tigrinum* sperm is needed in order to achieve ideal recovery. Salamanders naturally produce condensed spermatophores, which are deposited into the environment, sometimes in freezing temperatures [Duellman & Trueb, 1994; Vitt & Caldwell, 2009]. This suggests that salamander sperm is likely well suited to surviving freezing, especially when slow freeze methods are used. However, because the spermatophore is incredibly dense, salamander sperm may be required high concentrations for successful cryopreservation.

This is the first study to report effects of thawing rates on milt collected from salamanders which typically produce spermatophores. Previous studies have reported effects of cooling and thawing rates on spermatophores [Figel, 2013] and on milt collected from Cryptobranchids [Unger et al, 2013; Peng et al, 2013]. In previous studies, there was no significant effect of thawing rate on viability of salamander sperm or on abnormalities [Figel, 2013]. Previous studies have shown a difference in cooling rates and recovered viable sperm, but these result varied by species. In spermatophore producing species, a slow cooling process was superior to a fast cooling process whereas in Cryptobranchid milt the fast freezing process resulted in higher viability [Figel, 2013; Unger et al, 2013]. In this study, cooling rates were kept constant, therefore addressing differences in cooling rates is a venue for future study in the freezing of salamander milt from animals that typically produce spermatophores. The thawing rates in this study were chosen from highly successful thawing rates in anurans [Langhorne, 2015]. However, as

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mentioned previously, many salamander species, including *Ambystoma tigrinum*, breed in winter months, sometimes at freezing temperatures [Duellman & Trueb, 1994; Vitt & Caldwell, 2009]. It is possible that salamander sperm needs lower thawing rates for ideal recovery from freezing. The 20ºC thawing temperature was more successful, which may be an indicator that lower temperatures are more successful at thawing salamander milt. Further exploration of the thawing rates of salamander sperm is needed to fully understand how to achieve ideal recovery rates from freezing.

The goal of the addition of cryoprotectants is to prevent major damage to cells and tissues during the freezing and thawing process. This study has characterized different common abnormalities seen in thawed sperm and the frequency of each abnormality upon thawing at different temperatures. This assesses how effective the cryoprotectant was at protecting the sperm from damage; it does not assess how much damage or toxicity the cryoprotectant may have caused. The toxicity of cryoprotective agents to sperm is always a concern. There is potential that cryoprotective agents may cause damage to cell membranes or cell DNA and fertility [Anchordoguy et al, 1987; Holt 1, 2000; Parks & Graham, 1992; Watson, 2000]. The mechanism by which sperm recovery was observed in this study was motility, which does not report whether sperm is alive and unmoving, or is damaged and moving. Using fluorescent stains, such as Hoechest, SYBR-14, and Propidium Iodide, can help determine which cells are alive and unmoving or moving and damaged. Using these types of stains, it can be determined if sperm has been damaged at either the membrane or the genetic level. This can show if damages will affect sperm fertility or the fitness of an potential progeny developed from frozen-thawed sperm [Garner & Johnson, 1995; Januskauskas et al, 2003, Shiroshiva et

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al, 2011]. Preliminary attempts were made to use these three stains in *Ambystoma tigrinum* sperm and were unsuccessful. Successful staining of *Ambystoma mexicanum* sperm with SYBR-14 and propidium iodide has been reported [Figel, 2013]. It is possible that species specific buffers in salamanders are necessary in order for these stains to be effective.

The cryoprotective agents utilized in this study were mostly hyperosmotic solutions. There was a question as to how these solutions would affect the activity of the salamander sperm. There is not much available information on the response of salamander spermatozoa to osmolality. Anuran sperm are well known to become activated by hypoosmotic solutions, and anuran sperm activity has an inverse relationship with osmolality [Inoda & Morisawa, 1987]. In 1986, Hardy and Dent showed that sperm from the red spotted newt was more active in hypoosmotic solution; however, the sperm was still active at isosmotic and hyperosmotic solution. Additionally, the sperm remained active for a longer period in the isosmotic solution than in the hypoosmotic solution. Preliminary study was attempted to observe the effects of osmolality on sperm from *Ambystoma tigrinum* (see *Appendix G*). These experiments showed that, while there was a drop in activity from seminal fluid when transferred to any of the solutions, that sperm remained active within hypoosmotic solutions (100 mOsm), isosmotic solutions (300 mOsm), and hyperosmotic solutions (600 mOsm), but were most active at isosmotic solutions composed of potassium and chloride ions. In the cryoprotective study, the salamander sperm remained very active in the hyperosmotic cryoprotectants, which were not diluted upon thawing. In preliminary studies, when the cryoprotectant was diluted upon thawing with phosphate buffered saline, the sperm broke, died, or became

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immobile. Much more study needs to be done in order to understand osmolality, ions, and their effects on salamander sperm. Potential future studies include observing the effects of ions and of osmolality as separate variables over time. Additionally, in this study the medium which the cryoprotectants were made in was sterile water. In the future, attempts at trying different media, such as phosphate buffered saline, amphibian Ringer's, or Holtfreter's solution, could be beneficial to the success of freezing and thawing salamander sperm.

This study shows that salamander milt obtained from species that typically produced spermatophores is capable of cryopreservation in multiple cryoprotective agents and when thawed at different temperatures. While further study is warranted to determine ideal methods for obtaining maximal viable sperm recovery, this study shows that the process has potential and lays groundwork for development of protocols for conservation of endangered species of caudates.

References

[1]   Anchordoguy TJ, Rudolph AS, Carpenter JF, Crowe JH. Modes of interaction of cryoprotectants with membrane phospholipids during freezing. Cryobiology 1987;24:324–31. doi:10.1016/0011-2240(87)90036-8.

[2]   Bansal AK, Bilaspuri GS. Impacts of oxidative stress and antioxidants on semen functions. Vet Med Int 2010;2011:686137. doi:10.4061/2011/686137.

[3]   Browne RK, Kaurova SA, Uteshev VK, Shishova N V., McGinnity D, Figiel CR, et al. Sperm motility of externally fertilizing fish and amphibians. Theriogenology 2015;83:1–13. doi:10.1016/j.theriogenology.2014.09.018.

[4]   Byrne PG, Dunne C, Munn AJ, Silla AJ. Environmental osmolality influences sperm motility activation in an anuran amphibian. Journal of evolutionary biology. 2015 Mar 1;28(3):521-34.

[5]   Calisi RM, Bentley GE. Lab and field experiments: Are they the same animal? Horm Behav 2009;56:1–10. doi:10.1016/j.yhbeh.2009.02.010.

[6]   Clulow J, Trudeau VL, Kouba AJ. Amphibian declines in the twenty-first century: why we need assisted reproductive technologies. Adv Exp Med Biol 2014;753:275–316. doi:10.1007/978-1-4939-0820-2_12.

[7]   Duellman DE, Trueb L. Biology of Amphibians. Baltimore USA: John Hopkins University Press; 1994.

[8]   Figel C. Cryopreservation of s perm from the axolotl Ambystoma mexicanum : implications for Conservation. Herpetol Conserv Biol 2013;8:748–855.

[9]   Fuller BJ. Cryoprotectants : the Essential Antifreezes To Protect Life in the Frozen State 2004;25:375–88.

[10]   Garner DL, Johnson L a. Viability assessment of mammalian sperm using SYBR-14 and propidium iodide. Biol Reprod 1995;53:276–84. doi:10.1095/biolreprod53.2.276.

[11]   Gurtovenko A, Anwar J. Modulating the structure and properties of cell membranes: the molecular mechanism of action of dimethyl sulfoxide. J Phys Chem B 2007;111:10453–60. doi:10.1021/jp073113e.

[12]   Hardy, MP Dent J. Hardy and Baronet.pdf. J Exp Zool 1986;240:385–96.

[13]   Holt W V. Basic aspects of frozen storage of semen. Anim Reprod Sci 2000;62:3–22. doi:10.1016/S0378-4320(00)00152-4.

[14]   Holt W V. Fundamental aspects of sperm cryobiology: The importance of species and individual differences. Theriogenology 2000;53:47–58. doi:10.1016/S0093-691X(99)00239-3.

[15]   Inoda T, Morisawa M. Effect of osmolality on the initiation of sperm motility in Xenopus laevis. Comp Biochem Physiol -- Part A Physiol 1987;88:539–42. doi:10.1016/0300-9629(87)90077-6.

[16]   Januskauskas A, Johannisson A, Rodriguez-Martinez H. Subtle membrane changes in cryopreserved bull semen in relation with sperm viability, chromatin structure, and field fertility. Theriogenology 2003;60:743–58. doi:10.1016/S0093-691X(03)00050-5.

[17]   Kouba AJ, Vance CK, Willis EL. Artificial fertilization for amphibian conservation: Current knowledge and future considerations. Theriogenology 2009;71:214–27. doi:10.1016/j.theriogenology.2008.09.055.

[18]   Langhorne, Cecilia. 2015. Assisted Reproductive Technologies for Endangered North American Amphibians. (Doctoral Dissertation)

[19]   Marcec R., Langhorne C, Vance C, Kouba A, and Willard S. Cryopreservation of Spermic Milt in the Model Species Ambystoma Tigrinum (Tiger Salamander) for Application in Endangered Salamanders. Cryobiology. 2014; 69(3): 515. doi:10.1016/j.cryobiol.2014.09.342.

[20]   Parks JE, Graham JK. Effects of cryopreservation procedures on sperm membranes. Theriogenology 1992;38:209–22. doi:10.1016/0093-691X(92)90231-F.

[21]   Peng LY, Xiao YM., Liu Y, Kouba AJ, Vance CK, Zippel K, Shishova NV. Effect of cryopreservation and short-term storage of Chinese giant salamander sperm. Reproduction, Fertility and Development. 2013; 25, 1134.

[22]   Shishova NR, Uteshev VK, Kaurova SA, Browne RK, Gakhova EN. Cryopreservation of hormonally induced sperm for the conservation of threatened amphibians with Rana temporaria as a model research species. Theriogenology 2011;75:220–32. doi:10.1016/j.theriogenology.2010.08.008.

[23]   Storey KB. Life in a frozen state: adaptive strategies for natural freeze tolerance in amphibians and reptiles. Am J Physiol - Regul Integr Comp Physiol 1990;258:R559–68.

[24]   Sztein JM, Noble K, Farley JS, Mobraaten LE. Comparison of permeating and nonpermeating cryoprotectants for mouse sperm cryopreservation. Cryobiology 2001;42:28–39. doi:10.1006/cryo.2001.2300.

[25]   Unger S, Mathis A, Wilkinson R. A comparison of sperm health in declining and stable populations of hellbenders (Cryptobranchus alleganiensis alleganiensis and C . a . bishopi). Am Midl Nat 2013;170:382–92.

[26]   Uteshev V, Kaurova S, Shishova N, Stolyarov S, Browne R, Gakhova E. In vitro Fertilization with Hormonally Induced Sperm and Eggs from Sharp-Ribbed Newts Pleurodeles waltl. Russ J Herpetol 2015;22:35–40.

[27]   Uysal O, Bucak MN. Effects of oxidized glutathione, bovine serum albumin, cysteine and lycopene on the quality of frozen-thawed ram semen. Acta Vet Brno 2007;76:383–90. doi:10.2754/avb200776030383.

[28]   Vitt LJ, Caldwell JP. Herpetology. 3rd ed. Singapore China: Elsevier; 2009.

[29]   Watanabe T, Itoh T, Watanabe A, Onitake K. Characteristics of sperm motility induced on the egg-jelly in the internal fertilization of the newt, Cynops pyrrhogaster. Zool Sci 2003;20:345–52. doi:10.2108/zsj.20.345.

[30]   Watanabe A, Takayama-Watanabe E, Vines CA, Cherr GN. Sperm motility-initiating substance in newt egg-jelly induces differential initiation of sperm motility based on sperm intracellular calcium levels. Dev Growth Differ 2011;53:9–17. doi:10.1111/j.1440-169X.2010.01216.x.

[31]   Watson, PF. The Causes of Reduced Fertility with Cryopreserved Semen. Animal Reproduction Science 2000; 60: 481–92. doi:10.1016/S0378-4320(00)00099-3.

[32]   Watson, PF and Holt WV, Ed. *Cryobanking the genetic resource: Wildlife conservation for the future?* London, Taylor and Francis, 2001.

[33]   Wildt DE. Genome resource banking for wildlife research, management, and conservation. ILAR J 2000;41:228–34.

CHAPTER IV

DEVELOPMENT OF HORMONE PROTOCOLS FOR THE INDUCTION OF

OVULATION AND OVIPOSTION IN FEMALE TIGER SALAMANDERS

(*AMBYSTOMA TIGRINUM*)

Introduction

Amphibians rely on reproductive cues from the environment in order to breed

naturally. These cues commonly include changes in in temperature, humidity, day length,

or barometric pressure [Duellman & Trueb, 1994; Vitt & Caldwell, 2009]. Environmental

cues are specific to each species; however, regardless of each species' needs, the separate

cues within the environment must coordinate and synchronize appropriately in order to

cause the resultant change in hormone levels which trigger breeding [Kim et al., 1998;

Norris & Jones, 1987; Norris & Lopez, 2011; Ogielska, 2009]. The specific cues which

regulate amphibian hormone levels and signal amphibian reproduction are particularly

important for the female amphibian reproductive system and oviposition [Browne &

Zippel, 2007; Grant et al., 2009; Oseen & Wassersug, 2002; Rudolf et al., 2004; Whittier

et al., 1987]. The cycle of developing oocytes is a long process that takes a number of

months in most species [Norris & Jones, 1987; Norris & Lopez, 2011; Ogielska, 2009].

External cues throughout this process are key to developing eggs that will be capable of

fertilization, eggs with yolks capable of supporting the developing embryo, appropriate

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ovulation, and appropriate oviposition [Browne & Zippel, 2007; Anderson et al., 1999; Whittier et al, 1987].

In amphibian captive breeding facilities, some of the biggest challenges which result in reproductive failure concern females. Frequently, these challenges are a result of an inability to recreate environmental cues to stimulate appropriate endogenous changes for breeding [Clulow et al., 2014; Kouba et al., 2009; Roth & Obringer, 2003]. There are three main challenges which captive breeding facilities face regarding female amphibian reproduction: females will have developed eggs within their ovaries but will not oviposit; females will oviposit but fertilization will not be achieved; or females will not develop eggs [Kouba et al., 2009; Reyer et al., 1999]. In captive anurans, males will frequently still perform amplexus with females which are not going to oviposit [Reyer et al., 1999]. These mating behaviors may lead to wasted energy and resources of males that are ready to breed [Kouba et al., 2009]. Additionally, and more critically, females of some species of anurans, such as the Panamanian Golden Frog or the Wyoming Toad, have died in captive breeding colonies due to either a failure to release eggs or egg sepsis [Wright & Whittaker, 2001; Ploog, Personal Comm, 2016]. Egg binding and egg sepsis are not commonly recognized in caudates; however this may be because there are fewer captive breeding programs for caudate species.

While there is some available information available on exogenous hormones in caudates and obtaining eggs from females [Khattak & Tanaka, 2009; Mansour et al., 2011; Trottier & Armstrong, 1975; Uteshev et al., 2015], these attempts focus on highly inbred laboratory species [Khattak & Tanaka, 2009; Mansour et al., 2011; Trottier & Armstrong, 1975], or species freshly caught from the wild during their breeding season

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[Stoops et al, 2014; Uteshev et al., 2015]. For the purposes of captive assurance colonies, it is critical to test animals that have been obtained from the wild and acclimated to their surroundings, or results may not be replicable [Calisi & Bentley, 2009].

Ultrasound has been utilized in amphibian reproduction in order to view the development of follicles and oocytes [Kouba et al., 2009; Roth & Obringer, 2003; Stoops et al., 2014]. However, in these instances the ultrasound was used to view the oocytes at single time points and the development was not tracked over the course of a season or over the course of an entire hormone treatment. In this experiment the follicles of eastern tiger salamanders (*Ambystoma tigrinum*) are observed over the course of three different length courses of hormone treatments, as well as throughout the year while they are not being treated. Ultrasound is used against the different treatments in order to observe follicular and oocyte development to determine the ideal timing for hormone stimulation and induction of oviposition.

Materials and Methods

All protocols for animal care and use were reviewed and approved by the IACUC at Mississippi State University.

Experimental Animals

Fifteen female eastern tiger salamanders (*Ambystoma tigrinum*) were obtained from private breeders. The female salamanders were maintained in tanks and conditions identical to the male salamanders described in Section 2.2.1. Treated females were not fed during oviposition in order to prevent regurgitation.

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Induction of ovulation and oviposition using exogenous hormones

Hormone treatments were scheduled such that animals only received hormones at intervals of three months apart or greater. The three month recovery period was selected based on previously reported recovery periods in amphibians [Calatayud et al., 2015; Kouba et al., 2009] and based on the apparent recovery of follicular cycling observed on ultrasound. This was to ensure appropriate time between treatments for elimination of hormones and for recovery of the ovaries from hormone effects. This does not eliminate the possibility of long term damage to the ovaries or fecundity, and longer recovery times are suggested for animals used in conservation practice [Kouba et al., 2009; McDonough et al., 2015].

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Table 4.1   Ultrasound grading scale for follicular and oocyte growth in amphibians

Grade	Image on Ultrasound	Description
G3 (High Grade)		Near Complete Development: -Follicles hypoechoic and rounded -Well defined, well spaced, hyperechoic oocytes
G2 (Mid- Grade)		Moderate development: -Follicles uneven areas of hypoechogenicity -Uneven spacing between moderately developed oocytes
G1 (Low Grade)		Poor Development: -Follicles and oocytes tightly clumped together -Small and poorly developed follicles and oocytes -Little to no areas of hypoechogenicity between follicles
G0 (No grade)		No Development: -Little to no obvious follicular or oocyte development

*All measurements taken with Image J Software (National Institutes of Health, Bethesda, Maryland, USA)

Oviposition hormone protocols were developed based on the priming sequences

used in some anuran species [Calatayud et al., 2015; Browne & Zippel, 2007; Kouba et

al., 2009; Wright & Whittaker, 2001] and based on the cycling of oocyte and follicular

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development that was seen on ultrasound in the tiger salamanders. The treatments A: Short, B: Middle, C: Long) are detailed in Table 4.2. The concept behind the treatment was that the level of hormones would be increased accordingly to fit the needs of the grade on ultrasound (see table 4.1). Animals given a grade 3 (G3) on ultrasound were almost ready to lay and it was hypothesized that they only needed a small hormone signal in order to oviposit. Animals given a grade 2 (G2) were in mid cycle and it was hypothesized that they needed a slightly longer treatment period to finish developing their oocytes before oviposition. Animals given a grade 1 (G1) did not have very developed oocytes, and it was hypothesized that they would need a longer treatment period in order to increase hormone levels, to cycle, and to oviposit. Preliminary trials were run on treatment groups of n = 9 animals each, where females of ultrasound G3 were given the short (C) treatment, females of ultrasound G2 were given the middle (B) treatment, and females of G1 were given the long (A) treatment. Of the G3 females, 100% oviposited; of the G2 females, 83.3% oviposited; and of the G1 animals, 33.3% oviposited. Females treated with a control injection of sterile PBS (n=15) did not oviposit.

The promising results from preliminary trials led to a more expansive experimental design. A 3x3 factorial design, with the treatments and the ultrasound grades as factors, was executed (See table 4.3). Animals were randomly assigned to their treatment by their allocated grade at weekly ultrasounds. Each treatment by grade combination was assigned n=6 female salamanders. This meant each level of each factor was received by n=18 females. The eighteen females in the laboratory's possession were reused in multiple treatments. No single animal received the same treatment by grade

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combination twice. For protocols on reconstitution and administration of hormones, see

Appendix B.

Table 4.2   Female Ultrasound Paired Hormone Protocols

Time of injection prior to ovulatory dose	Treatment		
	A:Long	B:Middle	C:Short
360 hr	1 IU/g hCG	-	-
192 hr	1 IU/g hCG	1 IU/g hCG	-
24 hr	2 IU/g hCG	2 IU/g hCG	-
Ovulatory Dose	4 IU/g hCG + 0.1 µg/g LHRH	4 IU/g hCG + 0.1 µg/g LHRH	4 IU/g hCG + 0.1 µg/g LHRH

The treatments were initially tailored for ultrasound grades. Long was tailored for G1, Middle for G2, and Short for G3.

Table 4.3   3x3 Factorial Layout

		Factor 1: Treatment			
		Long (A)	Middle (B)	Short (C)	
Factor 2: Ultrasound Grade	G1	G1 x A (n =6)	G1 x B (n=6)	G1 x C (n= 6)	n_{G1}=18
	G2	G2 x A (n =6)	G2 x B (n =6)	G2 x C (n= 6)	n_{G2}=18
	G3	G3 x A (n =6)	G3 x B (n =6)	G3 x C (n= 6)	n_{G3}=18
		n_L=18	n_M=18	n_S=18	Total N=54

n_x where x is the factor level and n is the number of animals

Ultrasound

Ultrasound was used in order to visualize the follicle and oocyte development of the female salamanders. At the time of each ultrasound procedure, the animal was weighed and the width of her abdomen at the widest point was measured using calipers to the nearest millimeter. At each ultrasound viewing, the development of the oocytes and follicles were assigned a grade (G0-G3) as detailed in Table 4.1. An ultrasound image of

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the ovaries was captured and all images were saved to a hard drive for measurement and assessment. Measurement of the diameter of the hyperechoic area of the oocyte was taken for each image with Image J software (National Institutes of Health, Bethesda, Maryland, USA) using the ultrasound scale bar as a reference scale. A total of five oocytes were measured in each image and averaged together for oocyte measurements.

For each ultrasound procedure, a Sonosite Micromaxx ultrasound machine with a 13-6 MHz, 38mm linear transducer was used to capture images of the animals' ovaries. The frequency was placed on the general setting, and a depth of 2.7 cm was used for all animals. Gain was adjusted as needed. For the ultrasound procedure, animals were held in dorsal recumbency and their abdomen was wetted thoroughly with dechlorinated, conditioned tap water for conductance. The linear probe was placed transversely across the widest part of the abdomen. The probe was slowly angled or adjusted so that an even, transverse image of the abdomen and ovaries was obtained. For more detailed instruction on ultrasound, see *Appendix B.*

Ultrasound was performed on the female salamanders once per week while they were not receiving treatment. When a female was receiving treatment, the animal had ultrasound performed prior to each hormone dose and every day during the observation period until either they stopped oviposition or the observation period was over. The final treatment-related ultrasound was performed just after the animals had completed final oviposition, or the observation period ended, and then animals returned to their normal weekly ultrasound schedule.

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Egg Collection

Egg collection and animal observation occurred over the course of 120 hours. For the first 24 hours, the females would be observed every other hour for egg laying behavior and oviposition. If no eggs were obtained after the first 24 hours, observation decreased to every 4 hours. Following 48 hours, if no eggs were obtained, the females was observed every 12 hours until either she oviposited or the 120 hours was over.

If oocytes were obtained at any observation, collection would be attempted hourly until the female could not produce eggs. If females were nearing the end of their clutch, and only a small amount of eggs ($\leq$10) were obtained at each collection, collection would be attempted every other hour until eggs were no longer obtained. If a female oviposited, after every collection the animal would be weighed, an ultrasound would be performed, and her abdomen width would be recorded. Regardless of whether a female oviposited, every 24 hours each female that received a treatment was weighed, given an ultrasound, and had their abdomen measured.

During the 120 hr observation period, the females were removed from their normal group tanks and placed in individual plastic containers identical to those described in section 2.2.3. During the daytime, the containers were placed on a gentle slope such that approximately half of the interior was submerged in water and the other half was dry. During the first 48 hours of observation, the water was removed during the night. This was to prevent any oviposition into the water and missed opportunity to utilize eggs for *in vitro* fertilization. After 48 hours, water was left in the tubs constantly and replaced every 24 hours, or as needed if an animal defecated in the water. As was

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true for the male salamanders, containers were covered with dark towels during the observation period in order to reduce animal stress.

At each observation, the tub was inspected for any eggs the female might have oviposited. If the animal had oviposited in the water, the eggs were counted and discarded. If the animal had oviposited on a dry surface within the last two hours, the eggs were counted and removed for further analysis.

If the animal had oviposited, an attempt was made to collect eggs from the female. If the female had not oviposited, she would be checked to determine if she was ready to oviposit and, if ready, an attempt to collect eggs would be made. A collector determined if a female was ready to oviposit by picking her up, gently placing her in dorsal recumbency, and inspecting her cloaca. If her cloaca was red, swollen, and slightly open, there was a chance she would be ready to oviposit. If the female oviposited or showed any of the above signs, collection would be attempted. First, the forefinger would be wetted and the cloaca would be gently stroked. If a female was very ready to oviposit, eggs may come out from this slight cloacal stimulation. If the cloacal stimulation did not produce eggs after approximately 10 seconds of stoking, expression was attempted. First the females hind limbs were delicately restrained so that they did not kick any eggs exiting the cloaca, rupturing them. Next a gentle pressure was places on either side of the female at the base of the abdomen, just above the hip bones. A massage was not utilized, for fear of damaging the large, fragile oocytes and causing sepsis. If a very gentle pressure did not cause oviposition after approximately 30 seconds, the female was left alone until the next observation. If she did begin to oviposit, gentle pressure was applied until eggs stopped freely coming out of the cloaca.

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Eggs were collected from the female cloaca directly into a deep petri dish. Little to no manipulation of the eggs was carried out in order to maintain the integrity of the egg structure. Once the female finished her oviposition, the eggs were counted.

Evaluation of morphometric changes to hormone stimulation

As noted, the width of the female salamanders' abdomen were measured at each ultrasound. This occurred whether or not they were undergoing treatment. This was in order to monitor if the animals became measurable swollen when they were gravid.

Statistical Methods

Data was assessed for normality using histogram plots and the Shapiro-Wilk test.

A two-way multivariate analysis of variance (MANOVA) was used to assess the effects of the treatments and the ultrasound grades on egg laying and related variables. Variables found significant were assessed post hoc by the Least Square Means procedure. In order to assess the binary outcome of how many animals laid eggs, a Fisher's Exact test was used.

Results

Results of the two-way MANOVA showed an overall significant difference in number of eggs laid (p=0.001; F=4.1). There was a significant interaction effect of treatment and ultrasound grade (p=0.01, F=3.75) and a significant main effect of ultrasound grade (p=0.001, F=8.36) on the number of eggs laid. There was also a significant difference in time (hours, relative to the ovulatory dose) to first oviposition (p=0.01, F= 2.27), with a main effect by grade (p=0.04, F=2.91).

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There was no significant difference in the difference in the weight (g) or the width (mm) of the animal before treatment to the time of the ovulatory dose. However, there was a significant difference in weight from the time of ovulatory dose to the weight after completion of observation (p0.05, F=2.14), with a significant main effect of ultrasound grade (p=0.005, F=6.03). Similarly, the difference in width of the abdomen from the time of the ovulatory dose to after completion of observation was significant (p=0.03, F=2.28) with a significant main effect of ultrasound grade (p=0.02, F=4.22). Wilk's Lambda test supported the finding that there was a main effect of ultrasound grade overall (p=0.005, F=2.28).

The least squares means procedure was performed on the variables that showed significant overall and main effects. The results of this procedure are displayed in Table 4.7.

Results of Fisher's Exact analysis showed that number of animals laying eggs significantly differed by grade (p<0.0001), however there was no significant difference between animals laying eggs by treatment (p=0.4). The number of animals laying eggs showed to be significantly different by grade even when controlling for treatment (p=0.002). No significant difference in number of animals laying eggs by treatment was not seen, even when controlling for grade.

Overall, regardless of treatment, animals that were given a grade 1 on ultrasound responded less frequently to treatment and produced significantly lower numbers of eggs than animals that were given grade 2 or grade 3. Grade 1 animals also oviposited significantly later, relative to the ovulatory dose, than animals given a grade 2 or a grade 3. Regardless of ultrasound grade, animals treated with treatment A oviposited at an hour

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much closer to the time of the ovulatory dose than animals treated with treatment B or treatment C. This is likely because treatment A had the longest set of priming doses which gave the oocytes the longest amount of time to prepare for oviposition, prior to the ovulatory dose. Animals given a grade 1 or grade 2 on ultrasound would be given more hormones prior to the ovulatory dose to help development and would be more likely ready for oviposition when reaching the final dose in this treatment than in other treatments. Some animals given a grade 3 on ultrasound oviposited prior to the ovulatory dose (e.g. oviposited when given a priming dose). This was recorded as a negative time point. Grade 3 animals that did not oviposit at a negative time point and grade 2 animals were likely to oviposit within a few hours of the final ovulatory dose of treatment A. Grade 1 animals which achieved oviposition were likely to oviposit within a normal 18-24 hour range post ovulatory dose. On average, this made animals likely to oviposit much sooner if given treatment A.

Table 4.4   The interaction between treatment and ultrasound grade on mean number of responders, egg number, and time to oviposition

	Treatment	% Responders ± SEM	Egg Number ± SEM	Time to oviposition (hr) ± SEM *
Grade 1	A	33.3 ± 21.1 ab	100.7 ± 68.6 a	29.5 ± 13.5 ab
	**B	16.7 ad	4.7 c	41.0 ab
	C	0.0 d	0.0 c	-
Grade 2	A	100.0 ± 0.0 c	300.3 ± 60.4 ab	2.0 ± 32.1 ab
	B	66.7 ± 21 b	290.1 ± 111.0 ab	19.5 ± 2.7 ab
	C	66.7 ± 21 b	98.3 ± 54.2 a	43.8 ± 20.1 a
Grade 3	A	100.0 ± 0.0 c	207.5 ± 49.6 ab	-122.0 ± 69.2 c +
	B	100.0 ± 0.0 c	216.7 ± 54.9 ab	-16.1 ± 32.0 abc +
	C	100.0 ± 0.0 c	635.5 ± 226.9 b	15.0 ± 1.7 ab

a,b,c Values with different superscripts are significantly different

*Time to first oviposition relative to ovulatory dose + Negative number refers to oviposition prior to ovulatory dose

**Values reported for Grade 1, Treatment 2 are for a single animal

Ultrasound grades reported are grades recorded at the initiation of hormone treatment

For full description of ultrasound grades, see Table 4.1

For full description of treatment grades, see table 4.2

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Discussion

The number of animals that responded to treatment and the number of eggs produced by animals was significantly affected by ultrasound grade. Overall, regardless of treatment, animals that were given a grade 3 on ultrasound responded more frequently to treatment and produced significantly greater numbers of eggs than animals that were given grade 1 or grade 2. Grade 3 animals also oviposited significantly sooner, relative to the ovulatory dose, than animals given a grade 1 or a grade 3. This is likely due to grade 3 animals, having more developed oocytes than grade 1 or grade 2 animals, made them more prepared for oviposition. In the females with less mature oocytes, it would take more time for the oocytes to complete development and for the female to oviposit. Timing to oviposition has been shown to have a critical impact on egg quality in amphibians, with animals laying after 24 hours having decreased fertility rates [Browne & Zippel, 2007; Kouba et al, 2009]. Additionally, the responses of the animals was a significant effect which supported the hypothesis that using ultrasound to observe development of oocytes and to adapt treatment protocols to the stages of development seen on ultrasound is beneficial for assisted reproductive technologies in amphibians.

There was a question about ultrasound grade 3 animals that oviposited early when given treatment A. Grade 3 was the only grade of animals that oviposited prior to an ovulatory dose, and treatment A was the only treatment in which grade 3 animals oviposited early. The animals that oviposited early, on average, laid fewer eggs than grade 3 animals that did not oviposit early. On ultrasound after the animals oviposited, there were no mature oocytes or follicles observed and there were no oocytes near the ovisac. Had mature oocytes been visualize on the ovaries or in the oviducts/ovisac,

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treatment would have continued as there would be potential for ovulation and/or oviposition. [Ogielska, 2009; Sever-1, 1991; Sever-2, 1991; Sever & Lynne, 1985]. However, as mature oocytes and follicles were not observed, for the safety of the animals, treatment was discontinued. It is possible that, had the treatment been continued and had these animals been given the ovulatory dose that they would have recruited more eggs. Because they laid fewer eggs than other grade 3 animals, it should be tested to see if grade 3 animals that oviposit on a priming dose of treatment A will recruit and lay more eggs by the time they are given the ovulatory dose.

Treatments B and C may not have provided enough hormone in order to help immature oocytes to develop and result in oviposition. In some cases, oviposition did not occur at all; for instance, no grade 1 animals oviposited when treated with treatment C. The treatment A had three primes of hCG. The hormone hCG acts as a analog to luteinizing hormone (LH), which is responsible for stimulating progesterone production, estrogen precursor production, and maintaining the corpus luteum until the oocyte is mature [Atger, 1995; Moyle, 1995; Norris & Jones, 1987; Norris & Lopez, 2011; Ogielska, 2009]. Providing three primes of this hormone, over a two week period, may have allowed the oocytes to fully complete development where necessary. Over the course of the treatment period, females that were a grade 1 or grade 2 that were treated with treatments A or B could be seen increasing in grade number as they received priming doses. Animals that did not oviposit when given treatment C had increased ultrasound grades when examined on ultrasound a week later.

There was, conversely, a risk of "overpreparing" the eggs by giving animals with mature eggs more hormone than necessary. Animals with an ultrasound grade 3 that were

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given treatments 1 or 2 that completed the full treatment (i.e. did not lay eggs when given a prime) most often laid eggs poor in quality. Egg quality was defined by parameters including egg consistency, egg size, number of eggs intact, and egg color. The amount of data collected on egg quality for this study was insufficient, and for future studies would benefit from data including egg quality measurements between animal treatments and animal ultrasound grades.

Treatment and ultrasound grade were also shown to have interacted to affect egg production. Animals with the highest developmental stage (ultrasound grade 3) paired with the shortest hormone protocol (treatment C) produced the largest amount of eggs, while the animals with the lowest developmental stage (ultrasound grade 1) paired with the shortest hormone protocol (treatment C) produced the smallest amount of eggs. The grade 1 animals, however, produced the highest number of eggs for that specific ultrasound grade when given the longest hormone treatment (treatment A). The hormone protocols that were meant for higher and lower stages of development had significant effects on egg number within the corresponding ultrasound grade. This interaction of ultrasound grade and treatment is consistent with the hypothesis that animals will be more responsive to hormone protocols which are adapted to the developmental stages of eggs that are seen on ultrasound.

The time at which oviposition occurred, in proximity to the ovulatory dose, was also affected by an interaction between ultrasound grade and treatment. Animals given a grade 3 that were treated with treatment A oviposited significantly sooner than all other animals given all other treatments. This is likely because animals given a grade 3 had eggs that were very developed and ready to oviposit, so the long priming protocol of

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treatment 1 was excessive. They therefore responded prior to the ovulatory dose (oviposited when given a priming dose), making this a negative value. Once more, this interaction supports the hypothesis that the use of ultrasound grading to refine hormone protocols for ideal results is beneficial. Pairing this with treatment, it would be possible to improve the timing of oviposition. Timing to oviposition has been shown to have a critical impact on egg quality in amphibians, with animals laying after 24 hours having decreased fertility rates [Browne & Zippel, 2007; Kouba et al, 2009]. Therefore, using ultrasonography techniques and treatment to improve the timing of this variable is very beneficial for the future of captive breeding and assisted reproductive technologies in amphibians.

These protocols were attempted in the regionally endangered blue spotted salamander (*Ambystoma laterale*). Ultrasound was extremely clear, despite animals being so small (7-14 g), and use of hormones resulted in 100% of female ovipositing. Unfortunately, results were not replicable on further attempts. This may be attributed to differences in cycling hormones or even in environmental differences. Regardless, initial success suggests that, with some adjustments, protocols developed for induction of oviposition in *Ambystoma tigrinum* models may be applied to endangered target species safely and successfully (See *Appendix F* for blue spotted salamander data).

The use of ultrasound techniques in amphibians is relatively efficient and involves minimal stress on animals. In animals that are not habituated, there is a greater potential for stress as the animals must be handled or maintained in a position where they are moderately still for accurate ultrasound readings. There is some concern that ultrasonic waves may actually harm the oocytes Additionally, ultrasonography takes trained

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personnel which, particularly for amphibians, are less readily available than ultrasound machinery. There is also concern of ultrasound causing damage to developing oocytes. Most studies have been concerned with developing mammalian fetuses, and thus have observed amphibians already in the embryonic state. Very little is known about how ultrasound affects oocytes. It is known that ultrasound, when delivered at high frequencies for an extended amount of time, or on modified animals, can cause damage to developing fetuses [Abramowicz, 2014; Fisher & Stratmeyer, 2012]. When ultrasonography is performed at most frequencies used in medicine under typical circumstances has not been shown to cause negative effects on development [Fisher & Stratmeyer, 2012]. That being said, there is a concern about the effects of ultrasonography on oocytes, as so little is known, and particular concern in amphibians, as their skin is so thin and their large oocytes fill their coelomic cavity when mature [Wright & Whittaker, 2001].

Despite these concerns about ultrasound, traditional methods of determining gravidity in amphibians have proven fallible. These methods include observing weight, girth, or looking at the underbelly to attempt visualization of oocytes through the skin [Altig, Personal Comm, 2016]. Ultrasound is an alternative that allows one to look inside the animals and visualize the development of oocytes. Using high frequencies ultrasound, it may be possible to distinguish even further details of oocyte development and to actually track the process of ovulation. In this study, it was possibly to generalize the maturity of the oocytes and to determine oviposition by observing oocytes moving into the ovisac, but detailed development of the oocytes within the ovary and ovulation were not observed. In this study, the general cycle of the *Ambystoma tigrinum* was observed

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over time. It took 57.1 ± 3.9 days for animals to reach an ultrasound grade 3 from an ultrasound grade 0, 28.4 ± 7.2 days to reach a grade 3 from a grade 1, and 8.3 ± 2.1 days to reach a grade 3 from a grade 2. After laying eggs, most animals dropped from the grade they were at receipt of the ovulatory dose to a grade 1 or a grade 0 and took the above stated amount of days to return to gravidity. Because these animals had been given hormones, and were not kept at natural temperatures, these reported cycles may not be consistent with the cycles of wild animals. Future studies should include comparing wild animal cycles to captive animal cycles, and to compare cycles across multiple species.

The use of a ultrasound grading scale of oocyte development and maturity to determine appropriate hormone treatment for induction of ovulation appears to be a successful method of improving egg number, timing of oviposition, and response to hormone treatment. It is recommended that ultrasound grading be used to determine maturity of eggs and to scale the amount of hormones the animal will receive, based on that grade, when breeding amphibians.

References

[1]   Abramowicz, J. S. Ultrasound in Reproductive Medicine: Is It Safe?. In *Ultrasound Imaging in Reproductive Medicine*. 2014 (pp. 3-16). Springer New York.

[2]   Altig, R. Personal Communication. June 6, 2016.

[3]   Anderson, Michael T., and Alicia Mathis. "Diets of Two Sympatric Neotropical Salamanders, Bolitoglossa Mexicana and B. Rufescens, with Notes on Reproduction for B. Rufescens." Journal of Herpetology 33, no. 4 (1999): 601–7. doi:10.2307/1565576.

[4]   Atger M, Misrahi M, Sar S, Le Flem L, Dessen P, and Milgrom E. Structure of the Human Luteinizing Hormone-choriogonadotropin Receptor Gene: Unusual Promoter and 5′ Non-coding Regions. Molecular and Cellular Endocrinology . 1995; 111.(2): 113-23.

[5]   Browne, R K Zippel K. Reproduction and Larval Rearing of Amphibians. ILAR J 2007;8:214–34.

[6]   Calatayud NE, Langhorne CJ, Mullen AC, Williams CL, Smith T, Bullock L, et al. A hormone priming regimen and hibernation affect oviposition in the boreal toad (Anaxyrus boreas boreas). Theriogenology 2015;84:600–7. doi:10.1016/j.theriogenology.2015.04.017.

[7]   Calisi RM, Bentley GE. Lab and field experiments: Are they the same animal? Horm Behav 2009;56:1–10. doi:10.1016/j.yhbeh.2009.02.010.

[8]   Clulow J, Trudeau VL, Kouba AJ. Amphibian declines in the twenty-first century: why we need assisted reproductive technologies. Adv Exp Med Biol 2014;753:275–316. doi:10.1007/978-1-4939-0820-2_12.

[9]   Duellman DE, Trueb L. Biology of Amphibians. Baltimore USA: John Hopkins University Press; 1994.

[10]   Fisher, B. R., & Stratmeyer, M. E. Center for Devices and Radiological Health. *Ultrasound: Medical Applications, Biological Effects, and Hazard Potential*, 2012 (pp 195-228).

[11]   Grant, R. A., Chadwick, E. A., & Halliday, T. The lunar cycle: a cue for amphibian reproductive phenology? Animal Behaviour,2009; 78(2), 349–357. http://doi.org/10.1016/j.anbehav.2009.05.007

[12]   Kim JW, Im WB, Choi HH, Ishii S, Kwon HB. Seasonal Fluctuations in Pituitary Gland and Plasma Levels of Gonadotropic Hormones In Rana. General and Comparative Endocrinology. 1998; 109 (1): 13-23.

[13] Khattak S, Tanaka EM. Axolotl (Ambystoma mexicanum) in vitro fertilization. Cold Spring Harb Protoc 2009;2009:pdb prot5263. doi:10.1101/pdb.prot5263.

[14] Kouba AJ, Vance CK, Willis EL. Artificial fertilization for amphibian conservation: Current knowledge and future considerations. Theriogenology 2009;71:214–27. doi:10.1016/j.theriogenology.2008.09.055.

[15] Krebs, Jessi. Personal Communication, March 2015.

[16] Mansour N, Lahnsteiner F, Patzner R a. Collection of gametes from live axolotl, Ambystoma mexicanum, and standardization of in vitro fertilization. Theriogenology 2011;75:354–61. doi:10.1016/j.theriogenology.2010.09.006.

[17] Mcdonough CE, Martin MW, Vance CK, Kouba JACAJ. Frequency of exogenous hormone therapy impacts spermiation in male Fowler ' s toad (Bufo fowleri). Reprod Fertil , 2015.

[18] Moyle WR, Campbell RK, Rao SNV, Ayad NG, Bernard NP, Han Y, and Wang Y. Model of Human Chorionic Gonadotropin and Lutropin Receptor Interaction That Explains Signal Transduction of the Glycoprotein Hormones. Journal of Biological Chemistry 1995; 270 (34): 20020-0031.

[19] Norris, DO., and Jones, RE. *Hormones and Reproduction in Fishes, Amphibians, and Reptiles*. New York: Plenum, 1987.

[20] Norris, DO., and Lopez, KH. *Amphibians*. Amsterdam: Elsevier, 2011.

[21] Ogielska, Maria. *Reproduction of Amphibians*. Enfield, NH: Science, 2009

[22] Oseen KL, Wassersug RJ. Environmental factors influencing calling in sympatric anurans. Oecologia 2002;133:616–25. doi:10.1007/s00442-002-1067-5.Dev 2015.

[23] Ploog, Christina. Personal Communication, March 2016.

[24] Reyer HU, Frei G, Som C. Cryptic female choice: frogs reduce clutch size when amplexed by undesired males. Proc Biol Sci 1999;266:2101–7. doi:10.1098/rspb.1999.0894.

[25] Roth, TL, Obringer AR. Reproductive research and the worldwide amphibian extinction crisis. In *Reproductive Science and Integrated Conservation.* Eds WV Pickard, AR Pickard, JC Rodger and DE Wildt. 2003 pp. 359–374.

[26] Rudolf, VHW, and Rödel MO. Oviposition Site Selection in a Complex and Variable Environment: The Role of Habitat Quality and Conspecific Cues. Oecologia. 2004;142(2): 316–25. doi:10.1007/s00442-004-1668-2.

[27] Sever DM. Comparative anatomy and phylogeny of the cloacae of salamanders (Amphibia: Caudata). 1. Evolution at the family level. Herpetologica 1991;47:165–93. doi:10.1002/jmor.1052070306.

[28] Sever DM. Sperm storage and degradation in the spermathecae of the salamander Eurycea cirrigera. J Morphol 1991;84:71–84.

[29] Sever DM, Brizzi R. Comparative biology of sperm storage in female salamanders. J Exp Zool 1998;282:460–76.

[30] Sever, DM, Lynne DH. Spermatophore Formation in Desmognathus ochrophaeus (Amphibia : Plethodontidae). Copeia 1985;1985:394–402.

[31] Stoops MA, Campbell MK, Dechant CJ. Successful captive breeding of Necturus beyeri through manipulation of environmental cues and exogenous hormone administration: a model for endangered Necturus. Herpetol Rev 2014;45:251–6.

[32] Trottier TM, Armstrong JB. Hormonal stimulation as an aid to artificial insemination in Ambystoma mexicanum. Can J Zool 1975;53:171–3.

[33] Uteshev V, Kaurova S, Shishova N, Stolyarov S, Browne R, Gakhova E. In vitro Fertilization with Hormonally Induced Sperm and Eggs from Sharp-Ribbed Newts Pleurodeles waltl. Russ J Herpetol 2015;22:35–40.

[34] Vitt LJ, Caldwell JP. Herpetology. 3rd ed. Singapore China: Elsevier; 2009.

[35] Wright, K. M., & Whitaker, B. R. 2001. *Amphibian medicine and captive husbandry*. Krieger Publishing Company.

[36] Whittier, JM., and Crews D. Seasonal Reproduction: Patterns and Control. In *Hormones and Reproduction in Fishes, Amphibians, and Reptiles*, edited by David O. Norris and Richard E. Jones, 385–409. Springer US, 1987. http://link.springer.com/chapter/10.1007/978-1-4613-1869-9_13.

CHAPTER V

DEVELOPMENT OF *IN VITRO* FERTILIZATION PROTOCOLS FOR TIGER

SALAMANDERS (*AMBYSTOMA TIGRINUM*)

Introduction

Amphibian populations continue to decrease, as a result of aging animals, disease, environmental contaminants, and lack of recruitment and breeding. Captive assurance colonies for declining populations help to maintain population numbers; however, an often overlooked and equally important role of these colonies is to help to maintain population genetic diversities [Kouba et al., 2009; Clulow et al., 2014; Roth & Obringer, 2014]. Captive assurance colonies are effectively a living biobank whereby selective breeding of animals in dwindling populations maintains valuable genetic diversity. The genetics of aging animals can easily be lost in wild populations where mating opportunities are limited. Furthermore, fragmentation of habitat can lead to lack of recruitment and decreased genetic diversity which results in a loss of fitness [Collins & Storfer, 2003; Gascon et al, 2005; Hayes et al., 2010]. The formation of managed assurance colonies where genetic representation from founder animals that may be aging, and animals that are from fragmented habitats that would otherwise not be able to breed, allow introduction of new genes into the population and an overall preservation of fitness.

One of the tools that is extremely beneficial in managing the genetics of captive populations is *in vitro* fertilization (IVF). Eggs and sperm can be selectively produced for

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optimized genetic pairings, either by hormonally stimulated gamete production, or by collecting gametes from naturally reproductively active animals [Browne et al., 2006; Kouba et al., 2009; Wildt & Wemmer, 1999]. IVF allows underrepresented genetic lineages to be incorporated into populations than might otherwise be limited in their genetic recruitment. Frequently, aging animals found in captivity have been reproductively isolated and unable to contribute to the population gene pool as it progresses through many generations. Such animals often represent genetic lineages that have been lost in the population and therefore hold unique gene contributions that can be incorporated and preserved by IVF breeding strategies. Moreover, animals in populations separated by distance can have their gametes shipped to numerous breeding sites without transporting the animal itself. This decreases stress on animals and, with ease of shipment of multiple gametes, increases the opportunity for multiple breeding attempts and genetic pairings within a breeding season. Increasing recruitment through multiple pairings of gametes can alleviate the stress and demand for incorporating genetics from valuable founder animals [Browne et al., 2006; Wildt & Wemmer, 1999].IVF also increases breeding success through control of variables such as sperm concentration, which can be manipulated in order to achieve maximal fertilization [Browne et al., 2006; Kouba et al., 2009].

IVF is particularly straight forward in amphibians, because most anurans (frogs/toads), and some caudates (newts/salamanders), undergo extremal fertilization. In contrast to mammals where surgical procedures may be required to conduct IVF, in amphibians collection of gametes can be largely done externally and the eggs and sperm

can be combined in a petri dish [Browne & Zippel, 2007; Browne et al., 2006; Kouba et al., 2009].

Most caudates undergo internal fertilization, presenting some challenges to the typical amphibian IVF protocol, but the eggs and sperm are still designed to withstand the external environment. Caudate sperm is naturally deposited in a "packet" called a spermatophore, which can be exposed to the environment for hours and even days without desiccation [Russell et al., 1981; Selmi et al., 1997; Sever & Lynne, 1985]. Caudate eggs, much like anuran eggs, are anamniotic and must be laid in ponds or in moist locations where they develop exposed to the environment [Duellman & Trueb, 1994; Vitt & Caldwell, 2009].

The sperm to egg ratio is very important to consider when performing an IVF. While it is intuitive that too dilute of sperm will have low fertility, it is very possible, particularly in lower vertebrates, that too high of a concentration will have low fertility as well. In lower vertebrates, including amphibians, polyspermy, where more than one sperm fertilizes the egg, occurs frequently. Too many sperm fertilizing the egg can lead to abortion of the fertilized egg. While most vertebrate oocytes have mechanisms to block polyspermy, lower vertebrates differ somewhat from mammals. The a "fast block" occurs almost immediately after fertilization in all vertebrates. In the fast block mechanism, contact of the spermatozoan with the oocyte membrane causes rapid depolarization of the oocyte. In lower vertebrates, this depolarization occurs much faster than in mammals. However, the oocyte also repolarizes much faster than mammals, and it has been determined that many lower vertebrates eggs are easily re-fertilized, especially in the presence of high sperm concentrations. While polyspermy in lower vertebrates may not

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cause ill effects when only one or two extra spermatozoa have fertilized the egg, if the sperm count is too high and dozens of sperm have fertilized the egg, the embryo will fail to develop [Harada et al., 2011; Iwao, 2012; Kouba et al., 2009].

An additional tool that can be utilized in conservation and captive assurance of endangered species is cryopreservation of gametes. Cryopreserved sperm can be stored for years in order for use in *in vitro* fertilization. Genetics of animals senescent or deceased animals valuable to the population can be utilized for many years after what would be possible through natural breeding alone. Additionally, with the use of banked gametes, the ability arises to perform artificial insemination or *in vitro* fertilization in the event that natural mating fails.

In this experiment, three concentrations of sperm of the eastern tiger salamander (*Ambystoma tigrinum*) were applied to similarly sized clutches of eggs to determine appropriate concentration of sperm for IVF in salamanders. These concentrations were paired with an undiluted control of sperm, in order to determine if the dilution media affected the fertilization rates, and a parthenogenic control, to ensure fertilization did not occur by chance. Developmental stages of tiger salamander embryos were observed.

Additionally, an experiment in order to determine the effects of thawing frozen-stored sperm on fertilization took place. Sperm which had been stored for over a month in one of two cryoprotective media was thawed at 20ºC and utilized in *in vitro* fertilization. Fresh sperm was diluted in the cryoprotective agents and also used in IVF in order to determine if there were toxic effects or fertility effects of the cryoprotectants.

Materials and Methods

All protocols for animal care and use were reviewed and approved by the IACUC at Mississippi State University.

Experimental Animals

Fifteen male and fifteen female eastern tiger salamanders (*Ambystoma tigrinum*) were obtained from private breeders via The Memphis Zoo. Males salamanders were maintained according to protocols described in the methods section 2.2.1 and females were maintained according to protocols described in the methods section 3.2.1.

IVF trials using frozen-thawed sperm, only five of the fifteen female salamanders (*Ambystoma tigrinum*) were utilized. These animals were maintained in conditions identical to section 4.2.1.

Fresh sperm *in vitro* fertilization

<u>Collection and evaluation of eggs</u>

Eggs were collected via exogenous hormone induction and assessed for quality using methods described in *Chapter IV*. An attempt was made to collect eggs and separate so that 15-30 eggs, were in each petri dish. Naturally laid eggs were not used for *in vitro* fertilization trials; only eggs that were obtained via collection were used.

<u>Collection and evaluation of sperm</u>

For consistency, only milt was used for *in vitro* fertilization trials.

Milt was collected from male tiger salamanders via exogenous hormone stimulation via methods discussed in *Chapter II*. Milt was analyzed for concentration, motility, and abnormalities using the same methods as discussed in *Chapter II, Section*

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*2.*24. As differences in concentrations and motility did not significantly vary across hormone treatments in males, and specific concentrations were used for IVF treatments, hormone treatments used for collection of milt in males varied.

Dilution of milt

In order to determine the ideal sperm to egg ratio, milt was diluted to three specific concentrations prior to application for *in vitro* fertilization. Initial concentration was determined and then, based on the initial concentration, diluted down to aliquots of $1x10^7$, $1x10^6$, and $1x10^5$ spermatozoa/mL. An amount of 50µL was placed onto each grouping of 10 eggs. This amounted to sperm to egg ratios of: 50,000 spermatozoa/ egg, 5,000 spermatozoa/egg, and 500 spermatozoa/egg, respectively.

Milt was diluted in a 10% Holtfreter's solution (see *Chapter II* for solution components). If milt was not used for *in vitro* fertilization immediately upon dilution, it was stored at 4ºC for up to 12 hours. After 12 hours, the sperm was potentially still viable (see *Appendix D*), however the quality was diminished and it was no longer considered useful for *in vitro* fertilization trials.

Application of milt to eggs

Eggs that were collected fresh from the female were used for IVF. Eggs were collected in groups of approximately 20 per Petri dish (no less than 15, no more than 30). Once in the Petri dish, milt was pipetted over the eggs at 50µL of milt per 10 eggs. The milt was allowed to sit on the dry eggs for five minutes. After five minutes had passed, the eggs in the dish were flooded with dechlorinated, conditioned tap water. The dishes were moved as little as possible for the first 24 hours after exposing the eggs to the milt.

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Developmental staging of embryos

There is not a standard developmental staging series for tiger salamander embryos. Stages were numbered as the stages for *Ambystoma mexicanum* (the Mexican axolotl) and *Ambystoma maculatum* (the spotted salamander) [Schreckenberg, 1975 ; Harrison, 1969]. Fertilization was difficult to determine, so fertilization was determined when eggs underwent their first cleavage (entered the two cell stage, stage 1). The timing of the tiger salamander stagers was slightly different from the axolotl stages reported; however, laboratory conditions, including temperature and humidity, may change the speed of the developmental process [Bradford, 1990; Horne et al., 1994; Smith et al., 2015]. Reported here are the stages of the tiger salamanders as they were consistently seen in the Mississippi State University amphibian laboratory. See figure 5.1 for the key stages used to mark development in this study and *Appendix E* for the full staging series.

Frozen-thawed sperm *in vitro* fertilization

Eggs and sperm were collected and evaluated as in *Section 5.2.2*.

Freezing sperm

Sperm from 10 of the 15 male salamanders used in the experiment was frozen after collection in a stock solution of either 10% DMSO or 10% DMSO with 0.5% BSA following protocols described n *Chapter III*. Cryoprotective agents were selected based on results of preliminary trials and the trials discussed in *Chapter III*.

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Thawing sperm

A single straw was thawed at 20ºC for one of each of the 10 males used for frozen-thawed IVF. One *in vitro* fertilization is represented between one male and one female, with 2 IVF replicates per each of the five females.

Application of milt to sperm

Post thawing, sperm was analyzed for motility and abnormalities as described in *Chapter III*. Once analyzed, sperm was deposited onto eggs and IVF took place as described in *Section 5.2.2*. Controls of fresh sperm, diluted in each of the two cryoprotectants, were used to inseminate eggs alongside their frozen counterparts in order to test for toxic effects of the cryoprotectants on fertilization. A control of fresh sperm, with no cryoprotective agent, was also utilized to ensure good quality egg fertilization. Finally, a parthenogenic control was used which was "fertilized" with a Holtfreter's solution and observed for parthenogenic activity.

Statistical Analysis

Data was assessed for normality using histogram plots and the Shapiro-Wilk test. Abnormal data was transformed via a logarithmic transformation.

For fresh sperm IVF, analysis of variance was used to compare effects of the different levels of concentrated sperm (treatments), effects of individual females used in IVF, and effects of individual males used in IVF and in *in vitro* success.

Analysis of variance was also used in frozen-thawed sperm IVF, comparing the differences in thawed sperm, with each different cryoprotectants, to fresh sperm.
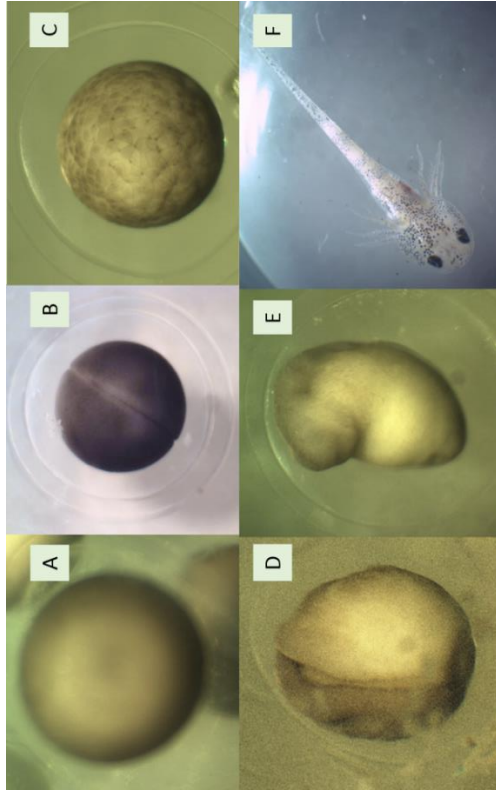
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Figure 5.1   Key developmental stages used to mark timing of growth in embryonic *Ambystoma tigrinum*

For images of all 45 stages and detailed descriptions of stages, see *Appendix D*

The time shown is the time the stage was first reached. The range of time (in hours) was variable due to temperature in the laboratory and individual variability.

Figure 5.1 A: No cleavage (0-18 hr)

Figure 5.1 B: Stage 2: 2 cell/first cleavage (5-18 hr)

Figure 5.1 C: Stage 7: Early blastula (18-36 hr)

Figure 5.1 D: Stage 18: Neurula (63-146 hr)

Figure 5.1 E: Stage 24 & 25: Tail bud (68-218 hr)

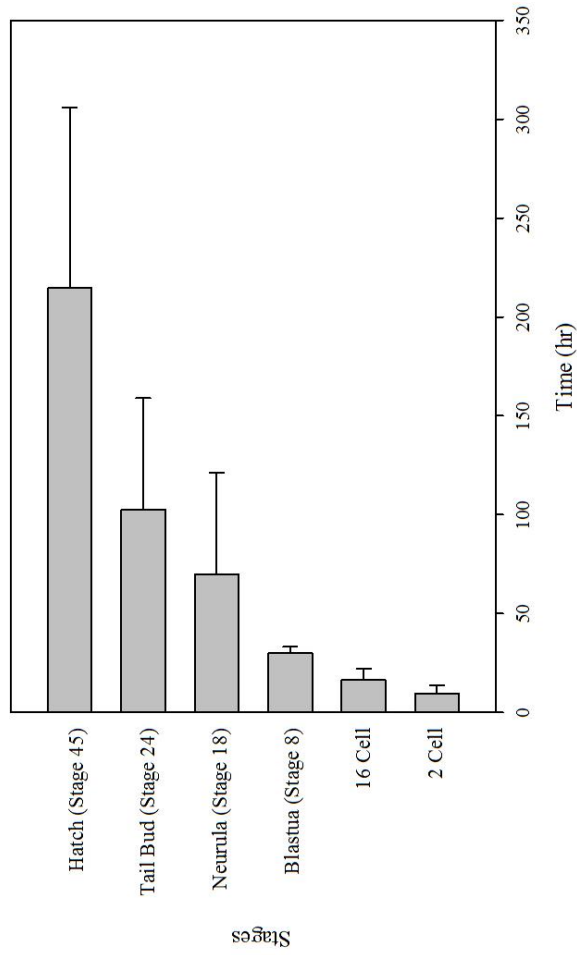*Figure 5.1 F*: Stage 47: Hatched (99-403)

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Figure 5.2   Mean time to key developmental stages of the eastern tiger salamander (*Ambystoma tigrinum*)

For detailed ranges of time for stages 0-45, see *Appendix E*. Variability in time seemed to increase at later stages. Much of the variation in time to specific stages was likely due to variable temperatures during development (temperature data not shown).

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Results

Results of the analysis of variance for fresh sperm IVF showed there no significant differences in percent of blastulas from cleaved eggs, or percent of larvae hatching between concentrations of sperm or between individual females. The percent of eggs cleaved was significantly different between sperm concentrations (p=0.02; F=4.3), with undiluted sperm having the highest amount of cleavage and 1×10^5 having the lowest amount of cleavage. There was a significantly different percent of cleaved eggs reaching neurula (p=0.01; F=3.5), and tailbud (p=0.001; F=5.7) between individual females, which may have had to do with egg quality.

Time to cleavage post IVF was affected by the individual female (p=0.002; F=7.3), the concentration of sperm (p=0.003; F=8.3), and the individual male (p=0.0002; F=12.5) with interaction effects between concertation of sperm and female (p=0.03) and the individual males and females (p=0.002; F=11.9).

Number of larvae hatched was significantly different according to concentration of sperm (p=0.006; F=4.7). The 1×10^6 sperm/mL concentration had significantly more hatched larvae from cleaved eggs than the 1×10^7 sperm/mL concentration. The developmental stages of blastula, neurula, and tailbud were also analyzed for differences based on sperm concentration, male, and female. Blastulas showed main effects by sperm concentration (p=0.0005; F=8.8) and female (0.002; F=4.5), with 1×10^7 sperm/mL having significantly less blastulas than all other concentrations. Neurulas showed a main effect by sperm concentration (p=0.0004; F=7.4) and female (p=0.004; F=3.9), with more neurulas occurring from undiluted sperm than from 1×10^5 sperm/mL or 1×10^6 sperm/mL. Tailbuds showed a main effect by both sperm concentration (p=0.02; F=3.3) and female

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(p=0.006; F=3.7) with both $1x10^6$ sperm/mL and undiluted sperm having more tailbud from cleaved eggs than $1x10^7$ sperm/mL (see Table 5.2).

The overall main effects on time to cleavage, fertilization, and developmental stages were statistically significant for concentration of sperm (Wilk's Λ p=0.06), individual female (Wilk's Λ p= 0.02), and individual male (Wilk's Λ p = 0.003). The overall male and female interaction effect was significant (Wilk's Λ p=0.03). However, the overall interaction between concentration of sperm and female (Wilk's Λ p=0.5) was not significant.

For the IVF using frozen-thawed sperm, there was a slight significant difference in the number of eggs cleaved (p=0.05, F=2.5). The eggs treated with of 5% DMSO and 5% DMSO with 0.5% BSA did not show significantly different cleavage rates than the control fresh sperm. The control fresh sperm that was diluted with 5% DMSO and 0.5% BSA had significantly higher cleavage than all other treatments except the control, but there were no significant difference in cleavage between any of the other treatments (See table 5.10). There was no significant difference in the time it took for the embryos to cleave between any of the treatments. There was no significant difference between treatment groups in the percent of embryos reaching the blastula stage (stage 7) or the neurula stage (stage 18; see Table 5.10). As for the tailbud stage (stage 24-25), there was an overall significant difference in number of embryos reaching this stage between IVF groups (p=0.02, F=3.12). Significantly more control embryos reached the tailbud stage than embryos fertilized with either fresh sperm or thawed sperm in 5% DMSO and 0.5% BSA. Significantly more embryos fertilized with fresh sperm and thawed sperm in 5% DMSO reached tailbud stage than embryos fertilized with thawed sperm in 5% DMSO

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and 0.5% BSA (see Table 5.10). There was an overall significant difference in the number of eggs hatched ($p=0.01$, $F=3.7$). In the control group, significantly more animals successfully hatched than in the fresh or thawed sperm treated with 5% DMSO and 0.5% BSA or the thawed sperm treated with 5% DMSO alone. There was no significant difference in the percent of animals hatching in either the fresh 5% DMSO or the fresh or thawed 5% DMSO with 0.5% BSA groups. The thawed sperm treated with 5% DMSO alone hatch a significantly lower percent of animals than all other groups (see table 5.10).

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Table 5.1   Effects of sperm concentration on fertilization and embryo development

Concentration (spermatozoa/mL)	Total Egg Number	Time to cleavage (hr)	Cleavage from eggs laid (%)	Blastula† (%)	Neurula† (%)	Tailbud† (%)	Hatched larvae† (%)
100,000*	218	10.5 ± 0.8 [ab]	6.9 ± 1.6 [c]	88.5 ± 10.3 [a]	53.8 ± 17.1 [b]	37.1 ± 15.4 [ab]	31.6 ± 14.5 [abc]
1,000,000*	258	7.9 ± 0.8 [bc]	28.8 ± 5.7 [b]	79.2 ± 9.3 [a]	63.5 ± 10.0 [b]	50.8 ± 9.7 [a]	46.9 ± 8.8 [a]
10,000,000*	247	7.7 ± 1.1 [c]	26.9 ± 6.5 [b]	54.0 ± 6.0 [b]	77.5 ± 13.2 [ab]	5.4 ± 1.5 [b]	5.0 ± 1.5 [bc]
Undiluted**	233	10.4 ± 1.1 [a]	42.1 ± 6.1 [a]	95.2 ± 3.0 [a]	85.9 ± 4.9 [a]	61.5 ± 11.6 [a]	42.2 ± 12.6 [ab]
Parthenogenic Control+	224	0.0 [d]	0.0 [c]	0.0 [c]	0.0 [c]	0.0 [c]	0.0 [c]

Means ± SEM shown in table

[abc] Values within the same column that have different letter superscripts are significantly different (p<0.05)

*Specific concentrations were fresh milt diluted in a Holtfreter's solution

** The undiluted control was non-diluted fresh milt; this milt ranged in concentrations between 1 x 10^6 - 9.3 x 10^7

+ Parthenogenic control consisted of eggs that were treated with PBS alone

† Blastula, neurula, tail bud, and hatched are all expressed as a percent of the number of cleaved eggs

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Table 5.2 Effects of thawed sperm *vs.* fresh sperm, comparing two different cryoprotectants, on fertilization and embryo development

CPA		% Motility		Total Egg #	Time to cleavage (hr)	Cleavage from eggs laid (%)	Blastula † (%)	Neurula † (%)	Tailbud † (%)	Hatched larvae † (%)
		Total	Progressive							
5% DMSO	Thawed	7.0 ± 2.3*	0.7 ± 0.5*	258	6.2 ± 1.8	16.0 ± 6.4 a	92.2 ± 7.8	81.6 ± 11.9	51.5 ± 24.3 ab	5.9 ± 1.0 a
	Fresh	59.0 ± 4.4	28.5 ± 3.9	253	5.0 ± 1.7	25.1 ± 5.1 a	84.3 ± 6.7	76.0 ± 8.0	59.3 ± 16.3 ab	57.3 ± 15.8 bc
5% DMSO + 0.5% BSA	Thawed	9.4 ± 2.1*	1.3 ± 0.7*	256	8.4 ± 1.4	25.0 ± 5.7 a	82.8 ± 7.2	58.1 ± 9.8	37.0 ± 10.2 c	33.6 ± 22.3 b
	Fresh	50.4 ± 3.8	25.4 ± 4.5	267	9.5 ± 3.0	47.4 ± 7.7 b	77.0 ± 9.8	67.3 ± 11.6	51.3 ± 12.5 bc	45.5 ± 12.5 b
Controls	No CPA	30.4 ± 4.8	11.1 ± 2.2	284	6.2 ± 1.1	26.8 ± 5.4 ab	94.9 ± 3.1	75.9 ± 9.0	70.3 ± 8.0 a	67.5 ± 7.1 c
	Parthenogenic	0	0	287	-	-	-	-	-	-

ab: Values with different superscripts within a column are significantly different from one another

Controls with cryoprotectants were fresh sperm diluted to 2,000,000 spermatozoa/mL and then diluted 1:1 with cryoprotectant

*Post-thaw motility of frozen sperm

Non-progressive motility not shown

† Blastula, neurula, tail bud, and hatched are all expressed as a percent of the number of cleaved eggs

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Discussion

Of the three different concentrations used for IVF that were diluted in Holtfreter's solution, the 1×10^6 concentration had the highest cleavage and the highest number of larvae hatched from eggs cleaved. However, fertilization rates were fairly low, even within the non-diluted control group ($42.1 \pm 6.1\%$). It is possible to attribute the low fertilization rates within the diluted groups to the Holtfreter's dilution medium and inappropriate osmolality and metabolic environment for the sperm. Osmotic parameters for salamander sperm are unknown. A study by Hardy and Dent from 1896 shows that hypoosmotic solutions may activate salamander sperm, although sperm are active in isotonic and hypertonic solutions and sperm remain active for the longest amount of time in isotonic solutions. Conflicting reports suggest that there is not a connection between osmolality and sperm activity [Siegel, Personal Comm 2012; Marcec, Unpublished data]. Because the 1×10^6 sperm/mL and the 1×10^7 sperm/mL treatments had significantly lower cleavage than the undiluted control, the 10% Holtfreter's medium played a role in the lower fertilization rates of the treatments. Holtfreter's solution is a salt medium (see *Chapter II, Section 2.2.4*) that has an approximately isotonic osmolality (267 mOsm). This solution is commonly used in the raising of Ambystomids in captivity [Gresens, 2004].

The concentration that had the highest success was not the highest concentration. The 1×10^7 concentration likely had lower success than the 1×10^6 concentration due to polyspermy, or the incident of multiple sperm fertilizing an egg. While amphibians undergo a fast and a slow block, much like mammals, to prevent polyspermy, the occurrence of polyspermy occurs much more frequently in amphibians. The primary

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difference in amphibians that allows for increased polyspermy lies in the fast block mechanism. The fast block is activated almost immediately after fertilization as contact of the spermatozoa with the oocyte membrane results in rapid depolarization of the oocyte. In amphibians and other lower vertebrates, depolarization occurs much faster than in mammals. This following repolarization of the egg also comes much faster than in mammals. It has been determined that, due to the rapid repolarization of the egg, many lower vertebrates can be re-fertilized much sooner than mammals. [Iwao, 2012; Harada et al, 2011; Toro & Michael, 2004]. In this study, often multiple fertilization sites could be seen prior to cleavage and, in these eggs, asymmetrical cleavage was noted followed by egg death.

The methods used for IVF were based off methods utilized in externally fertilizing frogs [Browne et al, 2006; Calatayud et al, 2015; Calatayud et al, 2016; Kouba et al, 2009]. The majority of salamanders are internally fertilizing [Sever-1, 1991; Sever-2, 1991; Sever & Brizzi 1998; Sever & Lynne, 1985] and thus may require a different process that that used n externally fertilizing frogs for optimal *in vitro* fertilization. There are reports of artificial insemination in caudates [Mansour et al, 2011; Osikowski, 2007; Trottier & Armstrong, 1975 ; Watanabe et al, 2007] and this has been a successful method used in the salamander laboratory at Mississippi State University [Marcec, Unpublished data]. In these attempts, timing of the deposition of the sperm into the cloaca was crucial in order to see fertilization. Exact timing for ideal fertilization in artificial insemination of *Ambystoma tigrinum* has yet to be determined. Both spermatophores and milt have been used in different species of internally fertilizing salamanders for artificial insemination, and spermatophores have proven more successful

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in this method [Osikowski, 2007]. It is suggested that the gel and proteins in the spermatophore are needed to react with the enzymes in the cloaca for appropriate sperm activation and fertilization [Osikowski, 2007; Sever-1, 1991; Sever-2, 1991; Sever & Brizzi, 1998]. However, artificial insemination attempts have proven less successful than IVF. Thus, IVF is still a promising technology and with further study may be improved. Some of the possible causes of low fertility in IVF in internally fertilizing salamanders could be attributed to the osmolality and the environment the sperm is placed in, as mentioned above. Indeed, very little is known or understood about the activation and ideal environment for caudate spermatozoa. The internal environment of the cloaca is often attributed to the activation and natural processes of sperm [Sever-1, 1991; Sever-2, 1991; Sever & Brizzi 1998; Sever & Lynne, 1985; Siegel et al, 2012], but this natural process, and thus natural success, has yet to be replicated to the fullest in artificial breeding of internally fertilizing caudates.

While using IVF techniques with milt in internally fertilizing salamanders may be unnatural processes, they is also highly practical. Milt is an effective media for assessment of sperm parameters, as it is in liquid form and can be easily diluted or concentrated as necessary. The gel that is present in spermatophores which is necessary for internal fertilization is not present in milt, making milt much easier to view clearly under the microscope. Spermatophores can be assessed, but they must be physically broken down into a solution which can cause excessive damage to sperm. The use of IVF is ideal for preventing transfer of disease between animals. In artificial insemination, sperm must be directly placed into the cloaca of a salamander. The spermatophore or milt may be a vector for diseases such as *Batrachocytrium spp.* or iridoviruses, which have

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been causing large declines in amphibians globally. Many animals are carriers of these diseases without showing symptoms. While some of these diseases, such as *Batrachochytrium spp*, can be tested for carrier status, others, such as most iridoviruses, do not have tests for carrier status. While it is still unclear if diseases of concern in salamanders can be transferred through gametes, milt can be collected and tested for diseases prior to deposition over eggs for IVF. IVF requires less handling of multiple animals and no direct contact of body fluids between animals and overall poses less risk for transmission of disease.

These data suggest both the male and the female are critical to the success of the IVF. This is intuitive, but it does remind one to observe the quality of the eggs involved in the procedure. The male had an effect on the time to fertilization and the stages of development, but not the number of larvae hatched. While it is likely both variables are important to each of these stages and significance was not found due to low numbers of animals, the results suggest that both quality of eggs and quality of sperm are equally important to the production of larvae.

In the trials utilizing the CPA in the fresh sperm, the fresh sperm diluted in the 5%DMSO and 5% BSA had higher cleavage than the control sperm, which was not anticipated. This may suggest that this CPA provides beneficial protection to live sperm that does not undergo a freezing process. BSA has been known to increase motility and viability of sperm in mammals [Harrison et al, 1982; Johnson et al, 2000], and a similar effect may have occurred in this study as the fresh sperm in the 5% DMSO without BSA had a cleavage rate that was not higher than the control sperm. When assessing the frozen sperm compared to the fresh sperm, the number of hatched larvae from cleaved

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eggs from frozen sperm was lower than that of fresh sperm. Because there was no significant difference in cleavage rates (with the exception of the fresh sperm in 5% DMSO and 5% BSA) between the fresh and frozen sperm, this suggests there is some damage to the DNA or there is some delay caused in development that results in embryos that cannot fully develop to hatching. The opposite of this phemononon is see in trout, where frozen-thawed sperm with damaged DNA has fertilization rates that are significantly lower than those of fresh, normal sperm with hatching rates that are similar to those of fresh sperm [Lewis & Aitken, 2005]. In humans, the pattern is similar to that seen in salamanders, where sperm with damaged DNA will still have similar fertilization rates, but abortion is far more common and embryos do not reach term as often as those of fresh sperm [Borini et al, 2006; Labbe et al, 2001].

The developmental staging of the eastern tiger salamander were fairly similarly to those reported in other Ambystomids, falling between the timing of *A. maculatum* and *A. mexicanum*. The range of time on either end of each stage was drastically larger than reported in other Ambystomid studies. This was likely attributed to having performed IVF throughout different seasons and fluctuating temperatures. Numbering the stages the same as reported by Shreckenberg and by Harrison was appropriate for the development of the animals.

Attempts were made at IVF in the regionally endangered blue spotted salamander (*Ambystoma laterale*). Attempts were made using both fresh sperm from *A. laterale* and frozen-thawed sperm from *A. tigrinum*. As this is a species that naturally undergoes kleptogenesis, which is a method of stealing sperm to activate a parthenogenesis-like reaction in the egg [Bogart et al, 2009], the use of sperm from *A. tigrinum* was a plausible

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method of fertilization. Fertilization was achieved, but no embryos survived passed the neurula stage. The lack of survival was attributed to egg quality, not to the method of IVF; however, replicates of the study will be necessary in order to determine if the method is functional in *A. laterale*. Because fertilization was achieved, this is encouraging that protocols developed for IVF in *A. tigrinum* may be applicable to endangered target species (see *Appendix F* for blue spotted salamander data).

References

[1]   Bogart JP, Bartoszek J, Noble DW a, Bi K. Sex in unisexual salamanders: discovery of a new sperm donor with ancient affinities. Heredity (Edinb) 2009;103:483–93. doi:10.1038/hdy.2009.83.

[2]   Bordzilovskaya, NP Dettlaff T. Table of stages of the normal development of embryos and the prognostication of timing of successive developmental stages at various temperatures. Axolotl Newsl 1979:2–22.

[3]   Borini A, Tarozzi N, Bizzaro D, Bonu MA, Fava L, Flamigni C, Coticchio G. Sperm DNA fragmentation: paternal effect on early post-implantation embryo development in ART. Human Reproduction. 2006 Nov 1;21(11):2876-81.

[4]   Bradford, DF. Incubation Time and Rate of Embryonic Development in Amphibians: The Influence of Ovum Size, Temperature, and Reproductive Mode. Physiological Zoology 1990:63(6), 1157–1180.

[5]   Brown, HA. The Time–temperature Relation of Embryonic Development in the Northwestern Salamander, Ambystoma Gracile.Canadian Journal of Zoology 1976: 54 (4), 552–58. doi:10.1139/z76-063.

[6]   Browne RK, Seratt J, Vance C, Kouba A. Hormonal priming, induction of ovulation and in-vitro fertilization of the endangered Wyoming toad (Bufo baxteri). Reprod Biol Endocrinol 2006;4:34. doi:10.1186/1477-7827-4-34.

[7]   Browne, R K Zippel K. Reproduction and Larval Rearing of Amphibians. ILAR J 2007;8:214–34.

[8]   Calatayud NE, Langhorne CJ, Mullen AC, Williams CL, Smith T, Bullock L, et al. A hormone priming regimen and hibernation affect oviposition in the boreal toad (Anaxyrus boreas boreas). Theriogenology 2015;84:600–7. doi:10.1016/j.theriogenology.2015.04.017.

[9]   Calatayud, NE, Shier, DM, Swaisgood, RR, & Durrant, BS. 119 HORMONE-ASSISTED REPRODUCTION IN A CAPTIVE MOUNTAIN YELLOW-LEGGED FROG POPULATION. Reproduction, Fertility and Development, 2016; 28(2), 189-190.

[10]   Clulow J, Trudeau VL, Kouba AJ. Amphibian declines in the twenty-first century: why we need assisted reproductive technologies. Adv Exp Med Biol 2014;753:275–316. doi:10.1007/978-1-4939-0820-2_12.

[11]   Collins JP, Storfer A. Global amphibian declines : sorting the hypotheses. Divers Distrib 2003;9:89–98.

[12] Duellman DE, Trueb L. Biology of Amphibians. Baltimore USA: John Hopkins University Press; 1994.

[13] Gascon, C Collins, JP Moore, RD Church, DR McKay, JE Mendelson J. Amphibian Conservation Action Plan. IUCN/SCC Amphib. Conserv. Summit 2005, 2005.

[14] Gresens J. An introduction to the Mexican axolotl (Ambystoma mexicanum). Lab animal 2004; 33(9): 41.

[15] Harada Y, Kawazoe M, Eto Y, Ueno S, Iwao Y. The Ca2+ increase by the sperm factor in physiologically polyspermic newt fertilization: its signaling mechanism in egg cytoplasm and the species-specificity. Dev Biol 2011;351:266–76. doi:10.1016/j.ydbio.2011.01.003.

[16] Hardy, Matthew P., and James Norman Dent. "Regulation of motility in sperm of the red-spotted newt." *Journal of Experimental Zoology* 240.3 (1986): 385-396.

[17] Harrison, RG. Organization and Development of the Embryo. S. Wilens, ed., pp. 44-66. Yale Univ. Press, New Haven, CT:1969.

[18] Harrison RP, Dott HM, and Foster GC. Bovine serum albumin, sperm motility, and the "dilution effect". Journal of Experimental Zoology 1982; 222.1: 81-88.

[19] Hayes TB, Falso P, Gallipeau S, Stice M. The cause of global amphibian declines: a developmental endocrinologist's perspective. J Exp Zool 2010;213:921–33. doi:10.1242/jeb.040865.

[20] Horne MT, Dunson WA. Exclusion of the Jefferson salamander, Ambystoma jeffersonianum, from some potential breeding ponds in Pennsylvania: Effects of pH, temperature, and metals on embryonic development. *Archives of Environmental* Contamination and Toxicology 1994: 27(3), 323–330. http://doi.org/10.1007/BF00213167

[21] Iwao Y. Egg activation in physiological polyspermy. Reproduction 2012;144:11–22. doi:10.1530/REP-12-0104.

[22] Johnson LA, Weitze KF, Fiser P, Maxwell WM. Storage of boar semen. Animal reproduction science. 2000 Aug 18;62(1):143-72.

[23] Kouba AJ, Vance CK, Willis EL. Artificial fertilization for amphibian conservation: Current knowledge and future considerations. Theriogenology 2009;71:214–27. doi:10.1016/j.theriogenology.2008.09.055.

[24] Labbe C, Martoriati A, Devaux A, Maisse G. Effect of sperm cryopreservation on sperm DNA stability and progeny development in rainbow trout. Molecular reproduction and development. 2001 Nov 1;60(3):397-404.

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[25] Lewis SE, Aitken RJ. DNA damage to spermatozoa has impacts on fertilization and pregnancy. Cell and tissue research. 2005 Oct 1;322(1):33-41.

[26] Mansour N, Lahnsteiner F, Patzner R a. Collection of gametes from live axolotl, Ambystoma mexicanum, and standardization of in vitro fertilization. Theriogenology 2011;75:354–61. doi:10.1016/j.theriogenology.2010.09.006.

[27] Marcec, Ruth. Motility of *Ambystoma tigrinum* sperm before and after contact with egg jelly. Unpublished raw data.

[28] Marcec, Ruth. Successful artificial insemination of *Ambystoma tigrinum*. Unpublished raw data.

[29] Mills, NE Barnhart M. Effects of hypoxia on embryonic development in two Ambystoma and two Rana species. Physiol Biochem Zool 1999;72:179–88.

[30] Osikowski A. Sperm transport after insemination in the Alpine newt (Triturus alpestris, Caudata, salamandridae). Folia Biol (Praha) 2007;55:109–14. doi:10.3409/173491607781492641.

[31] Roth, TL, Obringer AR. Reproductive research and the worldwide amphibian extinction crisis. In *Reproductive Science and Integrated Conservation.* Eds WV Pickard, AR Pickard, JC Rodger and DE Wildt. 2003 pp. 359–374.

[32] Russell LD, Brandon RA, Zalisko EJ, Martan J. Spermatophores of the salamander Ambystoma texanum. Tissue Cell 1981;13:609–21.

[33] Schreckenberg GM, Jacobson AG. Normal stages of development of the axolotl. Ambystoma mexicanum. Developmental Biology 1975:42(2), 391–400. http://doi.org/10.1016/0012-1606(75)90343-7

[34] Selmi MG, Brizzi R, Bigliardi E. Sperm morphology of salamandrids (Amphibia, Urodela): implications for phylogeny and fertilization biology. Tissue Cell 1997;29:651–64. doi:10.1016/S0040-8166(97)80041-9.

[35] Sever DM. Comparative anatomy and phylogeny of the cloacae of salamanders (Amphibia: Caudata). 1. Evolution at the family level. Herpetologica 1991;47:165–93. doi:10.1002/jmor.1052070306.

[36] Sever DM. Sperm storage and degradation in the spermathecae of the salamander Eurycea cirrigera. J Morphol 1991;84:71–84.

[37] Sever DM, Brizzi R. Comparative biology of sperm storage in female salamanders. J Exp Zool 1998;282:460–76.

[38] Sever, David M Lynne DH. Spermatophore Formation in Desmognathus ochrophaeus (Amphibia : Plethodontidae). Copeia 1985;1985:394–402.

[39]   Siegel, DM. Personal Communication. October, 2012.

[40]   Siegel DS, Murray CM, Wilson ER, Puleo ER, True K a., Aldridge RD. Sexual Kidneys in the Red-spotted Newt (Notophthalmus viridescens). J Herpetol 2012;46:136–44. doi:10.1670/11-013.

[41]   Smith GD, Hopkins GR, Mohammadi S, Skinner HM, Hansen T, Brodie ED, French SS. Effects of temperature on embryonic and early larval growth and development in the rough-skinned newt (Taricha granulosa). Journal of Thermal Biology 2015: 51, 89–95. http://doi.org/10.1016/j.jtherbio.2015.03.010

[50]   Toro E, Michael SF. In vitro fertilization and artificial activation of eggs of the direct-developing anuran Eleutherodactylus coqui. Reprod Biol Endocrinol 2004;2:60. doi:10.1186/1477-7827-2-60.

[51]   Trottier TM, Armstrong JB. Hormonal stimulation as an aid to artificial insemination in Ambystoma mexicanum. Can J Zool 1975;53:171–3.

[52]   Vitt LJ, Caldwell JP. Herpetology. 3rd ed. Singapore China: Elsevier; 2009.

[53]   Watanabe A, Takayama-Watanabe E, Vines CA, Cherr GN. Sperm motility-initiating substance in newt egg-jelly induces differential initiation of sperm motility based on sperm intracellular calcium levels. Dev Growth Differ 2011;53:9–17. doi:10.1111/j.1440-169X.2010.01216.x.

[54]   Wildt DE, Wemmer C. Sex and wildlife: The role of reproductive science in conservation. Biodivers Conserv 1999;8:965–76. doi:10.1023/A:1008813532763.

CHAPTER VI

METHODS FOR SEXING TIGER SALAMANDERS (*AMBYSTOMA TIGRINUM*) AND BLUE SPOTTED SALAMANDERS (*AMBYSTOMA LATERALE*)

Introduction

Caudates are currently experiencing the fastest extinction rates of all vertebrate taxa [Baillie et al., 2004; Gascon et al., 2005]. As such, a worldwide effort to address the population crisis by generate captive assurance colonies is underway. The goal of these colonies is not only to facilitate reproduction, but to also maintain genetic integrity within these populations, and reintroduce offspring into the wild [Clulow et al., 2014; Gascon et al., 2005; Griffiths & Pavajeau, 2008; Kouba et al., 2009]. The success of the captive assurance colonies depends on knowing the sex of the animals in these breeding programs and in remaining wild populations. Without knowing the true sex of animals, groups may be housed inappropriately, irrelevant breeding attempts may be made, and time and effort may be put into obtaining gametes from a wrongfully sexed animal [Clulow et al., 2014; Germano et al., 2011; Kouba et al., 2009]. While sexing an animal may seem an obvious and simplistic goal, knowledge of the sex of an amphibian is not always easily obtained. Amphibians lack external genitalia and many species fail to exhibit dimorphic characteristics [Duellman & Trueb, 1994; Vitt & Caldwell, 2009]. Accurate, efficient, non-invasive methods for sexing amphibians need to be developed,

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particularly for field applications in which animals must be captured and released [Ollivier & Welsh, 2003; Romano et al., 2009].

Mississippi State University houses eastern tiger salamanders (*Ambystoma tigrinum*), which are used as model species for the development of assisted reproductive technologies for other rare and endangered caudate amphibian species. The blue spotted salamander (*Ambystoma laterale*) is an endangered species which has been a beneficiary of the reproductive technology developments made using the tiger salamander. A captive colony of blue spotted salamanders was founded by the Iowa Department of Natural Resources and Omaha's Henry Doorly Zoo and Aquarium (OHDZ) in 2007, with the purpose of breeding *A. laterale* in captivity for reintroduction to a now barren breeding site of the salamander.

Both *Ambtystoma tigrinum* and *Ambystoma laterale* are considered seasonally dimorphic, in that during the breeding season the males will develop cloacal papillae at the caudal base of the cloaca (see Figure 5.1). The expression of these seasonal papillae is found across many salamander families, including Salamandridae, Plethodontidae, Ambystomidae, and Proteidae [Sever, 1992]. These papillae are used as an indicator of the sex of caudate amphibians. Sexual size dimorphism (SSD) is an additional method that is commonly cited as an approach to sexing salamanders and newts [Bakkegard & Guyer, 2004; Bruce, 1993; Raymond & Hardy, 1990; Todd & Davis, 2007]. Females are often noted to be the larger sex in SSD, as is frequently the case with other amphibians, reptiles, and fish [Cox et al., 2007; Parker, 1992; Shine, 1979]. The hypothesis is that the sex does not cause a difference in size, but rather that size causes a difference in sex and is determined by lax sex determination genes [Shine, 1979]. As such, morphometric

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measurements of cloacal length, mass, snout vent length (SVL), tail length, head width, head length, and body width have all been reported as methods for determining sex in salamanders [Alcorn et al., 2013; Bakkegard & Guyer, 2004: Bruce, 1993; Hasumi, 2010; Raymond & Hardy, 1990; Todd & Davis, 2007; Williams et al., 2009].

Ultrasound is a quick, non-invasive procedure that can assist in determining sex of individual amphibians [Miller & Fowler, 2012; Li et al., 2010]. Ultrasound, like taking morphometric data, requires minimal handling and manipulation of animals. Moreover, portable ultrasound equipment is readily available and can be used to sex amphibians in field studies. Unlike morphometric data, ultrasound permits visualization of the sexual organs, and in females, follicular development can be directly followed as well [Kouba & Vance, 2009; Reyer & Battig, 2004; Roth & Obringer, 2009]. However, ultrasound may require specialized training, while collection of morphometric data does not require training beyond handling of animals.

Other methods of sexing include collection of urine or feces for hormone analysis, hormone induced gamete production, or endoscopic surgery [Kouba & Vance, 2009; Kramer et al., 1983; Roth & Obringer, 2009]. While urine or fecal analysis is non-invasive and practical in a laboratory or captive setting, it is more time consuming and is not as practical when assessing wild populations that must be captured and released for collections and identification. Endoscopic surgery is likely more accurate than other methods, but is invasive and poses a high risk for amphibians under anesthesia, especially when considering endangered species. Endoscopy is also not readily field applicable [Kouba & Vance, 2009; Roth & Obringer, 2009].

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In this study, eight male and eight female *Ambystoma tigrinum*, and six male and five female *Ambystoma laterale* were sexed via morphometric methods and ultrasound. Sex was confirmed via injection with hormones and resulting production of sperm or eggs.

Materials and Methods

Experimental Animals

All protocols for animal care and use were reviewed and approved by the IACUC at Mississippi State University.

Sixteen eastern tiger salamanders (*Ambystoma tigrinum*) were used in this study (eight males and eight females). These animals were obtained from private breeders via The Memphis Zoo and were maintained at Mississippi State University in conditions identical to section 2.2.1 and 3.2.1.

Eleven blue spotted salamanders (*Ambystoma laterale*), owned by the Iowa Department of Natural Resources and maintained by OHDZ were used in this study (six males and five females). The animals had been retrieved from the wild as larvae and raised over the course of seven years in captivity by the staff at OHDZ. All eleven animals were housed in a Waterland small tub enclosure (Waterland Tubs, Santa Ana CA, USA) measuring 139.70 cm x 63.5 cm x 30.48 cm. The enclosure was filled with organic soil, natural leaf litter, pieces of damp sheet moss, and terra cotta pot pieces for hiding. Animals were fed a variety of small worms, crickets and flies on a daily basis. Seasonally, a portion of the Waterland tub would be filled with reverse osmosis purified water that was then filtered through a system with biological, chemical and mechanical filtration before being chilled to the appropriate seasonal temperature.

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Morphometric data

All visual assessments and measurements were done by the same individual for consistency. Animals were held so their ventrum faced the investigator. The caudal base of the cloaca was assessed for the presence of papillae and presence was recorded as "yes" or "no." The cloacal length, width, and height was then measured in millimeters using calipers. Height of the cloaca was measured as the amount of swelling of the cloaca, from the base of the tail ventrally. Each animal's head length, head width (across the line of the eyes), body width (across the widest part of the abdomen), SVL, and tail length were also measured with calipers (mm). Calipers were cleaned with ethanol between animals that were not cohabitating.

Figure 6.1   Male papillae in an eastern tiger salamander (*A. tigrinum*)

The blue arrow points to the papillae at the caudal base of the cloaca. The swollen cloaca extends over the cranial base of the tail; the protrusions that extend over the cranial tail are the papillae.

Ultrasound

A Sonosite Micromaxx ultrasound machine, equipped with an HFL38x model (6-13 MHz) 38mm linear transducer, was used for performing ultrasounds. The frequency was set on the general setting, the depth was at 2.7cm, and the gain was adjusted as needed for all animals of all species. All ultrasounds were performed by the same individual who was trained in ultrasonography. For a more detailed description of the ultrasound procedure, see *Appendix B*.

Both species were held in the palm of the investigator's hand, in dorsal recumbency, for ultrasound. The animal's ventrum was wetted thoroughly with dechlorinated and conditioned tap water or reverse osmosis water for use as a conductor. The transducer was laid transversely across the thickest part of the animal's abdomen in order to examine for ovaries in females and over the pelvic area in order to examine for the thickened collecting ducts entering the cloaca in males.

The male ducts are usually seen in transverse cross-section, so will appear as intensely hypoecoic circles surrounded by a thickened hyperechoic membrane (Figure 5.2A; Figure 5.3A; Figure 5.4D). The thickness of the membrane and the diameter of the ducts will vary, dependent on reproductive activity. Females were identified by observing ovaries within the coelomic cavity. Ovaries of tiger salamanders and blue spotted salamanders were identified by hyperechoic (light), defined, round oocytes. The smaller oocytes were densely packed, with little to no hypoechoic (dark) area between them. The larger oocytes had large, evenly spaced regions of hypoechogenicity between them. The layman description that was used to describe ovaries in training sessions was that the oocytes appeared as "stars in a dark night sky" (Figure 5.2 B, Figure 5.3 B).
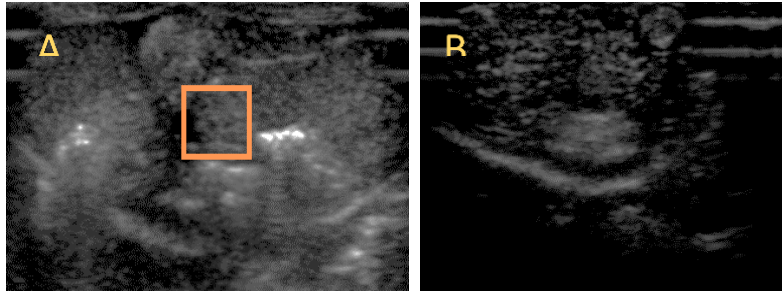
Figure 6.2   Ultrasound Sex Identification of Tiger Salamanders (*A. tigrinum*)

6.2 A: Ultrasound image of a male tiger salamander's pelvic region. The orientation of this image is such that the animal's dorsum is facing the top of the figure and the animal's ventrum is facing the bottom. Within the orange box, the hypoechoic (dark) pattern that can be identified is the mesonephric ducts entering the cloaca. *6.2 B*: Ultrasound image of a female tiger salamander's coelomic cavity. The orientation of this image is opposite of that in 6.2 A- the ventrum of the animal is facing the top of the figure and the dorsum of the animal is facing the bottom. The hyperechoic (light) specks across the screen are the oocytes. Note that both of her ovaries are developed. In some cases only one ovary may be developed or one ovary may be at a different stage of development than the other.
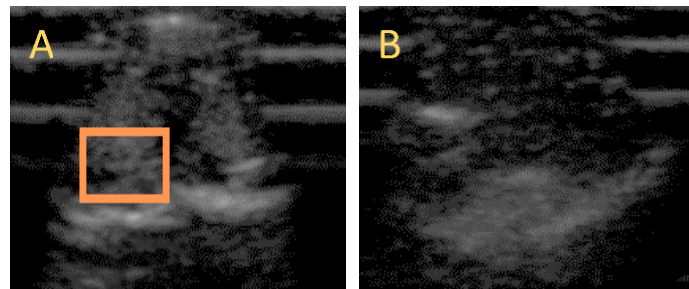

Figure 6.3   Ultrasound sex identification of blue spotted salamanders (*A. laterale*)

6.3 A: Ultrasound image of a male blue spotted salamander. Within the orange box, the hypoechoic pattern that can be identified corresponds to the mesonephric ducts entering the kidney. *6.2 B*: Ultrasound image of a female tiger salamander's coelomic cavity. The hyperechoic specks across the screen are the oocytes. Note both of her ovaries are developed. Both images 6.3 A-B are oriented the same way: the ventrum of the animal is facing the top of the figure and the dorsum of the animal is facing the bottom of the figure.

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Statistical Methods

A multivariate analysis of variance (MANOVA) was used to determine if morphometric measurements could distinguish between the sexes within each species. A Fisher's Exact test was used to determine the reliability of the method in sexing the animal compared to the exact sexing method in which hormone induced gamete production. All significance was determined by a p-value of <0.05 unless otherwise stated. Standard error is reported along with results where appropriate.

Results

Both species shared significant differences between sexes for measurements of tail length, body width, cloacal length, and the binary variable of presence of papillae. For tail length and cloacal length, males of both species were significantly larger than females. As for body width, *A. laterale*, females were larger (p < 0.05) than males, but *A tigrinum* males were larger (p < 0.05) than females. Papillae expression was found in males of both species more frequently than females.

Other general morphometric parameters such as SVL, head length and weight were significantly different between the sexes only in *A. laterale*, but not in *A. tigrinum*. Female *A. laterale* were larger than male *A. laterale* in both dimensional measurements and in overall weight By contrast, the structural characteristics of the cloaca were more pronounced and differentiated the sexes in A. *tigrinum* but did not contribute to sexing *A. laterale*. Specifically, cloacal width and height were both smaller (p < 0.05) in the female, Table 5.2.

Table 5.3 lists the mean value for each morphometric parameter for each sex and within each species. , A mean value of each morphometric measurement was determined

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for each sex within a species, Table 5.3. Based on this mean, an animal was considered a male if it fell directionally to the male side of the measurement (i.e. cloacal length average for *A. tigrinum* was 7.9 ± 0.6 mm; animals with a cloaca measuring longer than 7.9 mm were considered males, animals whose cloacal length were shorter than 7.9 mm were considered females). The mean and variation of each significant morphometric parameter for each sex are shown in Figures 5.4 and 5.5 for *A. tigrinum* and *A. laterale*, respectively.

The reliability of the morphometric data in sex determination was calculated as the number of animals that fell into the correct sex category compared to the true sex of the animals, determined by hormone induced gamete production. Morphometric data is often generalized to all species for sex determination, and Table 5.4 demonstrates that the most reliable indicator of sexing in either species is cloacal length, where 100% of females and > 90% of males are accurately identified. Table 5.4 shows the accuracy of each morphometric measurement in predicting the sex of the animal, represented as a percent correct prediction. Results of Fisher's exact analysis showed that the method to determine animal sex was similar to hormone induced gamete production within a species for males (*A. tigrinum* p = 0.56; *A. laterale* p = 0.45) and females (*A. tigrinum* p = 0.47; *A. laterale* p = 1.0). However, as an overall method for sex discrimination for combined species data, the morphometric data gave much poorer accuracy in identifying males (p = 0.04), while females were still reliably identifiable (p < 1.0) using either ultrasound or morphometric analysis.

Table 6.1 Differences in morphometric characteristics between sexes of *Ambystoma tigrinum*, and *Ambystoma laterale*

	A.tigrinum			*A. laterale*		
	Male (N=8)	Female (N=8)	p	Male (N=6)	Female (N=5)	P
SVL (mm)	102.1 ± 2.4	100.5 ± 2.4	0.7	62.6 ± 0.8	69.1 ± 1.1	0.0006*
Tail Length (mm)	91.9 ± 4.1	83.1 ± 1.3	0.05*	60.8 ± 1.0	57.5 ± 0.9	0.03*
Body Width (mm)	20.5 ± 0.7	18.4 ± 0.6	0.01*	8.2 ± 0.5	10.0 ± 0.2	0.02*
Weight (g)	39.6 ± 2.6	37.3 ± 1.7	0.5	8.0 ± 0.5	9.6 ± 0.3	0.02*
Head Length (mm)	22.6 ± 1.0	21.1 ± 0.5	0.3	10.1 ± 0.4	11.1 ± 0.1	0.04*
Head Width (mm)	18.3 ± 2.0	16.9 ± 0.5	0.3	8.2 ± 0.4	8.6 ± 0.3	0.4
Cloacal length (mm)	10.6 ± 0.8	5.6 ± 0.2	<0.0001*	7.0 ± 0.1	4.8 ± 0.4	0.0001*
Cloacal width (mm)	9.1 ± 0.5	6.5 ± 0.3	<0.0001*	4.1 ± 0.1	4.3 ± 0.5	0.5
Cloacal Height (mm)	4.1 ± 0.4	2.3 ± 0.2	<0.0001*	1.8 ± 0.2	1.9 ± 0.1	0.1
Presence of Papillae (%)	63.6 ± 15.2	23.1 ± 12.2	0.01*	100.00	60.0 ± 24.5	0.05*

Results of linear regression analysis shown for each species
* Indicates significance (p < 0.05)

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Table 6.2 Percent of correct classification of sex for morphometric measurements and ultrasound against the hormone confirmation control

	Ambystoma tigrinum		Ambystoma laterale		Both Species	
	Male	Female	Male	Female	Male	Female
	N=8	N=8	N=6	N=8	N=14	N=16
Morphometric Measurements	73.0 ± 14.1^a	76.9 ± 12.2^a	83.3 ± 16.7^a	80.0 ± 0.0^a	76.5 ± 6.0^b	77.8 ± 3.9^b
Ultrasound	91.1 ± 9.1^a	100.0 ± 0.0^a	100.0 ± 0.0^a	100.0 ± 0.0^a	94.1 ± 5.9^a	100^a
Hormone Confirmation	100.0 ± 0.0^a	100.0 ± 0.0^a	100.0 ± 0.0^a	100.0 ± 0.0^a	100.0 ± 0.0^a	100^a

[ab]: Values with different letter superscripts within a column are significantly different from one another ($p < 0.05$)

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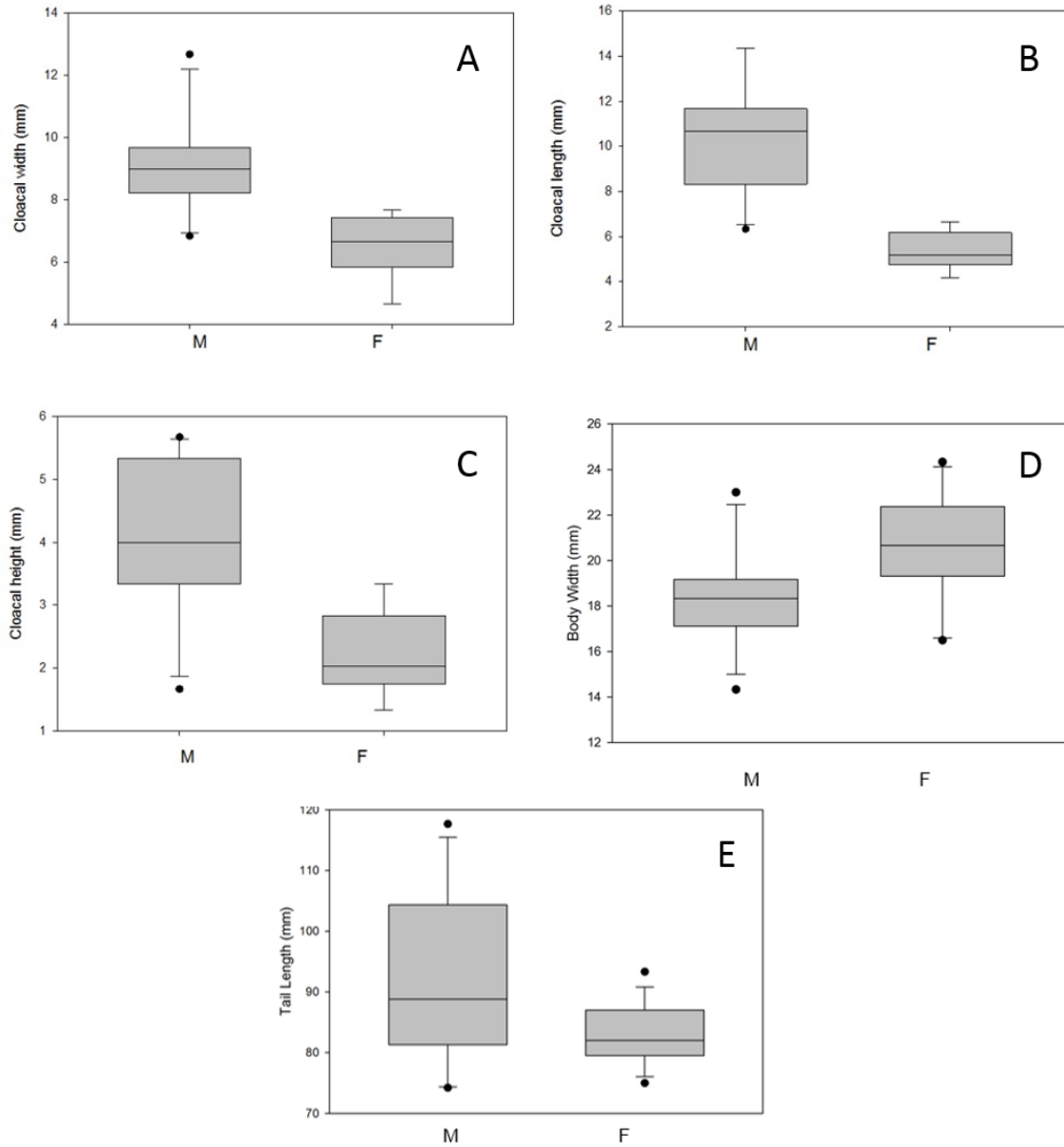
Figure 6.4   Box plots of significant differences in morphometric measurements
             between males and females of *A. tigrinum*

(A) cloacal width); (B) cloacal length; (C) cloacal height; (D) body width; and (E) tail
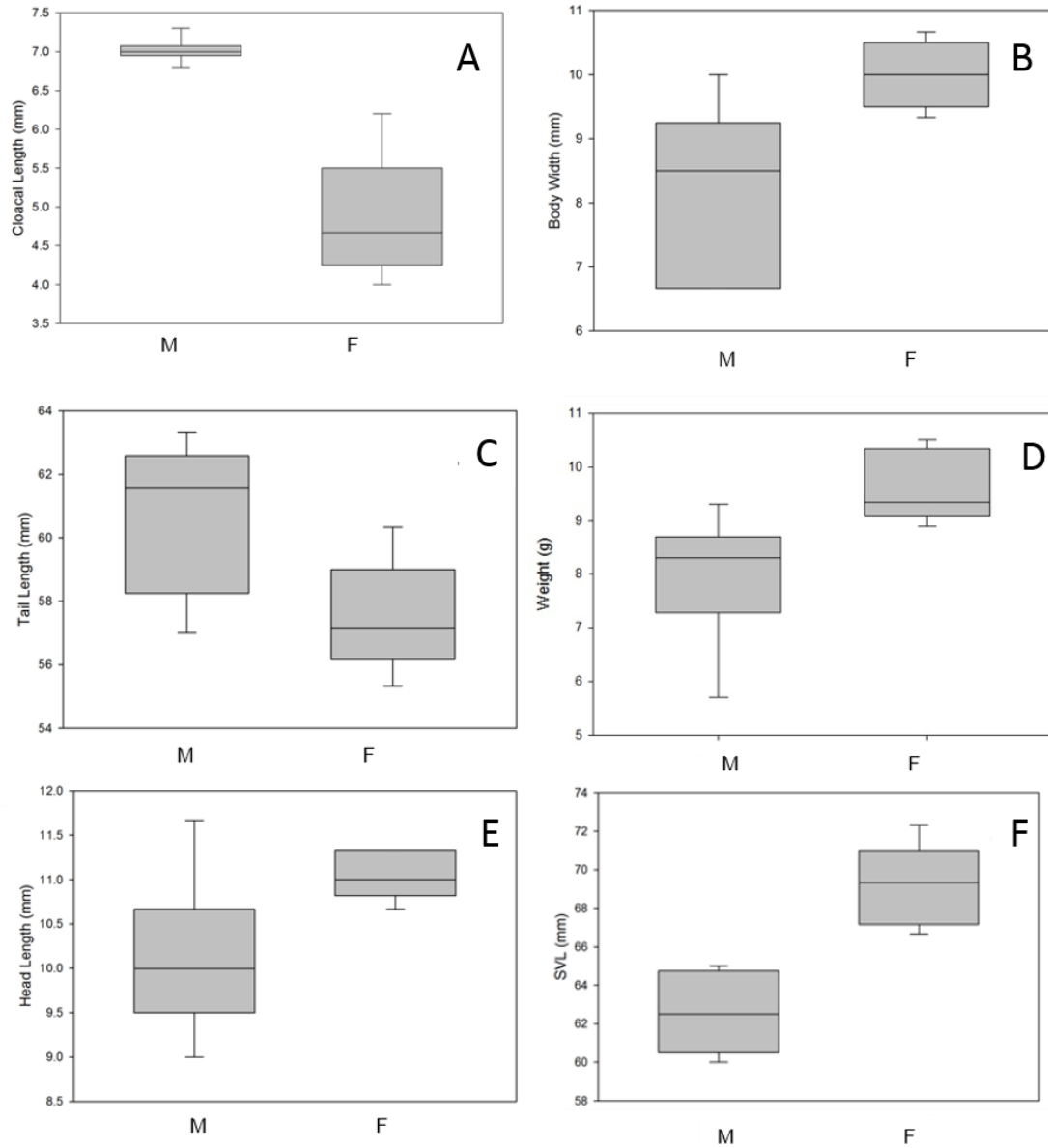length
*Black dots signify outliers

Figure 6.5   Box plots of significant differences in morphometric measurements
               between males and females of *A. laterale*

(A) cloacal length; (B) body width; (C) tail length; (D) weight; (E) head length; and (F)
SVL

Discussion

Sexing animals using morphometric parameters has been a common practice for

years, yet other methodologies such as ultrasound and hormone induced gamete

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production are expected to more be accurate, although these approaches require more sophisticated equipment and training and are can be more invasive. Here we examined the differences in the overall reliability of these methodologies to determine animal sexes in two caudate species, the common eastern tiger salamander and the regionally endangered blue spotted salamander. Although hormone induced gamete production of sperm or eggs gives definitive sexual identification of an animal, this procedure is ideally reserved for the end goal of obtaining gametes for assisted reproduction, rather than as a screening method for initially determining animal sex. This is especially true for species which exhibit a single reproductive event per year, as inducing gametes for sexing purposes interferes with the possibility of successful managed breeding. Therefore, developing and measuring the reliability of alternative, non-invasive sexing methodologies is paramount to optimizing successful breeding attempts through animal pairing, gamete development, gamete synchronization and overall assisted reproductive efforts.

Most caudates do not have dimorphic qualities such as the nuptial pads that develop in many anuran species and the papillae, which are a weak dimorphic characteristic in many species of salamander [Sever, 1992], are either sometimes present in females or mistakenly identified in a swollen female cloaca. Thus, visually sexing caudates may rely on taking other measurements, which can be variable based on many environmental factors including age, diet, and annual rainfall [Adams & Rolf, 2000; Blouin & Brown, 2000; Sotiropoulos et al, 2008; Walls et al 1, 1993; Walls et al 2, 1993]. This makes morphometric measurements understandably variable.

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Overall, sexing an animal using ultrasound or morphometric parameters produced similarly reliable results within each species. The accuracy of the parameters when sexing animals correctly for both species showed morphometric sexing was generally lower than ultrasound sexing by about 20% in males and up to 13% in females. Although this trend was observed from the current data, it is not significant in either single species with the limited numbers of animals which data was collected on, although a larger sampling of animals across species may yet yield significant differences in the two techniques. As such, the trade off in accuracy for animal sexing using easily obtained morphometric data may be reliable enough for breeding attempts where an ultrasound system and expertise in its use is not readily available. However, ultrasound sexing was nearly as accurate as hormone induced gamete production, especially in females from both species, and in over 90% of males.

Whereas the combined morphometric data yielded similar accuracy to ultrasound and gamete production in sex determination of salamanders, the individual morphometric parameters did not. The only morphometric characteristic that gave consistently good results in sexing animals in either species was cloacal length. The other parameters typically relied on for sexing animals are tail length, body width, and presence of papillae, were inconsistent in the classification of males and females. Parameters like weight, cloacal width, and cloacal height were highly accurate in one species or the other but not in both species simultaneously. Although, it is beneficial to have sexual dimorphism within a species, specific parameters that differentiate sexes are not generalizable across the genera.

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Two of the morphometric measurements were consistent in their directional significance between males and females across species. These measurements were tail length (males > females) and cloacal length (males > females). Relying on consistent features to sex animals over multiple species is both more efficient and empirical than using multiple and inconsistent methods. On average, *A. tigrinum* males had measurements with values that were greater than those of females. This was consistent with previous reports of cloacal measurements and tail length in Ambystomids, but was contrary to reports of body width [Shine, 1979; Todd & Davis, 2007]. It is normally expected that female caudates will be wider than males, as they will either be gravid and filled with eggs or will be storing fat in order to begin producing eggs [Duellman & Trueb, 1994;Vitt & Caldwell, 2009]. It is possible that the contrary finding of females thinner than males was a result of the body width in this group of *A. tigrinum* was artificially altered due to the females having been repeatedly induced to oviposit for experimental purposes. In the event that this caused altered results, these females would have been thinner than expected. Head width was not significantly different for either *A. laterale* or *A. tigrinum*.

Ultrasound was an efficient and accurate method to determine sex in Ambystomid salamanders , and for trained personnel ultrasound was subjectively easier to perform than morphometric measurements. Because the animal was only restrained for approximately one minute for ultrasound, it was likely less stressful than morphometric measurements which on average required handling the animal for close to three minutes However, the sex of an amphibian can be very difficult to determine, even by ultrasound, if it is not a gravid female because non-gravid females can be interpreted as males when

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no eggs are present. Furthermore, amphibian male organs are extremely difficult to detect on ultrasound because they are small and lacking fluid filled features such as eggs, making sex determination difficult even for the trained sonographer.

Of the two methods utilized for sexing in this study, both appeared to be suitably accurate and efficient for the sexing of Ambystomid salamanders for certain situations. While ultrasound may be more accurate in given circumstances, it is a more expensive tool that requires additional training to perform. Multiple morphometric measurements, used individually and in combination were found to be as accurate as ultrasound in determining animal sex in these two species and are comparable to gamete production by hormone induction

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References

[1]   Adams DC, & Rohlf FJ. Ecological character displacement in Plethodon: biomechanical differences found from a geometric morphometric study. Proceedings of the National Academy of Sciences of the United States of America. 2000; 97(8): 4106–11. http://doi.org/10.1073/pnas.97.8.4106

[2]   Alcorn MA, Deitloff J, Graham SP, Timpe EK. Sexual Dimorphism in Head Shape, Relative Head Width, and Body Size of Eurycea Aquatica and Eurycea Cirrigera. Journal of Herpetology. 2013; 47(2): 321–27. doi:10.1670/11-317.

[3]   Baillie J, Hilton-Taylor C, and Stuart SN. *2004 IUCN Red List of Threatened Species: A Global Species Assessment*. IUCN, 2004.

[4]   Bakkegard, KA & Guyer C. Sexual Size Dimorphism in the Red Hills Salamander, Phaeognathus Hubrichti (Caudata: Plethodontidae: Desmognathinae). Journal of Herpetology. 2004:38(1): 8–15. doi:10.1670/145-02A.

[5]   Blouin MS, & Brown ST. Effects of temperature-induced variation in anuran larval growth rate on head width and leg length at metamorphosis. Oecologia. 2000; 125(3), 358–361. http://doi.org/10.1007/s004420000458

[6]   Bruce, RC. Sexual Size Dimorphism in Desmognathine Salamanders. Copeia. 1993;2: 313–18. doi:10.2307/1447131.

[7]   Cox R, Butler M , John-Alder H. 2007. The evolution of sexual size dimorphism in reptiles. *Sex, Size and Gender Roles: Evolutionary Studies of Sexual Size Dimorphism*, 38–49. http://doi.org/:10.1093/acprof:oso/9780199208784.001.0001

[8]   Clulow J., Trudeau VL, & Kouba AJ. Amphibian declines in the twenty-first century: why we need assisted reproductive technologies. *Advances in Experimental Medicine and Biology*. 2014; 753: 275–316. http://doi.org/10.1007/978-1-4939-0820-2_12

[9]   Duellman DE, Trueb L. Biology of Amphibians. Baltimore USA: John Hopkins University Press; 1994.

[10]   Gascon C, Collins JP, Moore RD, Church DR, McKay JE, Mendelson J. (2005). Amphibian Conservation Action Plan. In IUCN/SCC Amphibian Conservation Summit 2005.

[11]   Germano J, Cree A, Bishop P. Ruling out the boys from the girls: Can subtle morphological differences identify sex of the apparently monomorphic frog, Leiopelma pakeka? New Zealand Journal of Zoology. 2011; 38:161-71.

[12]   Griffiths, RA, & Pavajeau, L. Captive breeding, reintroduction, and the conservation of amphibians. Conservation Biology: The Journal of the Society for Conservation Biology. 2008; 22(4): 852–861. http://doi.org/10.1111/j.1523-1739.2008.00967.

[13]   Hasumi, Masato. Age, Body Size, and Sexual Dimorphism in Size and Shape in Salamandrella Keyserlingii (Caudata: Hynobiidae). Evolutionary Biology. 2010; 37(1): 38–48. doi:10.1007/s11692-010-9080-9.

[14]   Kouba, A. J., & Vance, C. K. Applied reproductive technologies and genetic resource banking for amphibian conservation. Reproduction, Fertility, and Development, 2009; 21(6): 719–737. http://doi.org/10.1071/RD09038

[15]   Kramer L, Dresser B, Maruska E. Sexing aquatic salamanders by laparoscopy. In.; 1983; 193-4. Proceedings Am Assoc Zoo Vet Ann Meeting San Diego, CA.

[16]   Li P, Zhu B, Wang Y, Xiang X. Sex identification of Chinese giant salamander (Andrias davidianus) by Doppler B-ultrasound method. Journal of Biology. 2010; 1.

[17]   Miller RE, Fowler ME. Fowler's Zoo and Wild Animal Medicine. St. Louis, MO: Saunders-Elsevier; 2012.

[18]   Ollivier L, Welsh Jr. HH. Determining sex and life stage of Del Norte salamanders from external cues. Northwest Nat 2003;84:129–34.

[19]   Parker, GA. The Evolution of Sexual Size Dimorphism in Fish. Journal of Fish Biology. 1992;41: 1–20. doi:10.1111/j.1095-8649.1992.tb03864.

[20]   Pauly GB, Piskurek O, Shaffer HB. Phylogeographic concordance in the southeastern United States: The flatwoods salamander, Ambystoma cingulatum, as a test case. Molecular Ecology. 2007;16(2), 415–429. http://doi.org/10.1111/j.1365-294X.2006.03149.

[21]   Raymond LR & Hardy LM. Demography of a Population of Ambystoma Talpoideum (Caudata: Ambystomatidae) in Northwestern Louisiana. Herpetologica. 1990;46(4): 371–82.

[22]   Reyer H-U, Bättig I. Identification of reproductive status in female frogs-A quantitative comparison of nine methods. Herpetologica. 2004; 60:349-57.

[23]   Romano A., Bruni G., & Paoletti C. Sexual dimorphism in the Italian endemic species Salamandrina perspicillata (Savi, 1821) and testing of a field method for sexing salamanders. Amphibia-Reptilia. 2009; 30(3):425–434. http://doi.org/10.1163/156853809788795128

[24]   Roth T, Obringer A. Amphibian Reproduction. In Edited by Holt W, Pickard A, Rodger J, Wildt D. Reproductive Science and Integrated Conservation. Cambridge, UK: Cambridge University Press; 2009. 359-74

[25]   Sever, DM. Comparative Anatomy and Phylogeny of the Cloacae of Salamanders (Amphibia: Caudata). Herpetologica. 1992;48(3): 318–29.

[26]   Sever DM, Rania LC, Krenz JD. Reproduction of the Salamander Siren intermedia Le Conte With Especial Reference to Oviducal Anatomy and Mode of Fertilization. Journal of Morphology, 1996:227, 335–348. http://doi.org/10.1002/(SICI)1097-4687(199603)227:3<335::AID-JMOR5>3.3.CO;2-E

[27[Shine, R. Sexual Selection and Sexual Dimorphism in the Amphibia. Copeia 1979;2: 297–306. doi:10.2307/1443418.

[28]   Siegel DS, Sever DM, Aldridge RD. The pelvic kidney of male Ambystoma maculatum (Amphibia, urodela, ambystomatidae) with special reference to the sexual collecting ducts. Journal of Morphology. 2010:271(12), 1422–39. http://doi.org/10.1002/jmor.10883

[29]   Sotiropoulos K., Legakis A., & Polymeni R. M. Patterns of morphometric variation in the alpine newt (Mesotriton alpestris) at the southern limit of its distribution: environmental correlates. Integrative Zoology. 2008; 3, 123–133. http://doi.org/10.1111/j.1749-4877.2008.00074.

[30]   Todd BD & Davis AK. Sexual Dichromatism in the Marbled Salamander, Ambystoma Opacum. Canadian Journal of Zoology. 2007; 85(9): 1008–13. doi:10.1139/Z07-082.

[31]   Vitt LJ, Caldwell JP. Herpetology. 3rd ed. Singapore China: Elsevier; 2009.

[32]   Walls SC, Belanger SS, and Blaustein AR. Morphological Variation in a Larval Salamander: Dietary Induction of Plasticity in Head Shape. Oecologia. 1993; 96: 162-168.

[33]   Walls SC, Beatty JJ, Tissot BN, Hokit DG, & Blaustein AR. Morphological Variation and Cannibalism in a Larval Salamander (Ambystoma-Macrodactylum-Columbianum). Canadian Journal of Zoology-Revue Canadienne De Zoologie. 1993;71(8): 1543–1551. http://doi.org/Doi 10.1139/Z93-218

[34]   Williams RN, Gopurenko D, Kemp KR, Williams B, DeWoody JA. Breeding Chronology, Sexual Dimorphism, and Genetic Diversity of Congeneric Ambystomatid Salamanders. Journal of Herpetology. 2009; 43(3): 438–449. http://doi.org/10.1670/08-034R2.1

APPENDIX A

PRELIMINARY TRIALS FOR INDUCTION OF SPERMIATION IN TIGER

SALAMANDERS (*AMBYSTOMA TIGRINUM*) USING

EXOGENOUS HORMONES

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Materials and Methods

      Preliminary hormone trials were conducted for induction of spermiation in male eastern tiger salamanders. Twenty treatments were designed (see table A.1) based on previous studies in anurans and caudates. Five animals were treated with each hormone protocol and sperm was collected and assessed using methods described in *Chapter II*. From the results of these experiments, treatments were selected for further study; treatments selected, with their component parts, are discussed in *Chapter II*.

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Table A.1   Preliminary 20 hormone treatments for induction of spermiation in male
eastern tiger salamanders

Hormone	Code	Treatment
hCG	H1	100 IU hCG
	H2	200 IU hCG
	H3	300 IU hCG
	H5	500 IU hCG
	PH	Prime* 150 IU hCG Dose** 500 IU hCG
LHRH	L1	0.025 µg/g LHRH
	L2	0.05 µg/g LHRH
	L3	0.1 µg/g LHRH
	L4	0.25 µg/g LHRH
	L5	0.5 µg/g LHRH
	PL1	Prime* 0.025 µg/g LHRH Dose** 0.1 µg/g LHRH
	PL2	Prime* 0.05 µg/g LHRH Dose** 0.25 µg/g LHRH
	PL3	Prime* 0.05 µg/g LHRH Dose**0.1 µg/g LHRH
	PL4	Prime* 0.1 µg/g LHRH Dose** 0.25 µg/g LHRH
Combination	C1	100 IU hCG + 0.05 µg/g LHRH
	C2	200 IU hCG + 0.025 µg/g LHRH
	C3	100 IU hCG + 0.025 µg/g LHRH
	C4	200 IU hCG + 0.05 µg/g LHRH
	PC1	Prime* 100 IU hCG Dose ** 0.05 µg/g LHRH
	PC2	Prime* 200 IU hCG Dose** 0.025 µg/g LHRH

* Prime (priming dose) is given 24 hours prior to the dose (spermiation dose)
**Dose (spermiation dose) is given 24 hours following the prime (priming dose)

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Table A.2   Percent animals responding to respective hormone treatments in preliminary trials via milt, spermatophores, or both

Treatment	Animals responding to treatment (n = 5)	% Animals producing milt*	% Animals producing spermatophores**	% Animals producing milt and spermatophores
H1	60^+	16.7	100	16.7
H2	60^+	100	16.7	16.7
H3	60^+	66.7	33.3	0
H5	60^+	66.7	66.7	33.3
PH	20	0	100	0
L1	60^+	40	60	40
L2	60^+	33.3	66.7	0
L3	40	0	100	0
L4	60^+	66.7	66.7	33.3
L5	100^+	80	80	60
PL1	100^+	80	40	20
PL2	60^+	66.7	100	66.7
PL3	80^+	75	50	25
PL4	80^+	75	50	25
C1	100^+	20	100	20
C2	100^+	40	100	40
C3	60^+	0	100	0
C4	100^+	100	20	20
PC1	60^+	100	66.7	66.7
PC2	80^+	75	50	25
PBS	0	0	0	0

+: Indicates a significant difference from the control (within the first column)
*Percent of animals producing milt is taken from the number of animals that responded to treatment by producing milt, spermatophores or both
** Percent of animals producing spermatophores is taken from the number of animals that responded to treatment by producing milt, spermatophores or both

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Table A.3   Mean sperm parameters for analysis of milt in preliminary trials

Treatment	Time to first milt (hr)	Volume (μL)	Conc. (sperm/mL)	% Abnormal sperm	% Motility		
					Total	Progressive	Non-Progressive
H1*	2.0	500.0^a	tltc	tltc	tltc	tltc	tltc
H2	18.2 ± 11.6^{ab}	370.0 ± 215.8^{ab}	$4.0 \times 10^7 \pm 4.0 \times 10^7$	9.3 ± 9.3	57.7 ± 11.8^{ab}	32.7 ± 5.6^a	25.0 ± 6.8
H3	4.0 ± 1.2^b	18.0 ± 3.6^d	$4.7 \times 10^6 \pm 3.0 \times 10^6$	tltc	tltc	tltc	tltc
H5	12.0 ± 4.7^{ab}	25.7 ± 5.9^{cd}	tltc	tltc	tltc	tltc	tltc
PH	<	<	<	<	<	<	<
L1	24.5 ± 23.5^{ab}	28.7 ± 11.3^{cd}	$1.9 \times 10^7 \pm 8.5 \times 10^6$	0.2 ± 0.2	48.2 ± 4.6^{ab}	29.7 ± 2.5^a	18.5 ± 2.1
L2	18.6 ± 5.6^a	75.0 ± 55.0^{bcd}	$1.7 \times 10^7 \pm 1.4 \times 10^7$	1.0 ± 1.0	17.0 ± 17.0^{bc}	14.5 ± 14.5^{ab}	2.5 ± 2.5
L3	<	<	<	<	<	<	<
L4	3.5 ± 2.5^b	16.3 ± 2.4^d	$2.1 \times 10^7 \pm 1.0 \times 10^7$	0.3 ± 0.3	15.3 ± 7.9^{bc}	13.7 ± 6.8^b	1.7 ± 1.2
L5	10.0 ± 17.3^{ab}	13.9 ± 6.2^d	$4.6 \times 10^6 \pm 2.2 \times 10^6$	0.0	43.0 ± 18.6^{abc}	25.0 ± 8.1	18.0 ± 12.5
PL1	1.0 ± 0.0^b	17.3 ± 0.0^d	$1.3 \times 10^7 \pm 3.1 \times 10^6$	1.6 ± 0.4	32.6 ± 5.0^a	21.8 ± 3.1	1.6 ± 0.4
PL2	1.0 ± 0.0^b	243.5 ± 106.5^{ab}	$2.0 \times 10^7 \pm 8.7 \times 10^6$	2.7 ± 0.7	36.2 ± 6.5^a	25.3 ± 6.5	25.3 ± 3.8
PL3	1.0 ± 0.0^b	21.0 ± 9.7^{cd}	tltc	tltc	tltc	tltc	tltc
PL4	16.3 ± 7.7^a	53.3 ± 3.3^c	$1.1 \times 10^7 \pm 3.3 \times 10^6$	3.0 ± 1.5	7.7 ± 2.8^c	5.7 ± 1.9^b	2.7 ± 1.5
C1*	12.0^a	20.0^d	6×10^6	7.0	35.0^b	20.0^{ab}	25.0
C2	1.0 ± 0.0^b	100.0 ± 80.1^{bc}	$2.1 \times 10^6 \pm 8.5 \times 10^5$	9.0 ± 2.3	25.0 ± 4.3^a	14.4 ± 2.1^b	10.6 ± 2.3
C3	<	<	<	<	<	<	<
C4	7.8 ± 0.44^{ab}	10.2 ± 4.1^d	$5.5 \times 10^7 \pm 2.5 \times 10^7$	2.3 ± 1.7	35.0 ± 6.6^b	28.8 ± 4.6^{ab}	6.3 ± 2.2
PC1	1.0 ± 0.0^b	52.5 ± 19.8^{bcd}	$1.3 \times 10^7 \pm 5.1 \times 10^6$	3.8 ± 1.7	33.5 ± 6.9^b	24.3 ± 3.9^{ab}	9.3 ± 3.3
PC2	6.9 ± 0.1^{ab}	150.0 ± 0.0^b	$2.0 \times 10^7 \pm 6.0 \times 10^6$	0.0	65.0 ± 4.2^a	9.3 ± 2.1^b	56.3 ± 12.8

[abc]. Values with different letter superscripts are significantly different from one another within a column ($p < 0.05$)

tltc: sperm concentration was too low in order to assess concentration, motility, or abnormalities

< Signifies no animals responded by production of milt

*H1 and C1 represent the data of only one animal

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Table A.4   Mean sperm parameters for analysis of spermatophores in preliminary trials

Treatment	Time to first spermatophore (hr)	Number of spermatophores	Concentration (sperm/mL)	% Abnormal sperm	% Total motility
H1	11.0 ± 11.8	1.9 ± 0.3	$8.9 \times 10^5 \pm 3.1 \times 10^5$	9.2 ± 1.5	1.6 ± 1.0
H2*	24.0	2.0	1.2×10^6	16.0	0.0
H3	13.0 ± 15.6	1.4 ± 0.2	$2.3 \times 10^6 \pm 9.5 \times 10^5$	10.6 ± 2.5	4.6 ± 4.2
H5	1.5 ± 0.7	1.3 ± 0.2	$2.3 \times 10^6 \pm 8.2 \times 10^5$	7.6 ± 2.4	11.3 ± 4.0
PH	1.0	1.7 ± 0.3	$1.0 \times 10^6 \pm 4.9 \times 10^5$	6.5 ± 1.6	1.4 ± 0.9
L1	35.3 ± 18.8	2.3 ± 0.5	$1.1 \times 10^7 \pm 1.1 \times 10^7$	9.0 ± 2.2	2.5 ± 2.5
L2	12.5 ± 11.5	2.8 ± 1.0	$5.9 \times 10^5 \pm 1.8 \times 10^5$	3.3 ± 1.0	1.9 ± 1.5
L3	36.0 ± 12.0	1.5 ± 0.3	$2.1 \times 10^4 \pm 3.8 \times 10^3$	10.7 ± 5.4	5.0 ± 5.0
L4	39.0 ± 9.0	2.0 ± 0.0	$1.3 \times 10^4 \pm 4.3 \times 10^3$	15.3 ± 6.5	3.3 ± 3.3
L5	15.8 ± 5.1	1.7 ± 0.3	$6.4 \times 10^5 \pm 3.1 \times 10^5$	9.1 ± 3.9	4.6 ± 2.8
PL1	26.0 ± 22.0	1.6 ± 0.4	$5.2 \times 10^5 \pm 3.0 \times 10^5$	3.8 ± 1.1	1.3 ± 0.9
PL2	3.0 ± 1.5	1.4 ± 0.3	$2.0 \times 10^6 \pm 1.6 \times 10^6$	5.0 ± 1.1	0.0 ± 0.0
PL3	25.5 ± 22.5	1.0 ± 0.0	2.0×10^7	0.0	5.0
PL4	27.0 ± 21.0	1.5 ± 0.5	$5.0 \times 10^7 \pm 5.0 \times 10^7$	19.5 ± 10.5	13.0 ± 13.0
C1	21.8 ± 10.7	3.6 ± 0.8	$1.0 \times 10^6 \pm 2.5 \times 10^5$	4.4 ± 0.9	5.3 ± 2.1
C2	21.4 ± 24.4	2.1 ± 0.4	$9.9 \times 10^5 \pm 3.4 \times 10^5$	7.3 ± 1.3	6.5 ± 1.8
C3	1.4 ± 0.2	1.5 ± 0.3	$2.5 \times 10^6 \pm 3.9 \times 10^5$	5.5 ± 0.8	8.7 ± 1.6
C4	0.3 ± 0.05	1.0 ± 0.0	$1.6 \times 10^6 \pm 2.3 \times 10^5$	12.7 ± 2.3	18.0 ± 7.1
PC1	1.2 ± 0.2	1.3 ± 0.3	$1.9 \times 10^6 \pm 5.6 \times 10^5$	5.5 ± 2.3	16.0 ± 6.3
PC2	24.0 ± 0.0	1.0 ± 0.0	$1.5 \times 10^6 \pm 6.7 \times 10^5$	1.5 ± 0.6	6.2 ± 2.7

tltc: sperm concentration was too low in order to assess concentration, motility, or abnormalities

*H2 represents the data of only one animal

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APPENDIX B

ADDITIONAL METHODS FOR TIGER SALAMANDERS

Hormone preparation and administration for males

The LHRH was prepared by reconstituting 1.0 mg of LHRH in 1.0 mL of sterile phosphate buffered saline (PBS; Sigma Aldrich, St. Louis MO, USA). This solution was then separated into 100.0 µL aliquots for storage at -80ºC. The hCG was prepared by reconstitution of lyophilized hCG (2500 IU) with sterile PBS (2.5 mL) and the prepared vial was stored at 4.0 ºC.

All hormones were administered subcutaneously with a 26 gauge needle over the epaxial muscles. All primed hormone protocols were administered so that the priming dose (prime) was given over the animals' right side epaxial muscles and the spermiation dose (dose) was given 24 hours later over the animals' left side epaxial muscles.

- Individual dose LHRH category: Prior to administration of the hormone, the necessary dose of LHRH solution would be pipetted from a prepared aliquot with a sterile pipette tip and mixed with 50 µL of sterile PBS. It would then be loaded into a sterile 1 mL syringe for administration.

- Individual dose hCG category: Prior to administration of the hormone, the necessary dose of hCG was loaded from the vial into a sterile 1 mL syringe for administration.

- Individual dose Combination category: Prior to administration, the necessary dose of hCG would be pipetted from the prepared vial into a sterile microcentrifuge tube with a sterile pipette tip. The necessary dose of LHRH would be pipetted from a prepared aliquot with a sterile pipette tip into the hCG in the microcentrifuge tube. The hormones would be mixed by pipetting the solution up and down 10 times. The hCG and LHRH solution in the bullet tube was then loaded into a sterile 1 mL syringe for administration.

- Primed LHRH category: Each dose was prepared for administration as in the individual dose LHRH category. The prime was immediately loaded into a syringe for administration while the prepared dose was stored at 4ºC for 24 hours until time of administration.

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- Primed Combination: The prime was prepared for administration as in the individual dose hCG category and loaded into a 1 mL syringe for administration. The vial was returned to storage at 4ºC. After 24 hours, the dose was prepared as in the individual dose hCG category and loaded into a 1 mL syringe for administration.

Collection of Milt

At each collection time point, any spermatophores observed in the water were gently removed from the container via transfer pipette and immediately placed on a glass slide for further analysis. The milt collection procedure was performed by picking up the animal and holding it such that the ventral side of the animal faced the collector. The cloaca was gently wetted with water and stroked for approximately 5-10 seconds in order to stimulate the animal. The animal was observed for sexual behaviors and cloacal puckering at this time (see *Appendix C: Physical and Behavioral Analysis*). After cloacal stimulation, firm massage of the sides of the animal. After 1-10 seconds of massaging, a thick, white milt would be produced from the cloaca and pipetted directly into a microcentrifuge tube in 10 µL increments. If the animal did not produce milt after approximately 15 seconds of massage, a small piece flexible 0.86 mm x 1.32 mm micro medical tube (Scientific Commodities Inc., Lake Harvest City AZ) was used as a catheter and gently inserted 2mm into the cloaca. and carefully slid in and out of the cloacal opening in order to stimulate milt production by massaging the animals' sexual sensory glands. Milt filled the catheter tubing, which was subsequently emptied into a sterile microcentrifuge tube. Milt was allowed to fill the catheter tube a maximum of three times before discontinuing catheterization.

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Hormone preparation and administration for females

Lyophilized hCG and synthetic LHRH ([des Gly ^{10} D-Ala^6] LHRH ethylamide acetate salt hydrate) were obtained from Sigma Aldrich, St. Louis MO, USA. The LHRH and hCG were prepared as described in section 2.2.2 in the Hormone preparation and administration subsection. Hormones were administered subcutaneously with a 26 gauge needle over the epaxial muscles. Priming doses given 24 hours prior to the ovulatory dose were administered over the animals' right side epaxial muscles and the ovulatory dose was given over the animals' left side epaxial muscles. For preparation of doses prior to administration:

- hCG priming doses: Prior to administration of the hormone, the necessary dose of hCG was loaded from the vial into a sterile 1 mL syringe for administration.

- Combination ovulatory dose: Prior to administration, the necessary dose of hCG would be pipetted into a sterile microcentrifuge tube. The necessary dose of LHRH would be pipetted into the hCG in the microcentrifuge tube. After mixing the hormones by pipetting the solution up and down 10 times, the solution was loaded from the tube into a sterile 1 mL syringe for administration.

Ultrasound Procedural Detailing

A Sonosite Micromaxx ultrasound machine, equipped with an HFL38x model (6-13 MHz) 38mm linear transducer, was used for performing ultrasounds. The frequency was set on the general setting, the depth was set at 2.7cm, and the gain was adjusted as needed for all animals of all species. The transducer was cleaned off with ethanol between animals that were not cohabitating and the entire machine was thoroughly cleaned between species. All ultrasounds were performed by the same individual who was trained in ultrasonography. A linear transducer is highly recommended over a

curvilinear transducer for clarity and definition of the follicles and oocytes. The frequency was placed on the general setting, and a depth of 2.7 cm was used for all animals. The breast and tissue setting was used for all animals and gain was adjusted as needed. For the ultrasound procedure, animals were held in dorsal recumbency and their abdomen was wetted thoroughly with dechlorinated, conditioned tap water for conductance. The linear probe was placed transversely across the widest part of the abdomen (Figure B.1). The probe was slowly angled or adjusted so that an even, transverse image of the abdomen and ovaries was obtained.

Animals were held in the palm of the investigator's hand, in dorsal recumbency, for ultrasound. The animal's ventrum was wetted thoroughly with dechlorinated and conditioned tap water or reverse osmosis water for use as a conductor. The transducer was laid transversely across the thickest part of the animal's abdomen in order to examine for ovaries in females and over the pelvic area in order to examine for the thickened collecting ducts entering the cloaca in males.

The method of looking at the collecting ducts was found preferable to observing for the discrete testicular organ or the Wolffian duct in these species. Some species of amphibians have a discrete testicle more cranial in the coelomic cavity, close to the liver. In the species discussed in this experiment, the discrete organ is either extremely small or poorly defined [Sever]. The Wolffian duct is retained in male salamanders and used to transport sperm to the mesonephric kidney. This long, tortuous tube becomes engorged in males that are producing sperm and can sometimes be spotted on ultrasound. However, the Wolffian duct is very difficult to differentiate from intestine, posing confusion to the amateur on ultrasound. Additionally, females also retain their Wolffian ducts and utilize

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them for urine transport [Sever[2], Siegel]. Of the identifying ultrasonic qualities for males, preliminary trials showed the thicker collecting ducts of males to be the most reliable identifier of quality. The ducts are usually seen in transverse cross-section, so will appear as intensely hypoechoic circles surrounded by a thickened hyperechoic membrane (Figure 5.2A; Figure 5.3A; Figure 5.4D). The thickness of the membrane and the diameter of the ducts will vary, dependent on reproductive activity.

Females were identified by observing ovaries within the coelomic cavity. Ovaries of tiger salamanders and blue spotted salamanders were identified by hyperechoic (light), defined, round oocytes, measuring 0.36 ± 0.03 to 3.36 ± 0.73mm, and depend on of development level (see table 3.1). The smaller oocytes were densely packed, with little to no hypoechoic (dark) area between them. The larger oocytes had large, evenly spaced regions of hypoechogenicity between them. The layman description that was used to describe ovaries in training sessions was that the oocytes appeared as "stars in a dark night sky" (Figure 5.2 B, Figure 5.3 B).

Figure B.1   Ultrasound probe placement

The ultrasound probe is placed so that it lies transversely across the widest part of the animals' abdomen.

APPENDIX C

BEHAVIORAL AND PHYSIOLOGICAL ANALYSIS OF *AMBYSTOMA TIGRINUM*

IN RESPONSE TO HORMONE TREATMENT

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Materials and Methods for evaluation of male *Ambystoma tigrinum* behavioral responses to hormone treatments

All procedures and measurements were taken alongside the treatments performed in *Chapter II: Development of Hormone Protocols fot the Induction of Spermiation in Male Tiger Salamadners (Ambystoma tigrinum)*. For additional information on these treatments and the hormones delivered to the animals, please see *Chapter II*.

Cloacal Measurements

Animal weight was recorded immediately prior to each hormone treatment. For the first 24 hours of the observation period during which sperm collections were attempted, the collector would measure the cloaca length, width, and height with calipers prior to collection of milt. At the end of each observation period (see *Chapter II, Section 2.2 Materials and Methods*), the rate of change over the first 24 hours of observation, in mm, was calculated for the cloacal width, length, and height.

Behavioral Assessment

After cloacal measurements were obtained, the cloaca was wetted with water from the animal's own container and stimulated by gentle stroking with a gloved index finger for approximately 5-10 seconds. The animal was observed for behavioral responses, including a tail raise, clasping of the finger with the hind limbs, and a waving of the tail in a "tail dance" courtship-like motion. Behavioral responses were rated, as noted in the table below (Table B.1). At the end of each observation period, the behavioral response ratings were added together and a mean was taken. The mean behavioral response rating

calculated was used as the animal's overall behavioral response rating for the hormone treatment.

Table C.1   Rating scale of behavior for *Ambystoma tigrinum* treated with hormone

Rating	Behavioral response
0	None
1	Tail Raise
2	Clasping of finger
3	Tail Dance

Physical Response Assessment

During the 5-10 seconds in which the animal was stroked, the animal was observed for physical responses including cloacal opening and a foam response. If the cloaca was opened, exposing pink tissue and puckering, this was noted and the measurement of the opening was taken using calipers. After the observation period, the mean amount of the opening of the cloacal for the entire period was recorder. If a foam response was observed, noted by a frothy, yellowish substance appearing at the cranial base of the cloaca, this was recorded as a binary "yes" or "no" variable. Foam response at the end of the observation period was recorded as a "yes" or "no," also noting whether animals produced sperm during the observation period.

Statistical Analysis

Simple logarithmic regression was used to assess the relationship between the binary variable of production of foam upon cloacal stimulation (foam response) with the predictor variables of behavior, rate of change in cloacal measurements, response*, milt response*, spermatophore response*, amount of milt produced (μL)*, concentration of

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milt produced (spermatozoa/mL)*, total motility of milt (%)*, non-progressive motility

of milt (%)*, progressive motility of milt (%)*, abnormal spermatozoa in milt (%)*,

number of spermatophores overall*, number of each grade of spermatophore*, total

motility of spermatophores (%)*, abnormalities of sperm in spermatophores (%)*, and

concentration of spermatophores (spermatozoa/mL)*. (Variables noted with an * are

described in *Chapter II*).

Results

There was no significant difference between treatments width of cloacal opening

(mm) or the rate of change in the width or height of the cloaca (mm/hr). There was a

significant difference between treatments regarding rate of change (mm/hr) in cloacal

length ($p=0.03$; $F=1.96$; $MSE=0.3$; $R^2=0.2$). The PC resulted in a significantly greater

rate of change in cloacal length than in eight of the other treatments (L1, L2, L4, L5, PL1,

PL2, C1, and C2).

There was a significant difference between treatments regarding behavioral

response ($p=0.001$; $F=3.4$; $rMSE=1.0$; $R^2=0.2$). Animals given H3, L3, L5, PL1, or PL2

gave a behavioral response more frequently than those treated with three of the other

treatments (H1, H2, and C2)

The logistic regression model was significant for foam and response to treatment

($\chi^2=41.5$; $p<0.001$; CI 95%: 5.1-21.3) with animals that produced a foam response on

cloacal stimulation being 10.4 times more likely to respond to treatment than animals that

did not produce a foam response. In looking at responses by producing spermatophores or

milt, the model for foam production and milt response was significant ($\chi^2=56.5$;

$p<0.0001$; CI 95%: 6.8-26.4) with animals producing foam being 13.4 times more likely

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to produce milt than animals that did not produce foam. The logistic model for foam response and spermatophore response, however, was not significant. Foam response was also associated significantly with behavioral response (χ^2=19.8; p<0.0001; 95% CI: 0.2-4.1).

Logistic regression showed a significant model for overall number of spermatophores and foam response (χ^2=7.4; p=0.006; 95% CI: 0.1-0.7). Animals producing a foam response were 0.3 times more likely to produce a higher number of spermatophores than those that did not produce a foam response. Similarly, the model for foam response and number of grade 3 (χ^2=5.1; p=0.02; 95% CI= 0.3-0.9) and grade 4 spermatophores was significant (χ^2=4.5; p=0.034; 95% CI: 0.1-0.9). An animal that produced a foam response was 0.5 times more likely to produce a grade 3 spermatophore, and 0.3 times more likely to produce a grade 4 spermatophore than one who did not produce a foam response on cloacal stimulation.

Logistic regression models for foam response and amount of milt (μL), concentration of milt (spermatozoa/mL), total motility of milt (%), non-progressive motility of milt (%), progressive motility of milt (%), abnormalities of spermatozoa in milt (%), number of grade 1 spermatophores, number of grade 2 spermatophores, number of grade 5 spermatophores, concentration of spermatophores (spermatozoa/mL), the total motility of spermatophores (%), and spermatozoa abnormalities in spermatophores (%) were not significant.

The model between spermatophore response and behavioral response was also significant (χ^2=9.5; p=0.002; CI 95%=1.5-2.0). Animals responding by spermatophore production were 1.5 times more likely to produce a behavioral response than animals that

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did not respond by spermatophore production. For response by production of milt, the logistic model with behavioral response was also significant (χ^2=35000; p<0.0001; CI 95%: 2.7-7.2). An animal that responded by production of milt was 4.4 times more likely to produce a behavioral response than those that did not respond by production of milt.

There was no significant relationship see on logistic regression between spermatophore response or milt response and rate of change in cloacal width (mm/hr), rate of change in cloacal height (mm/hr), or rate of change in cloacal length.

The model for response and behavioral response, however, was significant (χ^2=43.8; p<0.001; CI 95%: 3.4-9.8). Animals that responded to treatment were 5.8 times more likely to have a behavioral response than those that did not response to treatment.

No significance was seen in logistic models for response and rate of change in cloacal width (mm/hr), rate of change in cloacal height (mm/hr), or rate of change in cloacal length (mm/hr).

There was a significant relationship between the induced behavioral response and overall response to treatment, as well as milt production and sperm production. Hormones including sex steroids, prolactin, and arginine vasotocin are associated with courtship behavior in amphibians [Kim et al, 2013; Moore et al, 1992; Rose et al, 1995; Toyoda et al, 2004; Toyoda et al, 1996]. The role of gonadotropins in regulating sexual behaviors primarily appears to be in the regulation of the levels of the sex steroids [Moore et al, 2005; Moore et al, 1992]. Thus the hCG protocols likely induced behavioral effects in the male eastern tiger salamanders by increasing levels of circulating testosterones. While an increase of LHRH would also play some role in regulation of the sex steroids, there has been some implications of LHRH as a direct regulator of sexual

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behavior [Norris & Lopez, 2011; Moore et al, 2005], potentially playing a role in pheromone regulation [Norris & Lopez, 2011] Further investigation into the effects of the hCG and the LHRH doses used in this study on the neuroendocrinology of the animals is needed to fully understand the behavioral response seen. However, the behavioral response was a significant indicator to show that animals were likely to produce a general response, a response by production of milt, or a response by production of spermatophores. While further study is necessary to determine the implications of the behavioral responses that were elicited during this study, these responses may be used as an indicator to determine if a male salamander that has been injected with hormones is responding to the treatment and if it is likely to produce sperm. The production of foam upon cloacal stimulation was significantly related to the behavioral response, occurring more frequently in animals that also showed a behavioral response.

The production of foam was also a significant indicator of whether an animal would respond to treatment, as well as of the quality of the sperm that would be produced. The induction of a foam response upon stimulation of the cloaca was associated with an overall response to treatments, a spermatophore response, and a milt response. Foam response was significantly associated with higher numbers overall of spermatophores, and specifically of G3 and G4 spermatophores. It has been noted that males within some species of quail (genus *Coturnix*) are known to produce a foam from a proctodeal gland in their cloaca when they are ready to breed [Mohan et al, 2002; Mohan et al, 2005; Singh et al, 2010]. The biochemical composition of this foam has not been entirely characterized, but has been found to contain a composition of protein, enzymes common to blood serum, lactate mucopolysaccharides, transaminase, and phosphatase.

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The foam produced by the birds is recognized as playing a role in male fertility, enhancing sperm motility, and disaggregation of sperm [Mohan et al, 2002; Singh et al, 2010]. The foam that was seen produced from the male eastern tiger salamander cloaca in this study may have a similar function to that seen in quail species. Salamanders are recognized as having proctodeal glands [Norris & Jones, 1987], although the function and true classification of these glands has been disputed [Sever, 1991]. Further analysis of the content of the foam that was produced with these hormone protocols, and of the cloacal glands from which the foam originated, is warranted for a better understanding of the foam and its indications. Foam was also noted to be significantly associated with a behavioral response. As noted with the behavioral response, while a better understanding of these reactions is necessary to grasp the implications, they may be used in order to observe a male salamander's response to hormone treatment and to predict sperm response.

Table C.2   Mean behavioral and physiological parameters for the final thirteen hormone treatments and control PBS treatment

	Mean Behavioral Response	Cloacal Opening	Rate of chance cloacal width	Rate of change cloacal length	Rate of change cloacal height	Animals producing foam (%)*	Animals producing foam and sperm (%)*
H1	1.0 ± 0.07 [bc]	0.4 ± 0.09	-0.1 ± 0.1	0.06 ± 0.06 [ab]	-0.007 ± 0.06	20.0 [bc]	20.0 [bc]
H2	1.0 ± 0.07 [bc]	1.0 ± 0.08	0.007 ± 0.07	-0.007 ± 0.06 [ab]	-0.02 ± 0.06	26.7 [bx]	26.7 [bc]
H3	1.5 ± 0.07 [a]	0.7 ± 0.06	-0.04 ± 0.1	0.007 ± 0.1 [ab]	0.01 ± 0.07	40.0 [ab]	26.7 [bc]
L1	1.3 ± 0.07 [ab]	1.0 ± 0.08	0.008 ± 0.08	-0.05 ± 0.07 [b]	0.06 ± 0.07	46.7 [ab]	33.3 [abc]
L2	1.1 ± 0.07 [ab]	0.8 ± 0.07	-0.04 ± 0.06	-0.3 ± 0.2 [b]	0.0 ± 0.0	33.3 [abc]	20.0 [bc]
L3	1.4 ± 0.07 [a]	0.5 ± 0.06	-0.04 ± 0.07	0.02 ± 0.08 [ab]	-0.04 ± 0.8	53.3 [ab]	46.7 [ab]
L4	1.1 ± 0.08 [ab]	0.6 ± 0.06	0.04 ± 0.07	-0.07 ± 0.06 [b]	-0.04 ± 0.07	46.7 [ab]	46.7 [ab]
L5	1.4 ± 0.09 [a]	0.6 ± 0.06	0.003 ± 0.09	-0.05 ± 0.06 [b]	-0.003 ± 0.09	73.7 [a]	66.7 [a]
PL1	1.3 ± 0.06 [ab]	0.8 ± 0.07	0.02 ± 0.07	-0.03 ± 0.07 [b]	0.02 ± 0.07	73.4 [a]	66.7 [a]
PL2	1.3 ± 0.07 [a]	0.6 ± 0.07	-0.06 ± 0.08	-0.03 ± 0.05 [b]	0.03 ± 0.08	66.7 [ab]	60.0 [ab]
C1	1.3 ± 0.07 [a]	0.6 ± 0.06	-0.3 ± 0.07	-0.04 ± 0.07 [b]	-0.2 ± 0.3	53.3 [b]	33.3 [abc]
C2	0.7 ± 0.3 [c]	0.7 ± 0.07	-0.06 ± 0.07	-0.05 ± 0.06 [b]	0.007 ± 0.07	66.7 [ab]	40.0 [abc]
PC	1.1 ± 0.06 [ab]	0.4 ± 0.05	-0.03 ± 0.08	0.1 ± 0.1 [a]	-0.03 ± 0.07	53.3 [b]	46.7 [ab]
PBS	0.01 ± 0.009 [d]	0.02 ± 0.02	-0.008 ± 0.3	-0.02 ± 0.02 [ab]	0.04 ± 0.03	0.0 [c]	0.0 [d]

For list of treatment codes, see Table 2.2 in *Section 2.2.2*

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APPENDIX D

SHORT TERM COLD STORAGE OF TIGER SALAMANDER

(*AMBYSTOMA TIGRINUM*) SPERM

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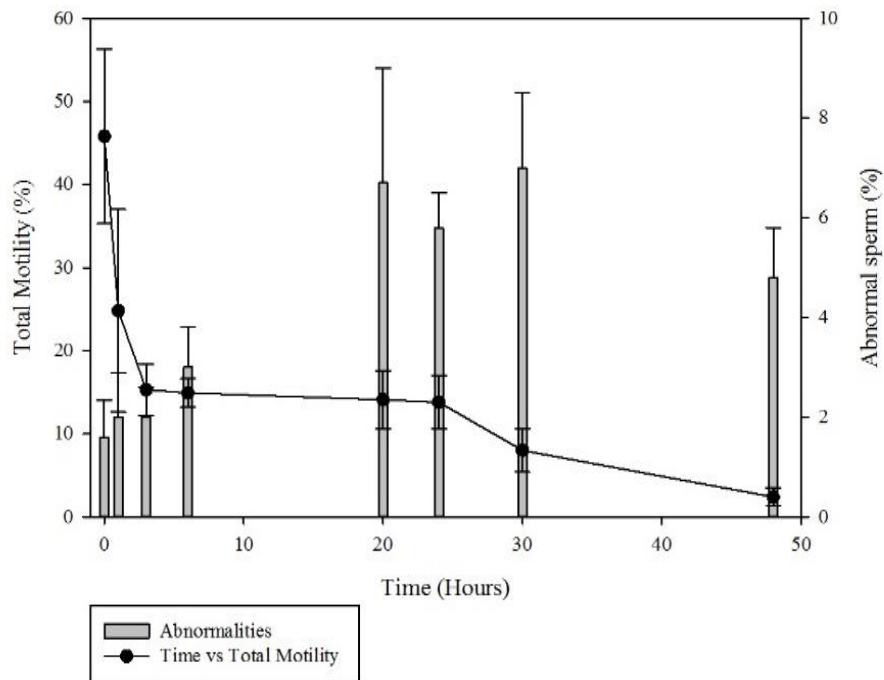
Figure D.1   Motility and abnormalities of fresh collected milt over the course of 48 hours when stored at 4ºC

Sperm was stored, starting at the collection point (time 0), at 4ºC for 48 hours. Missing data points accounted for by: multiple collections stopped moving prior to the termination of the 48 hour observation period; some collections had no visible motility at one time point and then would have visible motility again later. A collection would not be read as fully "immotile" until three subsequent readings had zero motility.

Materials and Methods

Milt was collected as described in *Chapter II*. Once collected and assessed, milt was stored at 4ºC for 48 hours in order to determine the longevity of sperm motility over this time period. Sperm was assessed every other hour for the first eight hours and then ever four hours for the remainder of the 48 hour period for motility and abnormalities. Assessment took place as described in *Section*
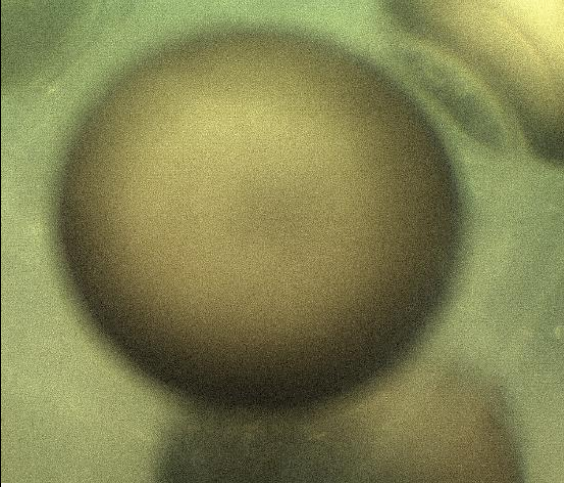
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APPENDIX E

DETAILED DEVELOPMENTAL STAGES OF THE EASTERN TIGER

SALAMANDER (*AMBYSTOMA TIGRINUM*)

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Materials and Methods
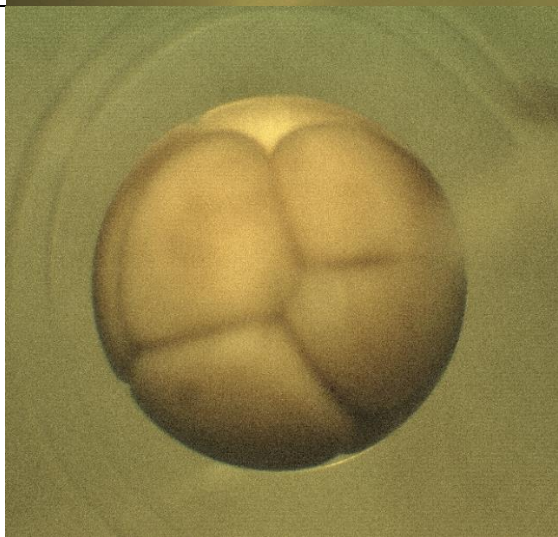
Embryos were produced via *in vitro* fertilization using methods described in *Chapter V*. Development of embryos was observed under a stereoscope both manually and digitally (via camera stop motion capture) such that they were observed every 2 hours during development for the first 48 hours and then every 6 hours thereafter. Stages were labeled and numbered similarly to those observed in *Ambystoma mexicanum* and *Ambystoma maculatum* by Harrison and by Shreckenberg.

Table E.1   Developmental staging of the eastern tiger salamander (*Ambystoma tigrinum)*

Stage	Hour Occuring Post IVF	Description	Image
1: No Cleavage	0-18	Fertilization is difficult to determine. Occasionally a spot at the animal pole (polar bodies) can be visualized, signifying reorganization of organelles	
2: 2 Cell	5-18	The cleavage furrow splits the embryo.	
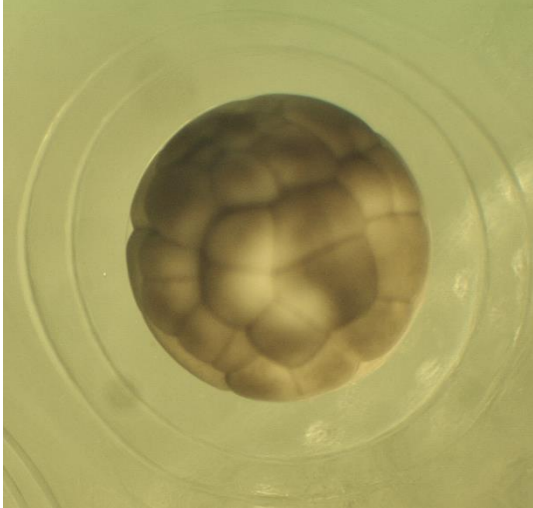
Stages 1 and 2; To 2 cell

Table E.1 (continued)

3: 4 Cell	8-21	A second cleavage furrow forms at a right angle to the first.	
4: 8 Cell	8-21	The cells are no longer evenly divided.	
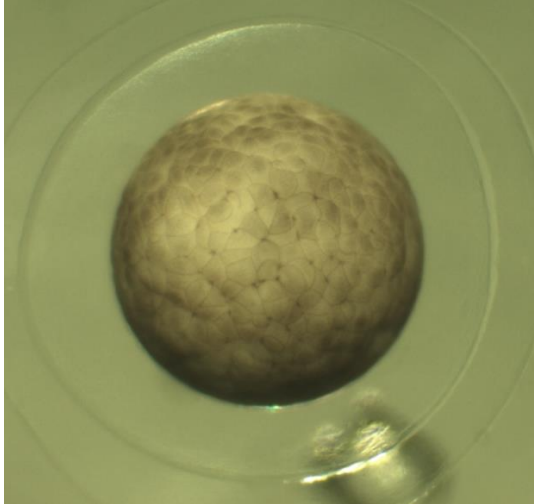
Stages 3 and 4; To 8 cell.

Table E.1 (continued)

5: 16 Cell	12-26	Sixteen cells crown the animal pole.	
6: 32 Cell	12-32	Approximately thirty two cells.	
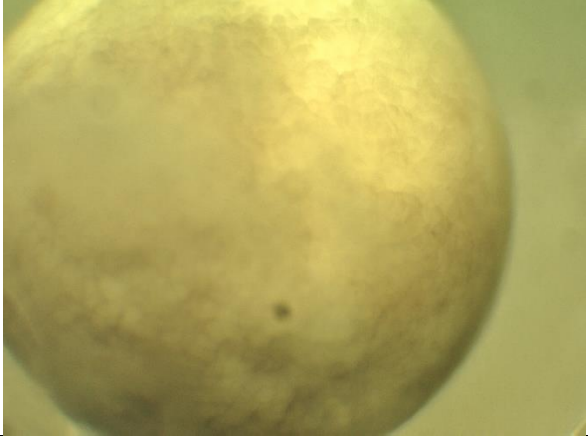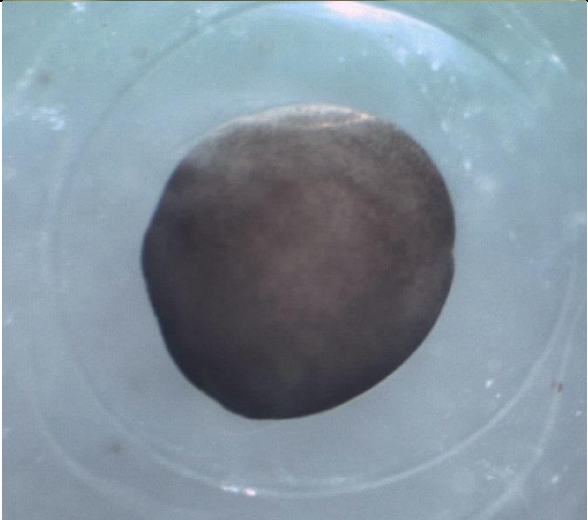
Stages 5 and 6; To 32 cell

Table E.1 (continued)

7: Early Blastula	17-36	Animal pole cells are no longer cleaving at the same time.	
8: Blastula	21-48	Innumerable small cells	

Stage 7 and 8; To Blastula

Table E.1 (continued)

9-10: Dorsal Lip Invagination and Involution	32-64	Invagination of the dorsal lip begins at the vegetal pole	
11: Blastopore	36-81	Lip forms a semi-circle, yolk can be seen touching lip	

Stages 9 to 11; To Blastopore

Table E.1 (continued)

12: Yolk Plug	48-98	Egg shape becomes slightly elongated; blastopore is complete, forming a circle around a yolk plug	
13-14: Early neurula; Neural plate formed	57-111	Egg is flattened dorsally and elongated anteriorly	

Stages 12 to 14; to Early Neurula

Table E.1 (continued)

15:Closing neural plate	61-127	The neural plate is formed, surrounded by the prominent neural ridge	
16-17	63-132	The neural folds begin to raise and close	

Stages 15 to 17; To closing of the neural folds

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18: Closing brain plate	63-146	The neural folds raise higher and the gap between them narrows	
19: Closed neural folds	64-157	The neural folds are lined up side by side, but not yet sealed	
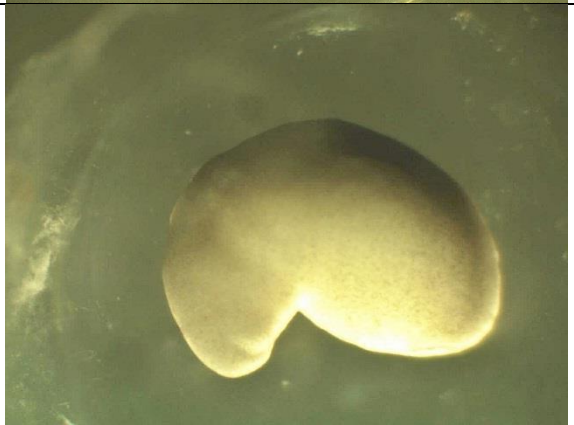
Stages 18 and 19; To closed neural folds

Table E.1 (continued)

20: Closing folds; beginning tail bud	66-171	The neural folds fuse	
21-23: Early tail bud	68-204	Folds are completely fused; the head becomes visible, along with the optic vesicles and the mandibular arch	

Stages 20 to 23; To Early Tail Bud

Table E.1 (continued)

24-25:	68-218	Head very prominent, branchial swelling appears	
26-27:	72-229	The body begins to elongate, the branchial swelling becomes more prominent	

Stages 24 to 27; to prominent branchial swellings

Table E.1 (continued)

28-30:	74-158	Pharyngeal pouches begin to show prominently; further elongation of the body; gill regions become more distinct	
31-32	72-268	Head begins to straighten pharyngeal pouches and gill regions more developed	

Stages 28-32; To Developing gill regions

Table E.1 (continued)

33-34	74-297	Head begins to straighten pharyngeal pouches and gill regions more developed; Tail straightens and elongates and heart develops	
35-36:	83-344	Pigment begins to appear; Gill sprouts become apparent; blood vessels develop	
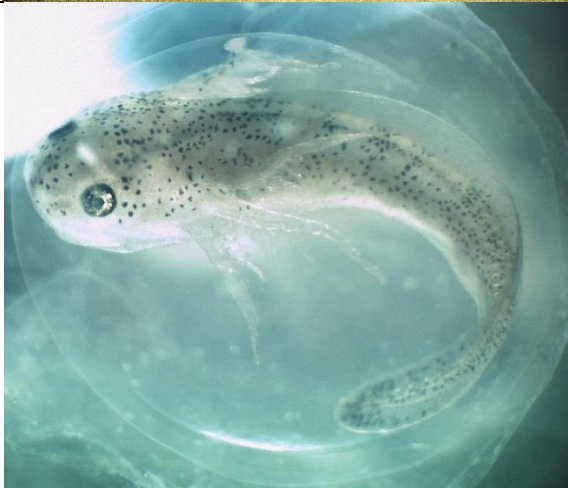
Stages 33 to 36; To blood vessel development

Table E.1 (continued)

37	86-382	Gills more prominent, increased pigment, increased blood flow	
38		Forelimb becomes visible; gills taper distinctly; mucus secretion begins	

Stages 37 and 38; To mucus secretion

Table E.1 (continued)

39:	91-391	The mouth becomes visible.	
40: Hatching	99-403	Gills are feathery with filaments; eye choroid is clear; embryo is prepared to hatch, mouth is opened	
45: Hatched	99-403	By stage 45, larvae is free swimming, has limb buds, yolk has been absorbed, and animal is eating	

Stages 39 to 45; To hatched

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References

[1]   Harrison, RG.Organization and Development of the Embryo. S. Wilens, ed., pp. 44-66. Yale Univ. Press, New Haven, CT:1969.

[2]   Schreckenberg GM, Jacobson AG. Normal stages of development of the axolotl. Ambystoma mexicanum. Developmental Biology 1975:42(2), 391–400. http://doi.org/10.1016/0012-1606(75)90343-7

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APPENDIX F

APPLICATION OF ASSISTED REPODUCTIVE TECHNOLOGIES DEVELOPED IN

EASTERN TIGER SALAMANDERS (*AMBYSTOMA TIGRINUM*) TO THE

THREATENED BLUE SPOTTED SALAMANDER

(*AMBYSTOMA LATERALE*)

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Introduction

Over the last decade, efforts have been made to increase captive breeding of threatened and endangered amphibians by the establishment of captive assurance colonies [Browne et al, 2006; Griffiths & Pavajeau, 2008; Obringer et al, 2000; Kouba & Vance, 2009]. Due to difficulty in replicating the very specific environmental cues needed for stimulation of breeding in these animals in a captive setting, these colonies frequently undergo failure [Griffiths & Pavajeau, 2008; Kouba & Vance, 2009; Obringer et al, 2000; Stoops & Campbell, 2014]. Because of the difficulty in simulating natural cues, captive assurance colonies are more commonly relying on assisted reproductive technologies (ART), including exogenous hormones and *in vitro* fertilization, in order to successfully breed animals. In order to ensure these technologies are safe for endangered species, they are frequently developed in model species that are not considered under threat. One of the many challenges of successfully developing ART is transferring protocols developed in model species to endangered species. Even when a protocol is developed in a species closely related to the target species, due to species specific differences the protocols may not successfully transfer. These protocols may need adapting to the target species. Successful transference and adapting to the target species is critical to the success of ART in captive assurance colonies.

In 2007, a captive assurance colony for the regionally threatened blue spotted salamander (*Ambystoma laterale*) was established between a partnership with the Iowa Department of Natural Resources and Omaha's Henry Doorly Zoo and Aquarium. In Iowa, *A. laterale* populations have declined and currently only breed at two temporal water bodies. A third temporal water body was selected for reintroduction of the species.

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For seven years, natural breeding of the captive *A. laterale* was attempted, but failed. In 2014, the partners decided to attempt ART in the blue spotted salamander captive assurance colony.

In these studies, protocols for the use of exogenous hormones to induce the deposition of gametes which had been developed in eastern tiger salamanders (*Ambystoma tigrinum*) were applied to the closely related, regionally endangered blue spotted salamander (*Ambystoma laterale*).

Materials and Methods

Experimental Animals

Eleven blue spotted salamanders, six males and five females, maintained at Omaha's Henry Doorly Zoo in their Amphibian Conservation Area were utilized in this study. Animals were maintained in small Waterland tubs, with moist cocofiber and recirculating RO water. Substrate was replaced monthly and cleaned as needed in between. Water was topped off as needed and a 20% water change was performed weekly. Temperature was maintained on a seasonal cycle. Animals were fed a variety of crickets, small worms, and flies five days a week which were cultured at the zoo and gut loaded with a nutritional diet.

Two animals, one male and one female, died during the course of the experimentation due to causes unrelated to experimentation. They were only used in the June 2014 trial and were not used in the remaining four trials. For the remainder of the trials, five males and four females were used. The data shown are raw data for these animals.

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Hormone induction of spermiation

Spermiation was induced in males by using one of two protocols described in *Chapter II*. The two protocols selected for use in the blue spotted salamanders were either the combination of 100 IU hCG and 0.05 µg/g LHRH or the single dose of 0.5µg/g LHRH. These treatments were replicated a total of four times: in June 2014, in November 2014, in March 2015, and in October 2015. In June 2014, the animals had not yet been sexed via ultrasound. Because it was unclear which animals were females prior to ultrasound sexing or determination of sex via gamete collection, all males also received a prime of 1 IU/g of hCG one week prior to the spermiation dose. Ultrasound sexing occurred three days after the priming dose and spermiation dose was delivered based on the sex noted on ultrasound (see *Chapter VI*). Sperm was collected and analyzed as described in *Chapter II*. Three out of the four spermiation attempts (June 2014, March 2015, and October 2015), all males produced sperm. Raw data from these three repetitions are displayed in figures F1-5. Figures F6-7 show a time curve of concentration and milt amount, respectively, collected over the course of 72 hours in June of 2014.

Hormone induction of oviposition

All females were treated with 1IU/g hCG one week prior to a prime of 2IU/g hCG followed 24 hours later by an ovulatory dose of 4IU/g hCG and 0.1µg/g LHRH. Attempts to induce oviposition were made four times, at the same time of year as listed for male salamanders above. In June of 2014, sex of females was unknown. The initial 1 IU/g of hCG prime was given prior to ultrasound sexing and ovulatory dose was administered based on sex determined via ultrasound (See *Chapter VI*). Females were observed via ultrasound for follicular development and eggs were collected following procedures

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described in *Chapter IV*. The grading scale for female *A. laterale* can be seen in Table F.2 and compared to that of *A. tigrinum* in Table 4.1. Only at one of the four attempts to hormonally induce oviposition in *A. laterale* did the females oviposit eggs. Raw data from this attempt (June 2014) is shown in Figure F.8.

In vitro fertilization

Procedures for *in vitro* fertilization were identical to those in *Chapter V*. Fresh sperm collected from *A. laterale* was very low in concentration and was not diluted prior to depositing onto eggs. Frozen-thawed sperm was used from *A. tigrinum* when fresh sperm was not available,. As this is a species that naturally undergoes kleptogenesis, these attempts were made in hopes that parthenogenesis would be induced by activation of the *A. laterale* eggs via the *A. tigrinum* sperm.

As previously mentioned, four attempts were made to hormonally induce oviposition in female blue spotted salamanders, and only at one of these attempts did the females oviposit eggs. Therefore, as eggs were only present in one out of the four trials, IVF was only attempted in one out of four trials. Raw data from this attempt (June 2014) is shown in table F.1.
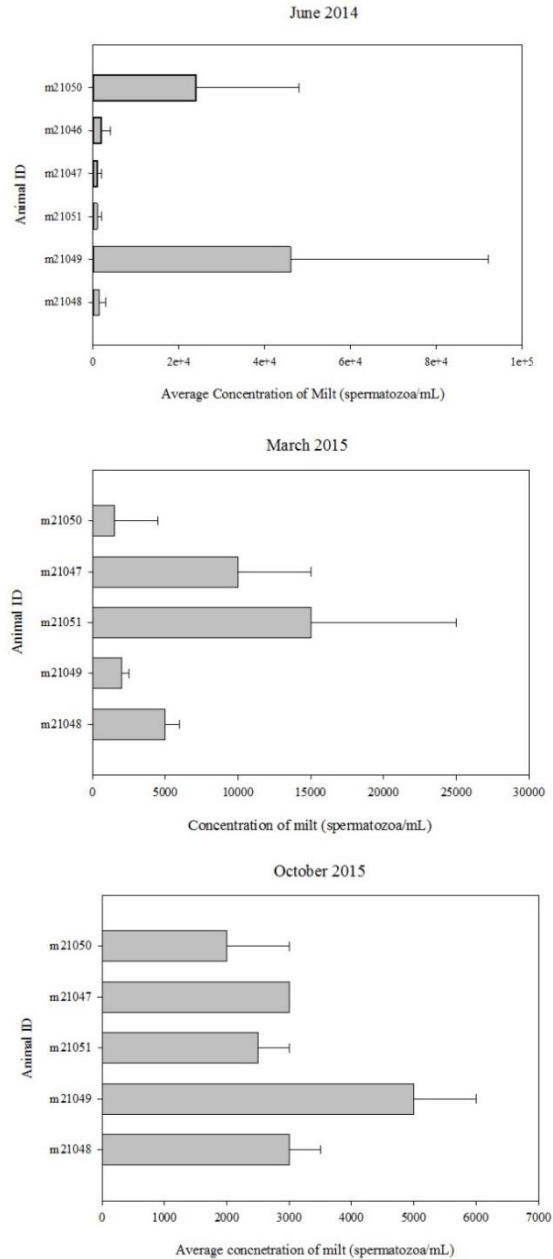
Figure F.1   Average concentration (±SEM) of milt produced by each male *Ambystoma
laterale* in June of 2014, March of 2015, and October of 2015

Each animal produced sperm collected at time points over the course of 72 hours. The
average concentrations for each trial are shown here. These concentrations were notably
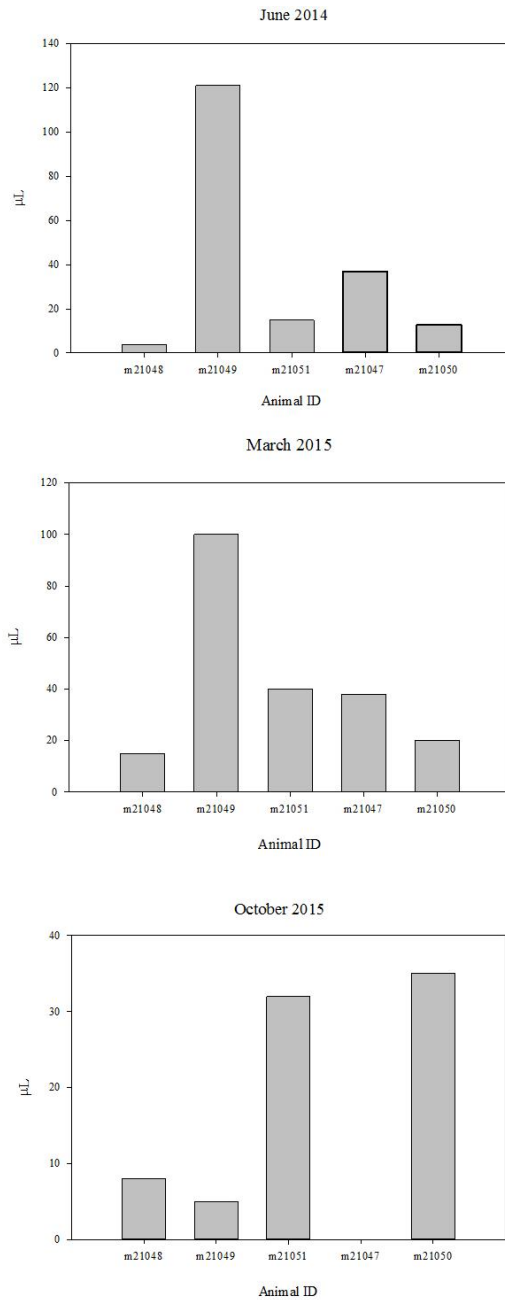lower than those seen in *A. tigrinum*.

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Figure F.2   Total amount of milt collected from each male *Ambystoma laterale* for
June 2014, March 2015, and October 2015

Each animal produced sperm which was collected at time points over the course of 72
hours. The total amount of milt collected for each trial is shown here.

Average concentration of milt produced per treatment
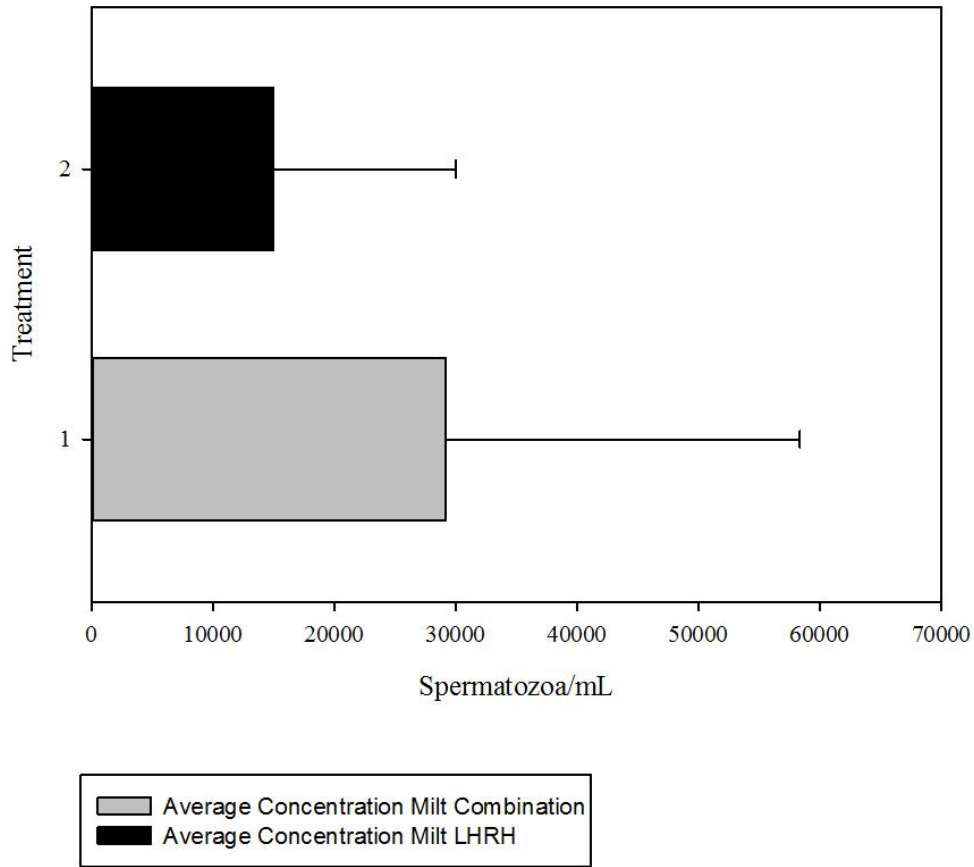
Figure F.3   Average concentration (±SEM) of milt collected for each treatment in *Ambystoma laterale* for June 2014, March 2015, and October 2015

This compares the average concentrations collected for each treatment (Combination: 100 IU hCG + 0.05 μg/g LHRH; LHRH: 0.5μg/g LHRH) for a single collection. While these concentrations are not significantly different, the LHRH produced milt with lower concentrations overall than the combination.

Average amount of milt collected per treatment
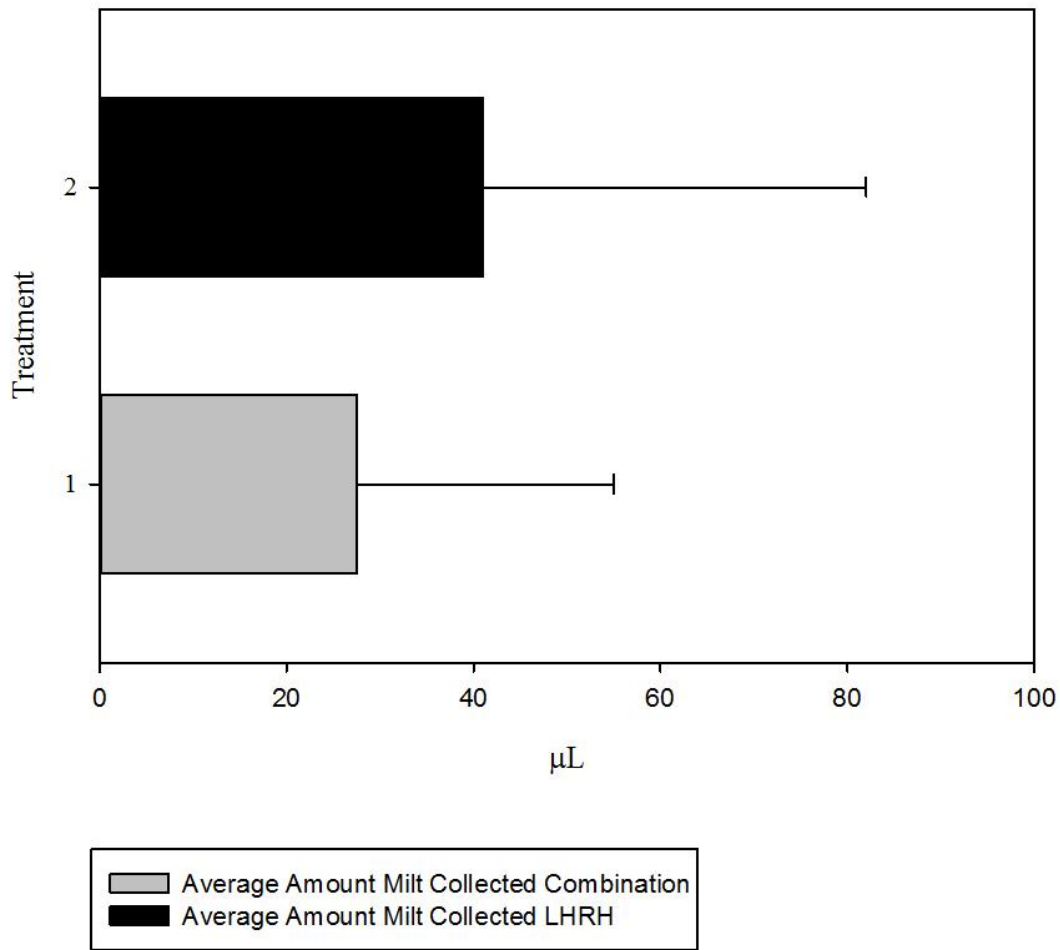
Figure F.4   Average amount of milt collected (±SEM) for each treatment in male *Ambystoma laterale* for June 2014, March 2015, and October 2015

This compares the average amount of milt collected for each treatment (Combination: 100 IU hCG + 0.05 µg/g LHRH; LHRH: 0.5µg/g LHRH) for a single collection. While these amounts are not significantly different, the LHRH produced milt with lower amounts overall than the combination.

March 2015
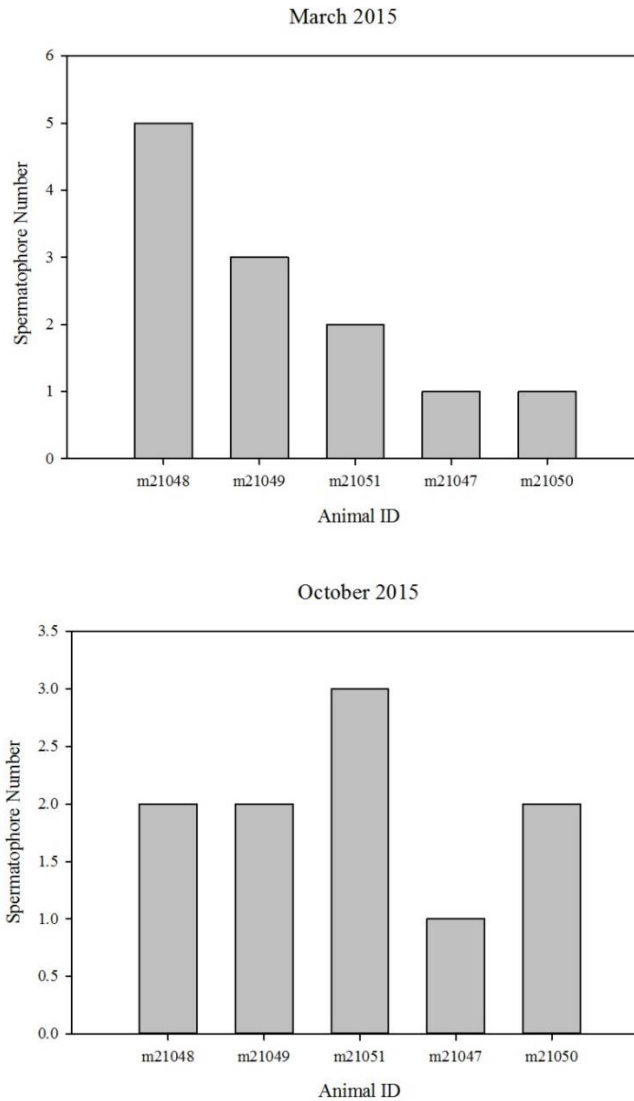
October 2015

Figure F.5   Total number of spermatophores collected from each male *Ambystoma laterale* for March 2015, and October 2015

Note: no spermatophores were collected in June 2014
Each animal produced sperm which was collected at time points over the course of 72 hours. The total amount of spermatophores collected for each trial are shown here.
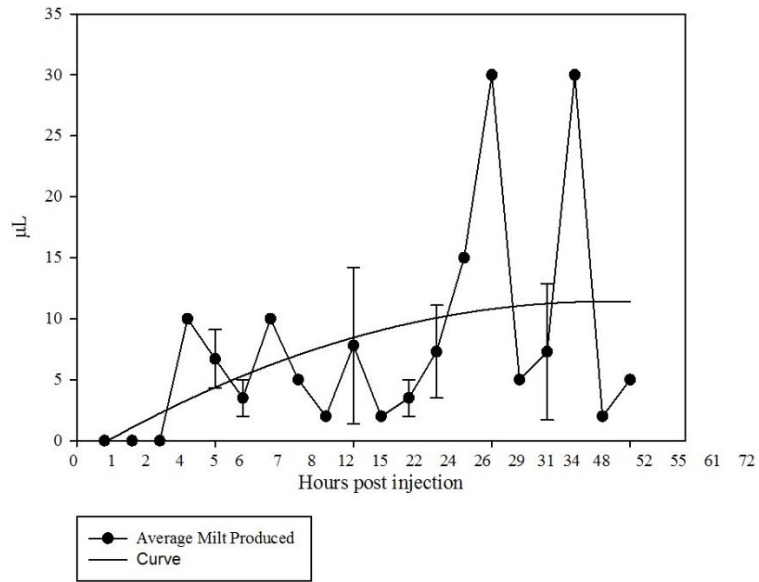
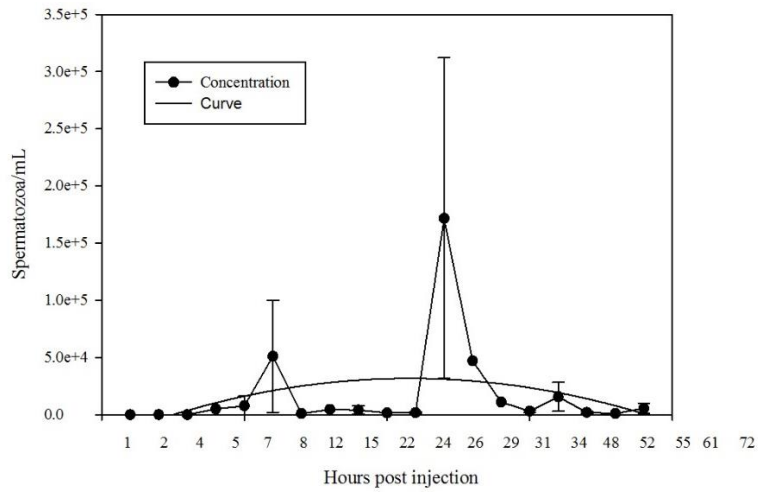Figure F.6   Amount of milt produced, in µL, by *Ambystoma laterale* over time for June of 2014

Figure F.7   Concentration of milt produced by *Ambystoma laterale* over time for June of 2014
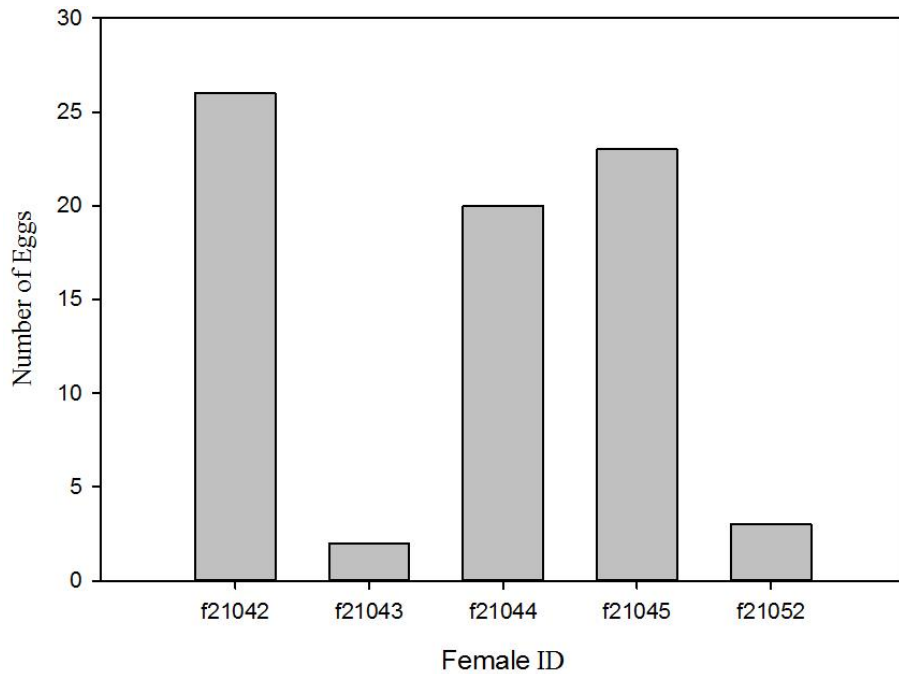
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Figure F.8   Total number of eggs produced per each female *Ambystoma laterale* for
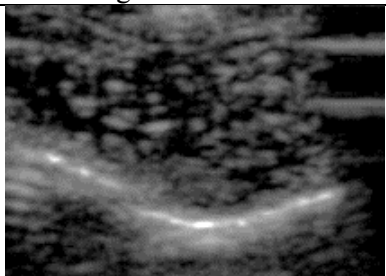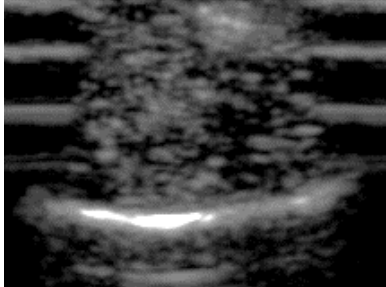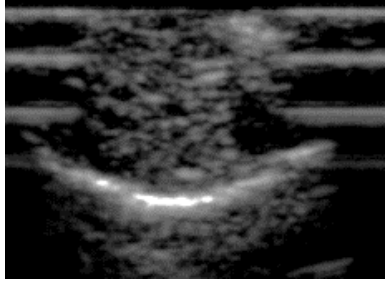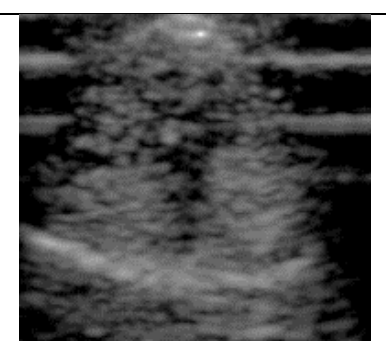June of 2014

Each animal oviposited at time points over the course of 72 hours. The total egg counts
are shown here. While these egg counts are far smaller than *A. tigrinum,* normal clutcxh
size for *A. laterale* is reported as anywhere from 2 to 500 eggs.

Table F.1 IVF attempts made in *A. laterale* in June of 2014 using both fresh sperm from *A. laterale* and frozen-thawed sperm from *A. tigrinum*

Sperm Type	Egg Number	Sperm Concentration	% Motility		% Cleaved from laid	%Blastula From Cleaved	% Neurula from cleaved
			Progressive	Non-progressive			
Fresh *A. Laterale*	2	1.1×10^4	14	14	100	100	0
	5	2.0×10^3	0	0	20	20	20
	6	1.3×10^4	0	0	0	0	0
	2	3.0×10^5	52	13	0	0	0
Total	15				20	20	6.7
Frozen—Thawed *A. Tigrinum*	9	1×10^6	2	6	33	33	0
	5	1×10^6	3	7	0	0	0
	3	1×10^6	4	7	33	33	0
Total	17				11.6	11.6	0

No embryos developed beyond the Neurula stage, likely due to poor quality eggs. Frozen-thawed *A. tigrinum* sperm was used in order to induce parthenogenesis.

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Table F.2   Ultrasound grading scale in *Ambystoma laterale*

Grade	Image on Ultrasound	Description
G3 (High Grade)		Near Complete Development: -Follicles hypoechoic and rounded -Well defined, well spaced, hyperechoic oocytes
G2 (Mid- Grade)		Moderate development: -Follicles uneven areas of hypoechogenicity -Uneven spacing between moderately developed oocytes
G1 (Low Grade)		Poor Development: -Follicles and oocytes tightly clumped together -Small and poorly developed follicles and oocytes -Little to no areas of hypoechogenicity between follicles
G0 (No grade)		No Development: -Little to no obvious follicular or oocyte development

The scale for ultrasound was based on the protocol for *Ambystoma tigrinum*. The principle for grading was the same. Oocytes appeared slightly more dense and closer together in *A. laterale* than in *A. tigrinum*.

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APPENDIX G

EFFECTS OF SOLUTIONS OF DIFFERENT OSMOLALITIES ON THE MOTILITY

OF *AMBYSTOMA TIGRINUM* SPERMIC MILT

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Materials and Methods

Milt was collected and assessed for initial concentration and motility as described in *Chapter II*. After initial assessment, milt was diluted 1:200 in solutions of either NaCl, KCl, or sucrose of 100, 300, or 600 mOsm or 1:200 in embryo transfer water (Sigma Aldrich, St. Louis MO, USA). Sperm was immediately assessed for changes in motility or changes in abnormalities. The diluted sperm solutions were then chilled at 4ºC and were observed every hour for 6 hours in order to observe the change in motility over time.

Table G.1   Raw data of the changes in motility and abnormalities seen when fresh milt
of *Ambystoma tigrinum* was exposed to solutions of different osmolalities

Osmolality	Solution	% Total Motility	Original % Total Motility	% Abnormalities	Original % Abnormalities
Water	H$_2$O	4.0 ± 1.9	59.1 ± 24.3	8.2 ± 5.4	1.7 ± 0.6
100 mOsm	KCl	9.5 ± 3.7	59.1 ± 24.3	0.8 ± 0.4	1.7 ± 0.6
	NaCl	11.8 ± 3.9	59.1 ± 24.3	6.2 ± 2.9	1.7 ± 0.6
	Sucrose	0.0 ± 0.0	59.1 ± 24.3	2.0 ± 1.3	1.7 ± 0.6
300 mOsm	KCl	13.7 ± 3.2	59.1 ± 24.3	0.4 ± 0.2	1.7 ± 0.6
	NaCl	11.8 ± 4.0	59.1 ± 24.3	3.2 ± 0.7	1.7 ± 0.6
	Sucrose	0.3 ± 0.3	59.1 ± 24.3	1.6 ± 0.8	1.7 ± 0.6
600 mOsm	KCl	9.2 ± 2.9	59.1 ± 24.3	0.8 ± 0.2	1.7 ± 0.6
	NaCl	1.7 ± 0.7	59.1 ± 24.3	10.0 ± 2.8	1.7 ± 0.6
	Sucrose	0.7 ± 0.7	59.1 ± 24.3	6.4 ± 2.2	1.7 ± 0.6

Original total motility and abnormalities represent the values of these parameters prior to
dilution with the KCl, NaCl, sucrose, or water.