Examining the Influence of Mating Systems on Testes Size in Salamanders

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by
Howard James Stanton II

Approved by

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ABSTRACT

Sperm competition theory predicts that relatively larger testes sizes evolve in animals with polygamous mating systems compared to those in monogamous mating systems due to sperm competition. Whereas intensity of sperm competition is the significant predictor of testes sizes in other taxa such as mammals, frogs, birds, insects, and fish, in salamanders the intensity of male-male competition in the transfer of spermatophores to females is predicted to be a critical factor. This is because males have to deposit more spermatophores to secure reproductive pay-off under higher intensity of male-male competition. I hypothesized that salamander species that breed explosively as groups possess increased proportional testes mass than those breeding in less competitive environments. I measured snout-vent length, body mass, and testes mass of *Ambystoma maculatum* (n=15), *A. opacum* (n=15), *A. texanum* (n=10), *A. tigrinum* (n=12), *P. glutinosus* (n=15) and *Notophthalmus v. viridescens* (n=14). I selected these species because they represented a variety of mating strategies with varied intensities of male-male competition. Accordingly, I predicted a gradient of proportional testes masses with *A. maculatum* having the greatest testes mass and *A. opacum* having the least. Testes were also examined microscopically, and the stage of spermatogenesis was classified to account for seasonal change in testes mass. The variables that I tested in relation to testes mass were species, body mass, stage of spermatogenesis, and their interactive effects. The best-fit generalized linear model was based on AICC and BIC. The results supported the hypothesis that increased male-male competition results in increased testes size, but other factors such as breeding season duration may also have an important effect on testes size.
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CHAPTER 1
INTRODUCTION

Overview

This study was undertaken to examine the relationship between mating strategies and testes size in salamanders. Previous studies have suggested, in various taxa such as mammals, birds, and insects, that species with high levels of sperm competition have evolved larger testes. This study is the first to examine such evolutionary relationship between mating strategies and testes size in salamander species. Salamanders are an interesting model because of the high variability in their breeding system. We analyzed four species in the genus *Ambystoma*, *Plethodon glutinosus*, and *Notopthalmus v. viridescens*. We focused on the genus *Ambystoma* to eliminate potential phylogenetic constraints, whereas the other two species were also examined in order to include a more diverse array of breeding strategies.

Sperm Competition and Testes Size

Evolution has driven the development of varied mating strategies among different species. Darwin (1871) was the first to note that females are generally the more selective sex. Bateman’s principle elaborates on Darwin’s observation. Male reproductive success is usually limited by the number of females they can inseminate, whereas female reproductive success is usually limited by the number of eggs they can produce and the energy they can expend on parental care (Bateman, 1948). This condition leaves the impetus on males to compete for female mates because females are the limiting resource.
Sperm competition is a post-copulatory form of male-male competition which occurs when a single female is inseminated by multiple males. Sperm from different males must compete to fertilize an ovum. Thus, it is predicted that increased levels of sperm competition will lead to evolutionary changes in sperm viability, morphology, and quantity (Harcourt et al. 1981; Harvey and Harcourt 1984; Kenagy and Trombulak 1986). Previous research has shown that a positive correlation exists between the intensity of sperm competition, due to multi-male breeding strategies, and relative testes size to body size in mammals, birds, insects, fish, and frogs (Short, 1979; Harcourt et al. 1981; Harvey and Harcourt 1984; Kenagy and Trombulak 1986; Moller 1989; Kusano et al. 1991; Pyron 2000). Increased sperm quantity associated with increased testes mass imparts a reproductive advantage to individuals with larger testes, allowing their genes to be passed on at a higher probability than rivals with smaller testes. However, this relationship is relatively understudied in amphibians and has not been previously studied in salamanders (Caudata). A previous study found that males of anuran species under intense sperm competition showed an increased testes mass compared to less competitive species (Kusano et. al, 1991).

This increased energy budgeting towards testes size has a lower cost-benefit ratio in species that rely on other varieties of mating competition to reduce risk of sperm competition. Male Gorillas (Gorilla sp.), for example, secure exclusive access to females through increased body size and combat (Harcourt, 1997). Sperm from the breeding male does not have to compete against those from other males within the female to fertilize the eggs. In Chimpanzees (Pan troglodytes), another ape, many males will breed a receptive female in a short period of time, leading to an increased risk of sperm competition. Accordingly, Chimpanzees have greater testes size than Gorillas relative to their body size (Short, 1979; Harcourt et al., 1981).
Kusano et al. (1991) examined the relationship between testes size and mating strategies of 19 species of Japanese anurans. A clear relationship was found between high levels of multi-male breeding and increased testes size. An alternative hypothesis, the sperm depletion hypothesis, which suggests that increased testes size is correlated with increased opportunities to mate over longer breeding season duration, was not supported (Cartar 1985; Kusano et al., 1991.)

Salamander Reproduction

Reproductive mode in salamanders is highly variable. It can range from internal vs. external fertilization; pair-mating with courtship vs. explosive group mating; and breeding seasons lasting only a week vs. multiple breeding seasons covering multiple month spans (Arnold, 1976; Verrell, 1989; Hasumi, 2001). Yet how these varied reproductive strategies affect the evolution of testes sizes has not been explored. For this study, I examined species utilizing internal fertilization with varying degrees of male-male competition and breeding season durations.

Most salamanders, including the focal species of this study (six species from three genera), use indirect internal fertilization. During breeding, males deposit spermatophores, which consist of a stalk topped with a gelatinous sperm cap. After deposition, the female straddles each spermatophore and takes the sperm cap into her cloaca. The sperm is stored in the spermatheca until it is used during fertilization, which occurs prior to oviposition. With this mode of insemination, receptive females can accept sperm from multiple males in one breeding season (Houck et al., 1985). This indirect fertilization leads to competition between males in delivering spermatophores (i.e., prezygotic male-male spermatophore competition) to a female. The spermatophore competition becomes intensive in populations with a male-skewed operational
sex ratio (OSR), meaning that there are more breeding males than breeding females in the area where breeding occurs. (Arnold, 1976; Verrell, 1989). It also leads to sperm competition within females that have accepted spermatophores from multiple males. As a result, a single clutch of eggs can have multiple sires (Houck et al., 1985; Liebgold et al., 2006). A successful insemination does not guarantee paternity for an individual male (Houck et al., 1985; Verrell 1989).

Different breeding strategies have evolved among salamanders in part in response to male-male competition and limited sperm quantities. Limited sperm quantity is a constraint for organisms with dissociated spermatogenesis. Whereas mammalians continually replace sperm, the salamanders’ cycle of dissociated spermatogenesis produces sperm only once throughout the course of the year leading into the breeding season. This cycle results in a limited amount available to deposit each season, as deposited sperm is not replaced until the following year. The investment of energy in courtship rituals by males can lead to higher success in spermatophore transfer to females (Arnold 1977). In species with elaborate courtship rituals, the number of spermatophores deposited is less than that of explosive breeders that exhibit limited courtship, but the insemination success rate is higher per spermatophore (Arnold 1976; Arnold 1977). Duration of breeding seasons is often associated with the pattern of spermatophore deposition. Ambystomatids, known for their short, explosive breeding seasons, often deposit their available sperm reserves in less than a week. Plethodontids have been shown to deposit, as few as, one spermatophore per week, but for months. (Arnold 1976).

Males of various salamander species will engage in behaviors to interfere with the reproductive success of competing males. For example, male *N. v. viridescens* that displays amplexus with a female often nudge or wrestle rival males off of females (Verrell 1989,
Takahashi et al., 2010). Males also mimic the courtship behavior of females in an attempt to lure them away from actual females and waste rival male’s spermatophores. Sexual interference by rival males has also been observed in *A. maculatum*, *A. texanum*, *A. tigrinum* and *P. glutinosus* (Arnold 1976; Verrell 1989; Wells 1980). *Ambystoma tigrinum* will isolate females by nudging them away from other males in a breeding pool (Arnold 1976.) In the terrestrial breeding *P. glutinosus*, males will defend a territory from rival males with physical aggression and biting (Wells 1980).

**Cycle of Spermatogenesis**

Spermatogenesis in temperate salamanders is a single cycle that produces spermatozoa over the course of a year leading into breeding season. Thus, in temperate amphibians, a large quantity of sperm is prepared by the initiation of breeding season and sperm supply will not be replenished by the end of the breeding season (Takahashi and Parris 2009). This mode of spermatogenesis is uniquely different from that of mammalian and avian species in which spermatogenesis occurs continuously and provides continuous sperm supply during the breeding season. In temperate amphibians, as the spermatozoa mature and are evacuated into the vas deferentia prior to breeding, the next cycle that provides sperm for the following breeding season begins with an influx of diploid spermatagonia into the posterior portion of each testis. The rest of the testis progressively fills with spermatogonia from posterior to the anterior (Werner, 1969; Uribe et al., 1994). The spermatagonia in the posterior develop first, going through a series of mitotic and meiotic cell divisions, ultimately leading to the formation of mature spermatozoa. The cyclic wave of spermatogenesis leads to different regions of the same testis hosting different stages of spermatogenesis at the same time (See Figures 5, 6).
Because of the cyclic nature of spermatogenesis, the size of testes can vary significantly throughout the year. Testes size is greatest when the testes are at the peak of spermatogenesis, prior to the testes evacuation that occurs as the breeding season begins (Highton, 1956; Werner, 1969). One study, examining *Plethodon cinereus*, showed that testes mass can vary from 1% to 4% of total body weight depending on the season in which the specimen was collected (Werner, 1969). Testes mass has also been shown to vary seasonally in the *Ambystoma* genus (Uribe et al., 1994; Norris et al., 1985). This seasonal variability in testes size must be taken into account during the analysis in order to make accurate comparisons between individuals collected at different stages of spermatogenesis and from different locales.

**Mating Strategies of Focal Species**

In this section, I will explain mating strategies of the salamander species that I examined. I chose the following species in order to encompass a variety of mating strategies. The four species of ambystomatids were chosen because they exhibit similar breeding behaviors with varying levels of male-male competition. *Notophthalmus v. viridescens* and *P. glutinosus* were included due to their highly developed courtship rituals and extended duration of breeding seasons. Their strategies are much different than the group-breeding ambystomatids. All of the chosen species utilize indirect internal fertilization.

*Ambystoma maculatum*

*Ambystoma maculatum* (Spotted Salamanders) breeding season is short, often lasting only a few nights, and takes place in early spring with the onset of rain and above-freezing temperatures. When conditions are optimal, males and females descend from their terrestrial habitats to vernal pools where breeding takes place in a free-for-all group fashion. Male-biased
Operational sex rations are well documented but vary between populations (Husting, 1965). The cause of this variance is most likely attributed to the increased stress of egg production in females, faster maturation of males, and increased predation on females due to the larger body size (Husting, 1965).

Male-male competition for females is extremely high due to the male-skewed OSR and the small temporal and spatial setting of the breeding season (Arnold 1976). When courtship takes place, it is very brief and frequently involves multiple males per single female. Females will take in spermatophores (~20), often from multiple males, until the spermathecae become filled (Arnold, 1976). Prior studies have shown that the female is often fully inseminated in less than one hour after entering the breeding pool (Arnold, 1976). In a laboratory setting, male A. maculatum in the presence of a female deposited a mean of 40.41 spermatophores. An average of 1.4 minutes of courtship was performed for each spermatophore (Arnold 1976). Almost all species of ambystomatids utilize an interference strategy known as spermatophore covering (Arnold, 1977; Verrell, 1989). In the presence of rival males, reproductive success is aided not only by producing an increased quantity of spermatophores, but also by interfering with spermatophores of rival males.

In matings in which multiple males are present, an individual male’s chance of a successful insemination correlates with the number of spermatophores placed (Arnold, 1976). The tactic of spermatophore covering increases a male’s chance of reproduction while simultaneously decreasing the probability of a competitor’s success (Arnold, 1976). A male will place his spermatophore directly on top of that of a competing male’s. The new spermatophore engulfs the previously placed spermatophore, making it unavailable to a female. Thus, this strategy doubles the males benefit. Arnold (1976) found that one-third of A. maculatum
spermatophores were placed upon previously deposited spermatophores. The documented high levels of competition, in conjunction with limited mate selection, led me to predict that *A. maculatum* would have the largest proportional testes of the species examined in this study.

**Ambystoma texanum**

*Ambystoma texanum* (Small-mouthed Salamanders) breed in aggregation in lentic areas, including roadside ditches, ephemeral pools, and swamps (Kraus and Petranka 1989). Breeding occurs during nighttime rain events in late winter and early spring (Kraus and Petranka 1989; Petranka, 1984). The duration and intensity of courtship can vary by locality. Wyman (1971) found that, in a laboratory setting, observed courtship lasted between 21 and 34 minutes and involved males amplexing females and guiding them to spermatophores. Garton (1972) found courtship to be of a lesser extent, consisting of nudging by the males followed by spermatophore deposition.

Studies have found the average number of spermatophores deposited during a single courtship by a single male to be in the 20-40 range (Garton, 1972; Harris, 2008). Spermatophores from competing males are often deposited in the same small area, leading to concentrations of up to 100 spermatophores (Garton, 1972; Petranka, 1982). Males stack spermatophores on top of their competitors previously placed spermatophores, thus blocking that spermatophores’ potential (Harris 2008; Arnold 1976). Harris (2008) found females to mount 19 ±9.5 spermatophores with no selection given toward those deposited by certain individuals. This method of reproduction with a high degree of sperm competition led me to predict a high proportional testes size, similar to that of *A. maculatum*.

**Ambystoma opacum**
*Ambystoma opacum* (Marbled Salamanders) employ a somewhat different strategy than *A. maculatum* and *A. texanum. Ambystoma opacum* are one of the few ambystomatids known to mate and oviposit on terrestrial substrate (Krenz and Scott, 1994). Whereas most ambystomatids are early spring breeders, *A. opacum* breed in the fall when vernal pools are partially dry (Noble and Brady, 1933). Males begin migrating to the pool up to two weeks prior to the arrival of females. Courtship and breeding takes place in the dry vernal pool or prior to entering the pool area (Krenz and Scott 1994). The female will oviposit within the boundaries of the pool and remain with the eggs until they are submerged.

One study found that 31-49% of females were viably fertilized before reaching the breeding pool (Krenz and Scott, 1994). By courting females outside of the wetland boundaries, males are able to reduce competition pressure from other males presumably by allowing the opportunity for pair mating without rival interference. It has also been shown that the first-breeding male is likely to sire more offspring than a male that subsequently breeds the female (Tennessen and Zamudio 2003).

Breeding competition within the boundaries of the wetland can be high due to a male-skewed operational sex ratio, meaning the number of reproductively active males is greater than actively reproductive females. This ratio is exaggerated in the early parts of the breeding season due to the difference in timing of arrival of males and females. Krenz and Scott (1994) found that in early breeding season the OSR was as high as 85 males to 1 female, but the season total was closer to a 6:1 ratio.

*Ambystoma opacum* are expected to have less male-male mating competition than the other ambystomatids examined, due to their terrestrial mating strategy and paired courtship.
behavior. Therefore, I predicted they would have the lowest proportional testes mass of the ambystomatids.

*Ambystoma t. tigrinum*

*Ambystoma t. tigrinum* (Eastern Tiger Salamanders) breed in ephemeral wetlands similar to other species of ambystomids but will also utilize permanent aquatic ponds (Petranka 1998). The breeding season is extended compared to other species of ambystomids. Breeding occurs from November through May, though the courtship season may only last up to two weeks. (Petranka 1998; Arnold 1976).

Breeding takes place in water as with *A. maculatum* and *A. texanum*. However, it does not usually occur in a large congress. Semlitsch (1983) documented the operational sex ratio as 1.9:1 and 1.2:1 in two separate populations. Breeding takes place in small groups of two to three individuals spread throughout the available habitat. Males will sequester females from competing males by nudging them to secluded areas. This behavior separates their breeding strategy from that of the explosive breeding ambystomatids (Arnold 1976).

The male begins courtship by initiating a tail-nudging walk. During this ritual, the female follows the male nudging his cloaca while the male maintains contact with the female using his tail. The male will deposit spermatophores, which are then mounted by the female. Arnold (1976) found *A. tigrinum* deposited one spermatophore per every 4.5 minutes of courtship. The result being a mean of 20.1 spermatophores deposited per courtship event, with a range of 8-37 (Arnold 1976). Competing males are known to use multiple methods of breeding interference, including female mimicry and spermatophore covering (Arnold 1976). Although male-male mating competition can be intense, the success of each courtship is expected to be less reliant on
sperm quantity and testes size compared to *A. maculatum* and *A. texanum*, and more reliant on body size and the ability of males to sequester females from the competition.

**Plethodon glutinosus**

*Plethodon glutinosus* (Northern Slimy Salamanders) are one of the larger species of lungless salamanders. Breeding in this species is much different from ambystomatids. All stages of reproduction occur on terrestrial substrate. Therefore, there is no mass breeding migration leading to an aggregate breeding site. Individuals find each other by following chemical scent cues. Lucas (2005) performed a sperm wave analysis on museum specimens from West Virginia and suggested that lower-elevation specimens deposit spermatophores in June-July. Highton (1962) also showed, from sperm wave analysis, that breeding *P. glutinosus* most likely mate over a one-two month period. This contrasts with the much shorter breeding season duration of the explosive breeding ambystomatids.

When a courting male encounters a female, he will initiate the courtship ritual by making contact using his mental gland. If the female is receptive, a complex courtship will ensue leading to the deposition of one to two spermatophores. (Organ 1960; Arnold 1976; Petranka 1998).

Plethodon glutinosus is expected to have smaller testes than the other salamanders tested, due to the emphasis on courtship during breeding, low male-male competition environment, and low spermatophore expenditure per mating event.

**Notophthalmus v. viridescens**

*Notophthalmus v. viridescens* (Eastern Red-spotted Newts) are common salamanders throughout the eastern US. They have a multiple-stage life history, from an aquatic larval stage,
to a terrestrial eft (i.e., juvenile) stage and to an aquatic adult stage. Adults inhabit almost any type of permanent or semi-permanent body of still or slow moving water, including pools, swamps, sluggish streams, and ponds (Bishop, 1943; Gates and Thompson, 1982). Breeding takes place aquatically beginning in the late fall and lasting through early summer. In some localities, the breeding season can last up to six months (Petranka 1998). Population sizes can vary greatly in each breeding pond. Gill (1978) documented a range from 6 to >2,600 breeding individuals per pond with a consistent OSR of 2 males: 1 female.

Red-spotted Newts have two methods of courtship. When a male approaches a female, he performs a display know as a “hula.” If the female responds by nudging his cloaca, he will deposit up to three spermatophores. If the female is unresponsive to his display, he will grasp her with his hind legs in amplexus. Once amplexed, the male will rub the female’s snout with his genial glands on his cheeks, release pheromones, and put on a further display. If the female is receptive, she will follow the male after he dismounts and the male will deposit a spermatophore, potentially followed by the deposition of 1 to 3 additional spermatophores. The abbreviated courtship is less used than the extended courtship and results in a much lower rate of actual sperm transfer to the female (Arnold 1977, Verrell 1989, Petranka 1998).

Courtship is important in *N. v. viridescens*. The rate of reproductive success is also correlated with increased body size and tail depth, which is a sexually dimorphic trait found in newts (Gabor et al., 2000). Verrell (1985) found that males also exhibit some degree of selection based on sight and smell for larger females, which is unusual amongst salamanders. Males with greater SVL and keeling of the tail were shown to be more likely to win access to females than smaller males with a lesser keel. They were also shown to have greater success at preventing sexual interference presumably due to their larger size (Gabor et al., 2000).
Although male-male competition is expected to be high due to the male-skewed OSR and high-density of breeding areas, sperm quantity may play a smaller role in *Notophthalmus viridescens* reproductive success than in the ambystomatids. The emphasis on courtship, coupled with a low-sperm expenditure mating strategy (1-3 spermatophores per event), led me to predict smaller testes in the *N. v. viridescens* than in the ambystomatids.

**Hypothesis and Predictions**

Based on the literature review, I predicted that explosive breeders with the highest levels of male-male competition and least amount of courtship would have the greatest proportional testes size when compared to species utilizing pair-mating or lower male-male competition breeding strategies. Of the ambystomatid species chosen for the study, I expected *A. maculatum* and *A. texanum* to have the highest proportional testes mass, *A. opacum* to have the lowest proportional testes mass, and *A. tigrinum* to fall in the mid-range. I also predicted that *N. v. viridescens* and *P. glutinosus* would have reduced testes mass compared to the ambystomatids examined, based on their high levels of courtship, mate selection, and lower number of spermatophores deposited per breeding event.
CHAPTER II

MATERIALS AND METHODS

Specimen collection

Specimens were obtained with a target of N=15 for each of the six chosen species. The objective was to obtain specimens collected prior to, or in early stages of, breeding season to eliminate as much seasonal variability among specimens as possible. Breeding seasons were determined for each species from prior literature. Wild caught specimens of *A. maculatum*, *A. opacum*, and *N. v. viridescens* were obtained through road searches during breeding migrations and the use of mesh funnel traps in vernal pools used for breeding. Preserved specimens of *A. tigrinum* were borrowed from The Carnegie Museum of Natural History. Individuals were identified as reproductive males based on the presence of noticeably swollen cloacae and other visible secondary sexual characteristics. These characteristics included conspicuous mental glands in *P. glutinosus* and patches of black horny nuptial excrescences on the inner hind thighs of *N. v. viridescens*.

*Ambystoma maculatum* (N=15) were collected between 3/12/10 and 3/16/10 during night time rain events. Most were captured during the breeding migration by visual searches of roads near known breeding sites in Cabell and Wayne counties of West Virginia. Specimens were also collected in a known breeding pool with mesh funnel traps. Both males and females were captured and eggs and spermatophores were found in the traps. The breeding event only lasted a few days. The first egg masses began to appear on 3/13/10 and many salamanders were observed in the pool. By 3/17/10 no new egg masses were noticed nor were additional salamanders observed in the pool.
*Notopthalmus v. viridescens* (N=14) were collected in a permanent pond in the Beech Fork Wildlife Management Area, Cabell Co., WV. Traps were placed in the pond on 11/14/10 and were removed 11/16/10. Only male newts were captured. Breeding season is known to occur in the late fall and spring. It is notable that males were observed in amplexus with other males. However, no spermatophores were found deposited in the traps.

*A. opacum* (N=15) were collected on 10/27/10 in Beech Fork WMA, Cabell Co., WV, during a nighttime breeding migration brought about by a rain event. All specimens were collected by visual searches of a road near known breeding sites. All captured specimens were reproductively active males.

All captured specimens were humanely euthanized the day of capture using a ventrally applied 20% benzocaine solution, available as the topical anesthetic Oragel™. Benzocaine application has been shown to be an effective, humane, and convenient method of euthanizing amphibians (Altig, 1980; McDiarmid, 1994; Chen and Combs, 1999) and is an accepted method of euthanasia as per American Veterinary Medical Association, American Society of Ichthyologists and Herpetologists, and Society for the Study of Amphibians and Reptiles guidelines. After euthanasia, specimens were fixed using 10% formalin before being washed and transferred to 70% ethanol.

Specimens of *A. tigrinum* (N=12), *A. texanum* (N=10), *P. glutinosus* (N=15), collected from throughout their native ranges were borrowed from Carnegie Museum of Natural History. Specimens with collection dates prior to, or during, their respective breeding seasons were used. These specimens were previously fixed with formalin and stored in 70% ethanol.
Data Collection:

The following data were collected for each specimen: snout vent length (SVL), body mass, individual testis mass, length of one testis, and stage of spermatogenesis. First, each specimen was measured for SVL, then blotted dry and weighed to the nearest mg. Once body mass was measured, the specimen was dissected.

An incision was made longitudinally down the middle of the abdomen. A transverse incision was made at each end of the longitudinal incision allowing the outer tissues to be folded back on each side. After access was made to the abdominal cavity, the lower intestine was severed allowing the viscera to be moved out of the way of the reproductive organs. The testes were then grasped with forceps and cut free from the vas deferens and any connective tissue holding them in place (see Figures 3, 4). Once removed from the body, testes were closely examined and all extraneous tissues were removed. Each testis was blotted dry and measured to the nearest 0.1 mg. In cases in which testes were very small or were broken in the extraction process, both testes were measured together.

Microscopic Examination of Spermatogenic Wave

In order to account for seasonal variability in testes size, each specimen was examined and classified based on the stage of spermatogenesis. A histological preparation was made from one testis of each salamander. Each testis was transferred from 70% ethanol to a solution of 30% sucrose – 70% phosphate buffered saline (PBS) for a period of at least 24 hours. Testes were then frozen in OCT™ compound and sectioned at 15µm using a cryostat. Sections were made of the entire length of the testis. Multiple slices were placed on each slide. After sectioning, specimens were allowed to air dry and were then stained using Erlich’s Hemotoxilyn. Slides
were washed with distilled water to remove excess stain and OCT™ compound. Each slide was then permanently mounted using Clear Mount™ solution.

Slides were examined under a variable power microscope and categorized based on the stage of spermatogenesis of the testis. This categorization was done by observing the presence of mature spermatozoa, spermatogonia, spermatocytes, spermatids, and evacuated ampullae (see Figures 5-10). Differentiating spermatogonia, spermatocytes, and spermatids proved to be difficult in some specimens. In cases in which the classification was unclear, vas deferentia were examined microscopically. This method of examination allowed me to make classifications of the stage of spermatogenesis based on the presence of spermatozoa in the vas deferens and inferences from documented natural history.

**Classification of stage of spermatogenesis**

I assigned a numerical classification to each specimen based on my observation of spermatogenic development. The classifications ranked developmental stage of the testes in order. The end of spermatogenesis, pre-spermioteleosis phase was ranked as stage 1 because it represented peak expected testes mass based on prior literature. (Werner, 1969; Uribe et al., 1994; Norris et al., 1985.) Because spermatogenesis is a continuous cycle with no distinct breaks, decimals were used to show the classification of a specimen appearing to be in transition between two spermatogenic stages.
<table>
<thead>
<tr>
<th>Spermatogenic Stage</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Mostly spermatids present.</td>
</tr>
<tr>
<td>2</td>
<td>Mature sperm finishing development.</td>
</tr>
<tr>
<td>3</td>
<td>Testes evacuating sperm.</td>
</tr>
<tr>
<td>4</td>
<td>Start of new spermatogenic cycle. Many ampullae empty or filled with spermatogonia.</td>
</tr>
<tr>
<td>5</td>
<td>Early stages of spermatogenesis.</td>
</tr>
<tr>
<td>6</td>
<td>Secondary spermatogonia turning into spermatocytes. Spermatocytes may be developing into spermatids.</td>
</tr>
</tbody>
</table>

**Table 1.** Numeric classification of varying spermatogenic stages used in analysis.

**Analysis**

Using the generalized linear model, the main effects of species, body mass (LGBmass), and stage of spermatogenesis and their interactive effects on testes mass were tested. The best-fit model was selected based on Akaike Information Criterion (AICc) and Bayesian Information Criterion (BIC). AICc was used in favor of AIC due to the relatively small sample sizes. The model that most explained our data tested included the effects of species, body mass, stage of spermatogenesis, and the interaction of species and body mass. Log$_{10}$ transformed body mass was used in the equation to eliminate variability due to the body mass difference between individuals (Moller, 1989).
<table>
<thead>
<tr>
<th>Model</th>
<th>AICC</th>
<th>BIC</th>
</tr>
</thead>
<tbody>
<tr>
<td>3 main effects + species x body mass</td>
<td>-67.5</td>
<td>-35.8</td>
</tr>
<tr>
<td>3 main effects + species x spermatogenesis</td>
<td>-67.9</td>
<td>-20.3</td>
</tr>
<tr>
<td>3 main effects + spermatogenesis x body mass + species x body mass</td>
<td>-64</td>
<td>-29</td>
</tr>
<tr>
<td>3 main effects + spermatogenesis x body mass</td>
<td>-61</td>
<td>-29</td>
</tr>
<tr>
<td>3 main effects + species x spermatogenesis + species x body mass</td>
<td>-52</td>
<td>-16.5</td>
</tr>
<tr>
<td>Factorial</td>
<td>-47.2</td>
<td>-17.4</td>
</tr>
<tr>
<td>3 main effects + species x spermatogenesis + species x body mass +</td>
<td>-40.1</td>
<td>-6.6</td>
</tr>
<tr>
<td>spermatogenesis x body mass</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3 main effects + species x spermatogenesis + spermatogenesis x body mass</td>
<td>-36.8</td>
<td>-1.3</td>
</tr>
</tbody>
</table>

*Table 2.* Best-fit model selection results using Akaike Information Criterion and Bayesion Information Criterion. The model using effects of species, body mass, stage of spermatogenesis, and the species and body mass interaction, was shown to most closely explain the results when using both AICC and BIC.
CHAPTER III

RESULTS

Results

I examined six species of salamanders (n=80) from three genera. All species examined use an indirect internal fertilization method but have varying breeding season durations, levels of mating competition, and courtship rituals. The best-fit model, selected using AICc and BIC, showed species, spermatogenic stage, body mass, and the interaction of species and body mass, all have a significant effect (p<.01) on testes mass.

<table>
<thead>
<tr>
<th>Source</th>
<th>Wald Chi-Square</th>
<th>df</th>
<th>sig</th>
</tr>
</thead>
<tbody>
<tr>
<td>(Intercept)</td>
<td>11.428</td>
<td>1</td>
<td>0.001</td>
</tr>
<tr>
<td>Species</td>
<td>18.445</td>
<td>5</td>
<td>0.002</td>
</tr>
<tr>
<td>Body mass</td>
<td>31.674</td>
<td>1</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Spermatogenesis</td>
<td>59.863</td>
<td>5</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Species x body mass</td>
<td>19.451</td>
<td>5</td>
<td>0.002</td>
</tr>
</tbody>
</table>

Table 3. Test of model effects: (Intercept), Species, Body mass, Spermatogenesis, Species x body mass, with dependent variable = testes mass. All variables were shown to have significant effect on testes mass.
Estimated Marginal Means 1: Species

The estimated marginal means were calculated for testes mass of each species within the best-fit model. The model was run with a fixed covariate of body mass = 3.9470, to eliminate the effects of variation in body mass among specimens on testes mass. The results showed that testes mass of *Ambystoma opacum* was significantly smaller than the rest of the species whereas there was no difference among the remaining species (Fig. 1).

![Estimated marginal means of testes mass by species. The results showed Ambystoma opacum (AO) to have significantly lower testes mass than the other tested species.](image)

**Figure 1.** Estimated marginal means of testes mass by species. The results showed *Ambystoma opacum* (AO) to have significantly lower testes mass than the other tested species.

Estimated Marginal Means 2: Stage of spermatogenesis

The estimated marginal means were also calculated for stage of spermatogenesis within the best-fit model. The model was run with a fixed covariate of body mass=3.947 to eliminate the effect of variation of body mass among specimens on testes mass. The results showed cyclic
variation consistent with my predictions. The peak testes size correlated with the category closest to stage 1. The following categories (3 - 4.5) decreased, corresponding with sperm evacuation and the beginning of a new spermatogenic cycle. The last stage (5.5) showed an increase as spermatogenesis progresses in the following cycle.

**Figure 2.** Estimated marginal means of spermatogenic stages. The data showed that testes size varied significantly according to stage of spermatogenesis.
CHAPTER 4

DISCUSSION AND CONCLUSIONS

Discussion

The results did not show a clear relationship between testes size and the amount of male-male competition during mating as hypothesized but suggested that the effects of sperm competition, the amount of which is affected by mating strategy, male-male competition, sperm quantity, and breeding season duration, does influence testes size. Of the six species tested, only *Ambystoma opacum* was shown to have significantly different testes mass from the rest of the species once variation due to spermatogenic stage and body size were accounted. The results supported my predictions that in a similar mating systems, increased male-male competition results in increased testes size but suggest that other factors also play a role.

*Ambystoma opacum* was the only species found to have significantly different testes mass than the other species. The lesser testes mass of *A. opacum* supports the hypothesis that both male-male mating competition and duration of breeding season influence testes size. *Ambystoma opacum* is one of the only species of ambystomatid that utilizes terrestrial mating. Krenz and Scott (1994) found that reproductive success of male *A. opacum* within the breeding ground was highly variable and that many females were inseminated prior to reaching the higher population density breeding site. This finding suggests that early breeding in low competition areas may be the most advantageous strategy for reproductive success for *A. opacum* males. By mating in areas with fewer males, the necessity of increased spermatophore production is lessened and an emphasis is placed on the ability to locate females early. This explanation may account for the lesser testes mass compared to other ambystomatids.
The intensity of sperm competition in salamanders may not be accurately assessed by the number of competing males in a given breeding event. Sperm competition was expected to be highest in congregational breeders with high male-male competition such as *Ambystoma maculatum*. However, the data did not show that congregational breeders have larger testes than pair breeders such as *Plethodon glutinosus*. The sperm depletion hypothesis may offer an explanation of the comparable testes size between the congregational breeders (*A. maculatum* and *A. texanum*) and the more selective breeders with longer breeding season duration (*A. tigrinum* and *P. glutinosus*) (Cartar, 1985; Kusano et al., 1991). Due to the ability of salamanders to store sperm for an extended duration, total length of breeding season may play an equally important role in creating sperm competition.

The two species that exhibit pair mating, *Notophtalmus v. viridescens* and *P. glutinosus*, were unexpectedly shown to have similar testes proportions to the explosive breeding ambystomatids. This result is likely because those species have longer breeding seasons than those species with explosive but short breeding seasons. The increased time for individual mating events, coupled with the female’s ability to store sperm, may lead to increased levels of sperm competition. This relationship between testes size and breeding season is unique to those species that exhibit dissociated spermatogenesis because of the limited number of breedings the male can participate in due to sperm quantity constraints. Female *P. glutinosus*, for example, may have the opportunity to accept spermatophores by many single males during their multi-month breeding season, just as *A. maculatum* do in their much shorter, congregational mating. The reproductive advantage of a male would be increased by having larger sperm reserves, allowing him to mate with more females each breeding season or by increasing the amount of sperm per spermatophore deposited.
The results showed that *A. opacum*, had lower proportional testes size than both *N.v. viridescens* and *P. glutinosus*. Due to the terrestrial breeding strategy of *A. opacum*, the amount of male-male competition undergone during each mating event may be more similar to *P. glutinosus*, than to the congregational aquatic breeding ambystomatid species. This strategy, coupled with the comparatively shorter breeding season, may explain the difference in testes size.

The non-significant difference in testes size between the remaining species may be related to the small sample size (n~15 per species). Though not significant, the results did show *P. glutinosus* to have smaller testes mass than the other species. This result would be in keeping with my predictions based on mating strategy. Phylogenetic differences may have also played a role. Future work should focus not only on increasing sample size, but examining additional species, especially from the *Notopthalmus* and *Plethodon* genera.

**Conclusions**

Sperm competition occurs when a female accepts sperm from multiple mates during a given breeding period. In taxa such as mammals and birds, with direct insemination and nearly instant fertilization, the amount of sperm competition can be estimated by the number of males that copulate with the female during a breeding event (Harvey and Harcourt, 1984; Moller 1989). However, the enormous amount of variation in breeding strategy between salamander species complicate making an accurate determination of the amount of sperm competition that takes place in each species. For salamanders with indirect fertilization, sperm competition is two-fold. Successful delivery of spermatophores to a female is affected by the number of spermatophores deposited by rival and focal males, especially in congregation breeding species (Arnold 1976). Once insemination is successful, competition takes place between the sperm of each male to
fertilize the ova. My results supported the hypothesis, in part. They showed that species with the highest male-male competition during a breeding season and least amount of courtship during mating, *A. maculatum* and *A. texanum*, do have increased testes size, compared to species with increased courtship and lesser male competition during mating, such as *A. opacum*. However, the study also showed that species with lesser male-male competition and increased courtship, but having longer breeding seasons (ie: *A. tigrinum*, *N.v. viridescens*, and *P. glutinosus*) had equivalent testes size to the aforementioned higher competition breeders.

In order to accurately assess sperm competition in a salamander with indirect fertilization and the capability of long-term sperm storage, the mean number of successful spermatophore transfers compared to the mean number deposited throughout the breeding season would need to be determined, as well as the average amount of sperm in each spermatophore. In addition, the mean number of spermatophores accepted by a female from different mates during the entire breeding season would need to be determined. Taking such measurements in the field is nearly impossible. A DNA analysis of spermatheca contents could yield beneficial data about the intensity of sperm competition, but the females would have to be collected very near the end of breeding season prior to fertilization.

This study also demonstrates the necessity of accounting for the stage of spermatogenesis when comparing testes size in species with seasonal spermatogenic cycles. The results showed that this variable can cause significant noise in testes mass data. It is also notable that specimens collected on the same night, such as *A. opacum*, showed a wide range of variation in the progression of spermatogenesis, despite being from the same locale. This variation may be caused by internal factors that vary individually such as physiology and development. Assuming that specimens collected during the same breeding event will be at similar stages of
spermatogenesis may lead to inaccurate comparisons, thus dictating the importance of examining each testis microscopically.
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APPENDIX
Figure 1. *Ambystoma opacum* showing reproductive system during dissection. Digestive system viscera has been pinned back exposing testes and vasa deferentia.
Figure 2. *Notopthalmus v. viridescens* showing reproductive system during dissection. Digestive system viscera has been pinned back exposing testes and vasa deferentia.
Figure 5. Longitudinal cross section of *Plethodon glutinosus* testis showing posterior to anterior progression of spermatogenesis. Ampullae containing mature spermatozoa are visible in the anterior-most portion of the testis.
Figure 6. Longitudinal cross section *Ambystoma opacum* testis showing posterior to anterior progression of spermatogenesis.
Figure 7. Close-up cross section of *Plethodon glutinosus* testis showing ampullae containing spermatocytes and spermatids
Figure 8. Close-up cross section of *Plethodon glutinosus* testis showing spermatids transforming into spermatozoa.
Figure 9. Close-up cross section of *Ambystoma opacum* testis showing ampullae containing mature spermatozoa.
Figure 10. Close-up cross section of *Ambystoma maculatum* testis, showing many ampullae packed with mature spermatozoa
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Date: May 14, 2013

Committee Chair Printed Name: Dr. Thomas K. Pauley

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The following application and protocol to use laboratory animals at Marshall University was reviewed and received final approval by the Institutional Animal Care and Use Committee (IACUC) on June 11, 2010

Title of application: “An examination of the evolutionary relationship between testes size and mating system in the Jefferson’s Salamander (Ambystoma Jeffersonianum).

IACUC Project No.: 451

Name of Principal Investigator: Dr. Thomas K. Pauley

Co-Investigator: H. James Stanton

Mizuki Takahashi

As a condition of approval, the Institutional Animal Care and Use Committee required the following modifications to the above-referenced application:

None

Monica A. Valentovic, Ph.D.
Chairperson, IACUC
Dear Sir/Madam:

The following application and protocol to use laboratory animals at Marshall University was reviewed and received final approval by the Institutional Animal Care and Use Committee (IACUC) on June 11, 2010.

Title of application: “An examination of the evolutionary relationship between testes size and mating system in Spotted Salamanders (*Ambystoma maculatum*).

IACUC Project No.: 452

Name of Principal Investigator: Dr. Thomas K. Pauley

Co-Investigator: H. James Stanton

Mizuki Takahashi

As a condition of approval, the Institutional Animal Care and Use Committee required the following modifications to the above-referenced application:

None

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Chairperson, IACUC
Dear Sir/Madam:

The following application and protocol to use laboratory animals at Marshall University was reviewed and received final approval by the Institutional Animal Care and Use Committee (IACUC) on June 11, 2010

Title of application: "An examination of the evolutionary relationship between testes size and mating system in Eastern Red-spotted Newts (Notophthalmus v. viridescens).

IACUC Project No.: 453

Name of Principal Investigator: Dr. Thomas K. Pauley

Co-Investigator: H. James Stanton

Mizuki Takahashi

As a condition of approval, the Institutional Animal Care and Use Committee required the following modifications to the above-referenced application:

None

Monica A. Valentovic, Ph.D.
Chairperson, IACUC