Extra-pair Paternity within the Female-defense Polygyny of the Lizard, *Anolis carolinensis*: Evidence of Alternative Mating Strategies

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ABSTRACT

Mate competition is a prominent component of sexual selection theory. Typically, males attempt to mate with the most females possible and females attempt to mate with the highest quality males possible. In the polygynous female-defense mating system of *Anolis carolinensis*, males compete directly for females through territorial behavior. Inter-male competition is intense due to an average polygyny ratio of 1 male to 3 females despite a 1:1 adult sex ratio. Through high levels of territorial behavior (e.g., 100 displays/h, 27 m patrol distances/h, 70% of day in defense-related activities), males attempt to exclude other males from resident females who, in turn, both store sperm and ovulate a single-egg clutch at weekly intervals over a 4-month breeding season. Paternity of hatchlings in 16 naturally occurring breeding groups was analyzed to determine the extent to which the territorial resident male was able to prevent other males from fathering offspring of his resident females. Lizards residing in or neighboring a resident male's territory were collected and RAPD-PCR was used to determine the paternity of hatchlings. Of the 48 hatchlings from 26 females, resident territorial males fathered 52% of hatchlings; 15% were fathered by a male whose territory bordered that of the resident male and 21% were fathered by a smaller male living covertly within the resident male's territory. Paternity for the remaining 12% of hatchlings belonged to an unsampled male. Given that females mated with multiple males, laboratory-based controlled matings were conducted where females were sequentially paired with two males and RAPD-PCR was used to analyze which of the two males fathered the subsequent hatchlings to determine the mechanism of sperm precedence. Regardless of mating order, only one male of the pair fertilized the eggs. Male *A. carolinensis* have reproductive strategies present in addition to defending resident females and female *A. carolinensis* have options in addition to simply mating with the resident male. While sperm precedence is present in this species, it is not based on mating order, but may involve both the number of sperm deposited in the female's tract as well as the quality of those sperm.

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Background and Objectives

Sexual selection theory

The success of an individual is measured by the degree to which its genes have been passed on to future generations. Depending on the number and quality of the mates that an individual is able to obtain, that individual's fitness will change. Andersson's (1994) definition of sexual selection states that sexual selection involves the "differences in reproduction that arise from variation among individuals in traits that affect success in fertilizations and competition over mates." Andersson has thus identified two areas where individual variation may translate into an increase in fitness, fertilizations and mate competition.

In terms of sexual selection, fertilizations must result from sexual reproduction with anisogamy or different sized gametes. The costs of sexual reproduction in sexual species are distributed differently for males and females (Andersson 1994). Female reproductive capacity is limited by the energy requirements necessary to provide nutrients to the embryo whereas male reproductive capacity is limited by his access to available females. Therefore, it is proposed that females are limited by their physiology and males are limited by their competitors (Daly & Wilson 1983). Physiological and behavioral strategies should evolve in order to insure a positive reproductive outcome in both males and females. Thus, selection should produce males who strive to mate with the highest number of females possible and females who insure that their mates have the best qualities possible.

Competition over mates occurs when one individual becomes less able to secure a mate because of the actions of another individual (Andersson 1994). As stated by Andersson (1994, p 11), there are many forms to mate competition. Scrambles occur when there is competition to be the first individual to find a mate. Endurance rivalry occurs when individuals remain at a breeding site for long periods of time, in lieu of foraging for example, to increase the number of potential mates that they encounter. Competition

based on contests results in selected traits for success in fights, such as large size or weaponry. Mate choice involves using traits and resources to attract mates. These diverse patterns of competition have in common the fact that they occur prior to copulation. In addition to the competitive interactions for mates described above, mate competition can occur following copulation in the form of sperm competition. Sperm competition has been defined as "competition between the sperm of two or more males to fertilize the eggs of a single female" (Parker 1970). Sperm competition results when a female mates with more than one male per breeding season (Birkhead & Moller 1992). In polygynous species, success in competition over mates is crucial for the fitness of the male (Andersson 1994), whether that competition is among individuals of the same sex for access to mates (intrasexual competition) or competition in which members of one sex choose specific mates of the other sex (intersexual selection) (Drickamer & Vessey 1992), so it is expected that male strategies will evolve to reduce the impact of extra-pair copulations.

Natural history of *Anolis carolinensis*

Anolis carolinensis exhibits a female-defense polygynous mating system. Despite a reported 1:1 adult sex ratio observed from census data taken in the field (Jenssen et al. 1995); Ruby (1984), Jenssen et al. (1995) and Nunez et al. (1997) have observed up to six resident females mated by a single territorial male. This means that two of every three adult males do not hold territories and therefore potentially do not have reproductive access to females. While *A. carolinensis* males defend territories that contain multiple female home ranges, peripheral females may be overlapped by more than one male. Females tend to mate with the male(s) who patrols their home range (Ruby 1984). This implies that females could potentially mate with multiple males during the breeding season. In addition to mating with the territorial male(s) who patrol her home range, a female may also mate with non-territory holding males who may be present. This potential for females to mate with more than one male would prevent any one male from being able to insure paternity of the offspring of his resident females.

At the start of each breeding season, male and female lizards move from the overwintering areas to set up their territories (Jenssen et al. 2001). Females set up small territories by choosing areas with ample resources and males choose territories such that they encompass as many females as possible (Jenssen & Nunez 1998). Males restrict access of other males to resident females by actively defending territories containing female home ranges. The majority of a male's time is spent patrolling and monitoring his territory for interloper males (Jenssen et al. 1995).

The breeding season of *A. carolinensis* extends from mid-April through mid-August (Gordon 1956). During this four-month breeding season, females continuously clutch a single egg every four to ten days (Andrews 1985). There is opportunity for a separate insemination of each egg that is ovulated (Hamlett 1952). Therefore, the paternity of 16-18 eggs is in contention for each female over the course of the breeding season.

In comparison to other species of *Anolis* observed under laboratory conditions, *A. carolinensis* has a relatively long coitus (Noble & Bradley 1933). Average copulation time in the laboratory has been recorded at 9 minutes (Valenstein & Crews 1977) and in the field at 15.8 minutes (Jenssen et al. 1995) and 26.4 minutes (Nunez et al. 1997). Parker (1984) hypothesized that prolonged copulation can function as a type of sperm competition by reducing the probability that a female will be remated prior to ovipositing. It is possible that long copulation duration in *A. carolinensis* is to prevent the female from having additional matings prior to oviposition.

Repeated mating of a female lizard by a resident male can be viewed as a strategy to dilute the sperm of competitors. These repeated matings may have evolved as a consequence of sperm competition (Devine 1984). *Anolis carolinensis*, however, does not seem to employ this strategy although a discrepancy is present over the number of times that a male *A. carolinensis* will copulate per day. A captive *A. carolinensis* male mates, on average, two times per day (Greenberg & Noble 1944). However, evidence from field data (Jenssen et al. 1995 and Nunez et al. 1997) shows that a resident male will mate

about once per day regardless of the number of females in his territory, therefore, a resident male is not likely to mate multiple times with a resident female.

If, by repeated matings, a male can increase the amount of sperm in the female tract and thus his chances at paternity, one might expect that males under natural conditions would average more than one copulation per day. Yet, there are reported instances of resident males passing up resident females who are soliciting courtship from them (Greenberg & Noble 1944, Jenssen et al. 1995, and Nunez et al. 1997). Both Jenssen et al. (1995) and Nunez et al. (1997) have found that resident males pass up 70% of their courtship opportunities with receptive females. If, however, copulation places the male in a situation where the likelihood of a territorial challenge increases, the male should reduce his risk of a fight by limiting the number of copulations (Jenssen et al. 1995).

Sperm storage is present in *A. carolinensis*. Fox (1963) initially reported the presence of sperm storage tubules in *A. carolinensis*. These tubules have been further described by Conner & Crews (1980) as being embedded in the wall of the utero-vaginal transition, having been formed by the folding and fusion of the oviducal epithelium. Conner & Crews (1980) also found evidence that sperm enter the sperm storage tubules between two to six hours after insemination, and if these sperm fertilize an egg, they enter the infundibulum six to 24 hours post mating.

The exact mechanisms of sperm release and transport from the sperm storage organs in females are unknown in anoline lizards. One proposed mechanism is that the female produces muscular contractions of the oviduct to force the sperm out of the sperm storage tubules (Conner & Crews 1980). If the release of sperm from the sperm storage organ is under the voluntary control of the female, then females would be able to influence male reproductive success after the male has copulated with the female (i.e. cryptic female choice, sensu Eberhard 1996).

Sperm storage is most likely functioning to allow the female to lay fertile eggs in the absence of the resident male (Crews 1973) and to lengthen the period of fertile egg laying

(Fox 1963). Although sperm have been found in the sperm storage tubules during the non-breeding season, sperm retention in *A. carolinensis* is proposed to be relatively inefficient for fertilization of eggs produced more than a few months post-copulation (Licht 1973). Unlike in snake sperm storage tubules in which the sperm are nourished, no nourishment is provided to the sperm once they have entered the storage tubules of *A. carolinensis*. However, *A. carolinensis* sperm longevity has been estimated at seven months in the sperm storage tubules (Fox 1963).

Sperm competition theory

Sperm competition theory is concerned with the physiological processes that occur within the female reproductive tract following multiple matings, as well as the behaviors of the individuals involved (Birkhead & Moller 1992). In mating systems in which sperm competition exists, males are selected for strategies that increase the probability that their sperm will fertilize the eggs of their mates. Parker (1970) described several adaptations which, if employed by a male, may increase the probability that his sperm will fertilize the eggs over the sperm of another male. Mating or copulatory plugs are temporary structures that are secreted (e.g. from the kidneys in brown water snakes, *Nerodia taxispilota* [Devine 1984]) by the male into the reproductive tract of the female to mechanically prevent insemination by additional males. Prolonged copulation (e.g. Mediterranean fruit flies, *Ceratitis capitata* [Saul et al. 1988]) and non-contact guarding phases (e.g. zebra finch, *Taeniopygia guttata* [Birkhead et al. 1989]) may also reduce sperm competition.

Intense intrasexual competition may lead to sperm competition between males due to the necessity for a given male to outcompete others to insure paternity of the offspring. A successful male should either displace potentially stored sperm in the female tract or place his sperm in a position such that they will fertilize the eggs. These strategies are necessary because within the reproductive tract of the female, sperm precedence may result in the sperm of one male being more likely to fertilize the eggs than the sperm of another male (Parker 1970). Displacement of sperm by a second male can be based on

specialized structures on the genitalia. Once a new mate removes the previous male's sperm, he then introduces his own sperm into the tract of the female (Parker 1970). Sperm precedence can also occur when the sperm of a male takes priority over the sperm of other males in fertilization of the eggs based on the order that the sperm was deposited into the female tract. In first male sperm precedence, it is the sperm of the male who is the first to mate with a female that fertilize the eggs preferentially over the sperm of males who mate after this male. First male sperm precedence is seen in the sierra dome spider, *Linyphia litigiosa* Keyserling, where structures in the female reproductive system appear to dictate the use of the first male's sperm to fertilize the egg over the sperm of additional mates, although the exact mechanisms are unknown (Watson 1991). Conversely, in last male sperm precedence, it is the sperm of the male who is last to mate with the female that fertilize the eggs preferentially over the sperm of previous mates. Last male sperm precedence is seen in the Namib desert beetle, *Onmacris unguicularis*, where the female ejects the sperm plug of a previous mate prior to mating with another (DeVilliers & Hanrahan 1991).

If a species possesses an evolutionary strategy involving sperm competition, a male that copulates with the female may not necessarily be assured paternity of the offspring (Eberhard 1996). Therefore, a male who is able to both introduce a large amount of sperm into the female tract and also prevent additional males from copulating with her would have a high selective advantage over males who employ only one of the above strategies (Parker 1970).

Intersexual competition may also lead to sperm competition because females may employ sperm competition as a strategy to obtain the greatest amount of sperm possible, from the greatest number of males possible prior to egg fertilization. Obtaining an excess of sperm from a large number of males would present a situation where the "most efficient" sperm fertilize the eggs. The resulting sons would then presumably have this most efficient sperm, which would insure the passing on of the mother's genes (Devine 1984).

A system involving sperm competition selects for any feature that allows a male to outcompete a rival male's sperm. The necessary requirements for sperm competition and sperm storage are present in *A. carolinensis*. Male *A. carolinensis* engage in long copulations possibly to restrict the amount of time a female has available to copulate with another male prior to oviposition. Resident males may frequently copulate with familiar females within a receptive cycle to insure that they will dilute any rival sperm that may be present in the female tract. Males restrict the access of other males to resident females by actively defending territories containing the home ranges of females. Females may also promote sperm competition by mating with more than one male to insure that the most effective sperm give rise to sons with effective sperm (Devine 1984, Olsson et al. 1996).

Research questions

Sperm competition has been documented in most animal groups, including insects, amphibians, reptiles, birds and mammals (see Smith 1984 for a review). When looking at potential mechanisms of sexual selection available to a given species, it is important to take into account the natural history of that species. Given the social organization and reproductive strategies of the lizard, *Anolis carolinensis*, sperm competition is a plausible mechanism for sexual selection in this species.

In the present study, I had two primary objectives for elucidating the role of sperm competition in *A. carolinensis*. First, I wanted to determine the extent to which males can behaviorally exclude other males from reproductive access to the females in their territories, thereby preventing sperm competition from occurring in the first place (Chapter 1). To do this I documented the natural occurrence of copulations between females and males other than the resident male through the use of field observations and molecular techniques. Second, I wanted to examine the role that females might play in promoting sperm competition (Chapter 2). To do this I conducted laboratory-controlled matings to determine the order of sperm precedence.

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Chapter 1

Paternity in naturally occurring breeding groups of *Anolis carolinensis*

Abstract

In the polygynous female-defense mating system of *Anolis carolinensis*, males compete directly for females through territorial behavior, where male body size positively correlates with size of male territory and number of females resident within the territory. Inter-male competition is intense due to an average polygyny ratio of 1 male to 3 females despite a 1:1 adult sex ratio. Through high levels of territorial behavior (e.g., 100 displays/h, 27 m patrol distances/h, 70% of day in defense-related activities), males attempt to exclude other males from resident females who, in turn, both store sperm and ovulate a single-egg clutch at weekly intervals over a 4-month breeding season. Furthermore, sedentary and seemingly passive females within male patrol areas may have other reproductive options in addition to simply mating with the territorial male. In spite of obvious selection for large, aggressive and conspicuous males, the defense of multiple females may not be the only mating strategy open to male *A. carolinensis*. Therefore, I used the techniques of RAPD-PCR to determine the paternity of hatchlings within polygynous breeding groups of naturally occurring lizards to test the following hypotheses: 1. extra-pair paternity should increase with size of male territory because effective female-defense behavior should decrease with size of territory; 2. extra-pair paternity should increase with decreasing body size of territorial males because neighboring males might be more likely to move into a territory patrolled by a small male than a territory patrolled by a large male; 3. females at the perimeter of a male territory should reflect greater extra-pair paternity than females in the interior of a territory because of increased opportunity for extra-pair contacts. The first hypothesis was supported. From 16 breeding groups, the territorial males fathered, on average, 52% of 48 sampled hatchlings from 27 females, with territorial male paternity being negatively correlated with male territory size. The second hypothesis was not supported. Smaller territorial males did not see more occurrences of extra-pair paternity than larger territorial

males. Surprisingly, the third hypothesis was also not supported. The greatest threat to the fitness of a territorial male was not from neighboring territorial males (15% of overall paternity), but from small inconspicuous males residing with the patrolled areas of territorial males (21%). Paternity for the remaining 12% of hatchlings did not belong to the territorial male, but whether the father was a neighboring male or an inconspicuous male was unable to be determined.

Introduction

The costs of reproduction in sexual species are distributed differently for males and females (Andersson 1994). Female reproductive capacity is limited by the energy requirements necessary to provide nutrients to the embryo, whereas male reproductive capacity is limited by his access to available females. Thus, it has been proposed that females are limited by their physiology and males are limited by their competitors (Daly & Wilson 1983). Physiological and behavioral strategies evolve in order to insure a positive reproductive outcome in both males and females. Therefore, selection should produce males who strive to mate with the highest number of females possible, and females who insure that their mates have the best qualities possible.

In polygynous species, success in competition over mates is crucial for the fitness of the male, so one would expect to see male strategies evolving to reduce the impact of extra-pair copulations (Birkhead & Moller 1992). *Anolis carolinensis* exhibits a polygynous mating strategy. Despite a reported 1:1 sex ratio observed from census data taken in the field (Jenssen et al. 1995), researchers have observed up to six resident females mated by a single territorial male, with an average of three females for every one male (Nunez et al. 1997). Males restrict the access of other males to resident females by actively defending territories containing the home ranges of females. The majority of a male's time is spent patrolling and monitoring his territory for interloper males. Males patrol at a rate of approximately 27 m/h and display approximately 100 times/h (Jenssen et al. 1995).

Therefore, while males defend territories that contain multiple female home ranges (Jenssen et al. 1995), the potential exists for extra-pair paternity due to temporary invasion by “floater” males or female home ranges being overlapped by more than one male (Ruby 1984). This information implies that females could potentially mate with multiple males during a reproductive cycle, thus increasing the uncertainty of male paternity because of this threat by outside males. *Anolis carolinensis* females have a continuous four-month breeding season, during which females produce a one-egg clutch every four to ten days (Andrews 1985). There is opportunity for a separate insemination of each egg that is ovulated (Hamlett 1952). Therefore, for each female, there are

approximately 16 to 18 ovulatory events during the breeding season which are in contention.

Anolis carolinensis has some unusual reproductive strategies. Males reject 70% of copulation opportunities with receptive resident females and average only one copulation per day (Jenssen et al. 1995). This may indicate a limitation of available sperm. In comparison to other species of *Anolis* observed under laboratory conditions, *A. carolinensis* has a relatively long coitus (Noble & Bradley 1933). Average copulation time in the laboratory has been recorded at nine minutes (Valenstein & Crews 1977) and in the field at 15 minutes (Jenssen et al. 1995) and 26 minutes (Nunez et al. 1997). It is possible that long copulation duration in *A. carolinensis* is to prevent the female from having additional matings prior to oviposition.

Even given the behavioral profile of *A. carolinensis*, there is no a genetic estimate of male fitness. Genetic evidence of paternity would document the extent to which males can behaviorally exclude other males from reproductive access to the females in their territories, thereby preventing sperm competition from occurring. Three hypotheses were tested in this study.

The first two hypotheses were centered on the resident male. First, I hypothesized that the larger a resident male's territory, the more incidences of a male other than the territorial male fathering the offspring would be observed. The larger the territory, presumably the more difficult it is for a territorial male to monitor all areas of his territory for intruding males. Second, I hypothesized that smaller territorial males would have more incidences of another male fathering offspring born to females on his territory than a larger territorial male would because a neighboring male might be more likely to attempt to move into a territory patrolled by a small male than he would a territory patrolled by a larger male.

The third hypothesis was centered on the resident female. I hypothesized that a female with a home range closer to the edge of her resident male's territory would have more of her offspring fathered by males other than the territorial male than would a female with

her home range in the center of the resident male's territory. The closer a resident female is to a resident male's territory boundary the easier it may be for a neighboring or floater male to move a short distance across a territory border to mate with a peripheral female without first being detected by the territorial male.

Methods

Field Methods

Observations were made on a naturally occurring population of *Anolis carolinensis* located along the Augusta Canal, Augusta Co., Augusta, Georgia, USA (latitude 33° N) from 22 June 1998 to 27 July 1998 and from 13 June 2000 to 21 July 2000, which is within the four month (mid-April through mid-August) breeding season (Gordon 1956) of this species. Data collection occurred in the morning (0800 - 1100 h) and again in the afternoon (1830 - 2000 h). Paternity estimates were made for six territorial males during 1998 and ten territorial males during 2000 by identifying a territorial male and capturing all lizards in or near his territory, collecting eggs oviposited from captured resident females and analyzing paternity of the resulting hatchlings (Appendix 1).

Only males who were territorial (i.e. bright green and conspicuous by frequent moves and displays) and resided in habitat of limited complexity were sampled so that I could more easily detect and observe the lizards. All observations were completed for a given male prior to moving to a new territorial male. A male was initially observed to determine the extent of his territory. Once a male had made two complete loops of his territory, I mapped the territory by drawing the trees and vegetation encompassed by his patrolling and the neighboring trees and vegetation. The territorial male was then marked with Testor's paint applied via a paint pole (Passek and Collver 2001) or with India ink applied via a squirt gun. This temporary marking of the territorial male enabled me to insure that I was observing the same lizard from day to day.

Each territory was kept under observation for an average of five consecutive days. During this time, I attempted to locate all lizards that resided within the boundaries of the territorial male. For each lizard, I noted its location within the territory, plotted that location on the map I had generated and then attempted to capture that lizard and remove it from the territory. Lizards were captured by hand or by noose and were individually placed into small zipper-type storage bags and then into a cooler until it was time to leave the field for that observation period.

Female lizards captured from within a male's territory were classified as either being on the periphery of the male's territory or centrally located within the male's territory. Whether a female was classified as peripheral or central depended upon whether she was closer to the edge of the territory (peripheral) or the center of the territory (central) when she was initially observed.

Once I was confident that I had removed all of the lizards residing within the territorial male's territory that I was able to capture, I then captured the territorial male. In some instances, I was able to observe additional lizards within the habitat, but was unable to capture them either because they were able to successfully evade my attempts or because they were present in an inaccessible area of the habitat (e.g. too high in the tree to reach). For these lizards, I simply marked their location on the territory map.

I measured territories for which I had completed observations and collections in order to get an estimate of territory volume for each resident male. Territories were measured on the x, y and z-axes. Territory length was measured by either using a measuring tape or by pacing off the distance. Territory height was measured by using a tangent height gauge (Forestry Suppliers, Inc.) to obtain the height of the tallest point patrolled by the resident male within his territory. Territory width was visually estimated for each territory observed. Territory width was fairly standard as it was constrained due to the presence of a dirt pathway on one side (which lizard territories were never observed to encompass or cross) and the Canal on the other.

Each captured lizard was given a unique identification letter and number to designate lizards residing within or near a given territory. Identifiers were written on their ventral surface using a Sharpie marker. Snout-vent length (SVL), total length, and mass were obtained for each lizard. A 25-100 μl blood sample was collected from the postorbital sinus of each lizard in heparinized microhematocrit tubes. Collected blood was placed into an Eppendorf tube containing 200 μl 1X TNE buffer (10 mM Tris, 10 mM NaCl, 2.5 mM EDTA, pH 8.0), pipetted up and down two times to mix and placed at –20 °C. Male lizards were then placed individually into Whirl Pak sample bags (Forestry Suppliers, Inc.) and then at –20 °C. Once the field season ended, samples (blood and whole lizard) were transported to Virginia Tech. During transport, samples were packed on dry ice and then transferred to –80 °C for long-term storage.

Female lizards captured from within a male's territory were placed individually into small plastic containers (33 x 18 x 11 cm^3) (Rubbermaid #221F) with plastic plants and a dish containing moist soil in which they could lay their eggs. Females were housed on a 14:10 light:dark cycle with a temperature ranging between 27-34 °C during the light cycles and 23 °C during the dark cycles. Females were fed crickets, grubs, or mealworms daily and boxes were misted daily to provide water. Dishes were checked daily for eggs. Each female was isolated after removal from the field site, so all eggs laid in captivity were fertilized with sperm deposited before capture.

Upon locating an egg in the soil dish, I would transfer it to a 266 ml plastic cup (Solo Co.) containing an equal mixture of 10 g of water and vermiculite. A small indentation was made in the water/vermiculite mixture and the egg was placed horizontally into this indentation so that half of the egg was below the mixture and half was above. The top of the cup was covered with a piece of plastic wrap held in place with a rubber band and cups, labeled with the identification number of the mother and a number to identify each hatchling from its siblings, were placed at into an incubator (temperature range 22.5 °C to 35.0 °C, average low temperature: 24.3 °C, average high temperature: 30.2 °C) for the duration of incubation. With this procedure, I had an 85% hatching success (n = 78).

Upon hatching, each hatchling's unique identification number was written on its ventral side. As hatching did not begin until the field season had ended, hatchlings were placed immediately into Whirl Paks and into –80 °C.

Molecular Techniques

Once all hatching was completed, paternity of hatchlings was investigated through the use of RAPD-PCR (Williams et al. 1990, Welsch & McClelland 1990). The wide use of RAPD-PCR as a tool for parentage analysis is partly because it can be employed with little knowledge of the biochemistry of the species (Welsch & McClelland 1990). DNA sequence information is not required for the design of amplification primers, primers of arbitrary nucleotide sequence are employed (Williams et al. 1990). In comparison with other molecular techniques used in parentage analysis, RAPD-PCR requires very small amounts of DNA (Tirado & Lewis 1997).

DNA was isolated from blood or muscle tissue of adults and from muscle tissue of hatchlings using a PUREGENE DNA Isolation Kit (Gentra Systems kit D-5000A). The protocol for DNA isolation from 5-10 mg mouse tail tissue was followed for tissue samples and the protocol for DNA isolation from 2 μl non-mammalian whole blood was followed for blood samples. Muscle tissue from one front leg of males, one rear leg of females or all four legs of hatchlings was chopped into small pieces and placed into 300 μl cell lysis solution (Gentra Systems) and ground using a pestle. Seventy-five μl of the mixture of whole blood suspended in TNE buffer was added to 300 μl cell lysis solution and pipetted up and down two times to mix. All samples, once added to cell lysis solution, were kept on ice until 1.5 μl proteinase K was added. Samples were then incubated overnight at 55 °C. Following overnight incubation, samples were cooled to room temperature, had 1.5 μl RNAse added, and incubated at 37 °C for 15 min to one hour. Once incubation with RNAse was complete, samples were again put on ice for 15 min prior to and immediately following addition of 100 μl protein precipitation solution (Gentra Systems) to insure tight protein pellets. Following centrifugation to pellet the proteins, supernatant was poured into 300 μl iced isopropanol. For 1998 samples, 0.5 μl

glycogen (Gentra Systems) was added to the isopropanol as a carrier. Glycogen was not added to the samples collected in 2000, as the glycogen did not appear to greatly enhance the visibility of the DNA pellet. Pellets were washed once with 70% EtOH and air-dried for 15 min. All samples were then resuspended in 50 μl DNA hydration solution (Gentra Systems) and left at room temperature overnight prior to storing at 4 °C.

Following storage at 4 °C for at least 24 h, samples were quantified using a spectrophotometer (Beckman DU-600) and quality was assessed by running a small amount of the DNA out on a 1% agarose gel containing ethidium bromide. Samples were then diluted to a concentration of 5 ng/μl for PCR amplification and RAPD analysis.

Sixty primers representing kits OPA, OPB and OPC (Operon Technologies, Inc.) were tested for polymorphism (Table 1.1). Each primer was used separately to amplify DNA fragments from eight adult male *A. carolinensis* located on our study site, but not used for behavioral observations. Of these 60 primers, 14 failed to amplify and 18 showed low amplification or poor resolution. Twenty-eight primers showed successful amplification. Of these 28 primers, 14 were non-polymorphic and 14 primers showed polymorphism. Of these 14 polymorphic primers, three were found to produce bands that allowed individual males to be uniquely identified from each other. These primers were OPA-16 (5' AGCCAGCGAA 3'), OPB-17 (5' AGGGAACGAG 3') and OPC-18 (5' TGAGTGGGTG 3').

The reaction mixture for RAPD-PCR contained 2.5 μl 10X PCR buffer (10 mM Tris-HCl, 50 mM KCl, 0.1%Triton X-100), 2.0 μl MgCl, 1.0 μl DNTPs (10 mM mix), 1.0 μl primer (0.125 μg/μl random 10-mer), 0.75 Units Taq polymerase in storage buffer B (Promega Corp.) and double distilled water to a total volume of 21 μl. Samples were placed into a PTC-100 Programmable Thermal Cycler (MJ Research, Inc). PCR cycles were as follows: 1 cycle at 95 °C for 5 min and 46 cycles at 94 °C for 10 sec (denature), 36 °C for 10 sec (anneal), 72 °C for 2 min (extend). Storage was at 4 °C.

Samples were amplified and separated in groups in order to keep all lizards residing within or near a given territory together. Three amplifications and separations (one for each of the three primers) were performed for each group. Two μl of 6X loading dye (Promega Corp.) was added to 12 μl of each amplified PCR. Samples were separated on 2% agarose gels containing 12 μl ethidium bromide in 1X TAE buffer (total gel volume 300 ml). Lane assignments were made so that hatchlings flanked potential fathers, while mothers were run in the outermost lanes. Gels were run at 50-60 V for 15-30 min and then at 100-110 V for 5-6 h. Following the initial run at 50-60 V, buffer was recirculated for the duration of the run using a pump. Separation of DNA fragments was visualized using the AlphaImager 2000 Documentation and Analysis System (Alpha Innotech Corp.) and photographed using a digital graphic printer (#UP-D890, Sony Corp.).

Gels were scored following the guidelines set in Scott et al. (1992). Band patterns were checked for reproducibility by repeating RAPD-PCR to insure that the same band patterns were achieved for each individual with each primer. Only diagnostic bands were used for scoring. Diagnostic bands were those bands unique to both a hatchling and one adult male (Fig 1.1 and Appendix 2). One criticism of using RAPD-PCR for parentage analysis has been that a true parent may be falsely excluded if there is a high percentage of non-parental bands in the offspring profile (i.e. bands unique to the offspring) (Reidy et al. 1992). If analysis is limited to only assigning paternity based on the presence of diagnostic bands (parentage by inclusion, Lewis et al. 2000), then presence of non-diagnostic bands will not affect paternity (Scott et al. 1992).

Because RAPDs are inherited in a Mendelian fashion (Williams et al. 1990), in sexually reproducing species, barring mutation, a hatchling's bands will come from its mother and father. Paternity estimates were made so as to be as conservative as possible. So, following Tirado and Lewis (2000), if a hatchling's RAPD profile lacked unique bands, I did not assign paternity to it (i.e. the father may have been present in those male lizards sampled, but I was not able to determine who he was using the primers that I employed). If a hatchling's RAPD profile contained unique bands, but these bands did not match any in the putative parents, paternity was said to be unaccounted for (i.e. the hatchling's father

was not among those males captured and removed from the field site). Despite having profiles for each lizard using three different primers, diagnostic bands were infrequent, and therefore, the presence of one diagnostic band was deemed sufficient to assign paternity.

Statistical Analyses

Grouped logistic regression using a chi-square approximation of the test statistic was performed to determine whether territory volume, location of females within the territory or size (mass) of the territorial male affected the incidence of extra-pair paternity. The proportion of hatchlings fathered by the territorial male was used as the dependent variable. Because the same protocols and sampling methods were followed for both the 1998 and 2000 field seasons, data from these field seasons were pooled.

Overall success of paternity was analyzed for each territorial male based on the number of offspring he fathered from any given female on his territory. Only hatchlings for which paternity was determined by presence of diagnostic bands (i.e., father was one of the males sampled) or hatchlings which had unique bands in their profile that were not present in the mother or any putative father (i.e., father not among those sampled and therefore resident male was not the father in these cases) were used in the analysis. Hatchlings for which paternity could not be determined were excluded from the analysis.

Investigating paternity in *A. carolinensis* differs from investigations of paternity in other lizards (e.g. Bull et al. 1998, Lewis et al. 2000) because unlike most lizards, *Anolis* species do not lay multiple-egg clutches. The challenge with *A. carolinensis* was obtaining enough eggs to make an overall estimate of male paternity. Given that I was only able to obtain one or two eggs from each female out of the 16-18 eggs during a breeding season, the overall measure of male *A. carolinensis* fitness may not be as robust as fitness measurements from non-Anoline species.

The eggs that a female laid immediately following her removal from the field were eggs that had been fertilized prior to my observations of that territory (Fig 1.2). An assumption of my study is therefore that the male who was the territorial male during my observation period was also the territorial male when the eggs in question were fertilized, i.e. no changes in territorial ownership occurred between when all eggs undergoing paternity analysis were fertilized and when I began observations.

Results

Incidence of extra-pair paternity

Fifty-four eggs were collected. I was unable to determine paternity for 6 hatchlings due to a lack of diagnostic bands in their RAPD profiles and these hatchlings were excluded from the statistical analyses. Diagnostic bands were present in the remaining 48 hatchlings. Six of the 48 hatchlings had a unique band that was not shared by any adult in the sampled population. Of the 48 eggs for which paternity was assigned, territorial males fathered 52% of the hatchlings of females residing within their territories. Of the hatchlings fathered by a male other than the territorial male, 15% were fathered by a neighboring male who shared a territorial border with the territorial male of interest and 21% were fathered by a non-territorial male who resided within the territorial male's territory (i.e., covert male). For the 12% of hatchlings that had a unique band that was not shared by any adult in the population, I was only able to conclude that their father was not the territory owner, but not whether their father was a neighboring male or a covert male (Fig 1.3).

Factors affecting territorial male paternity

The volume of a male's territory was positively correlated with the incidence of extra-pair paternity ($p = 0.04$, $\chi^2 = 4.32$, $df = 1$). However, the position of a female relative to the territorial male's patrol area (i.e., interior or periphery) was not significantly related to

the incidence of extra-pair paternity ($p = 0.19$, $\chi^2 = 1.71$, $df = 1$). Size (mass) of territorial males also did not affect the incidence of extra-pair paternity ($p = 0.75$, $\chi^2 = 0.10$, $df = 1$).

Size relationship between territorial males and covert males

I captured an average of 1.2 covert males per resident male territory. Wilcoxon rank sums tests indicated that these covert males were significantly smaller than resident males in both SVL ($p = 0.001$, $W = 3.38$, $df = 1$) and mass ($p = 0.0002$, $W = 3.67$, $df = 1$). Territorial male (n=17) SVL mean±SE = 65.8±1.09 mm; mass mean±SE = 6.04±0.29 g; covert male (n=12) SVL mean±SE = 59.2±1.11 mm; mass mean±SE = 3.97±1.03 g; resident female (n=27) SVL mean±SE = 54.4±0.68 mm; mass mean±SE = 3.87±0.19 g.

Observed turnovers of territories

Over the course of ~130 h of observation during the 2000 field season, I witnessed two instances in which territories changed ownership. In one instance I observed two adjacent, marked, territorial males exchanging displays. One male moved into the other's territory and the males began circling each other and jaw sparring. Twice, the invading male was thrown off of the perch following a bout of jaw sparring. The second time this occurred, the thrown male did not return to either his own territory or the territory he previously invaded. The following day the male whose territory was invaded was patrolling both his territory and that of the invading male. When I located the invading male on the following day, he was dark brown with fresh wounds on his head and was sitting without moving on the ground at the edge of the territory he had previously patrolled.

In the second instance of changing territory ownership, following two days of observation of a resident male on his territory, I became unable to locate him. After periodic observation of this territory over the next few days, I observed the adjacent resident male patrolling the territory of the absent male and subsequently located the

displaced male on the ground at the edge of his former territory. As occurred in the first observation, the displaced male was on the ground, not moving and dark brown in color.

Discussion

Male and female *A. carolinensis* exhibit strategies that allow them to maximize their respective reproductive success. My data provide an estimate of how effective male behaviors are for insuring paternity and greater insight into female behaviors to insure that the best sperm fertilize her eggs.

I hypothesized that the larger a male's territory, the more likely offspring of females residing within his territory would be fathered by males other than him because the larger the territory, the harder it would be for the territorial male to patrol all areas at one time, thus making it easier for a neighbor to move in undetected and mate with a resident female. My data provide evidence for larger territories having more incidences of extra-pair paternity, but the extra–pair paternity surprisingly is not from neighboring males, but from non-territorial males (i.e., covert males) living within the patrolled territory itself.

Territorial males appear to be successful in patrolling their territorial borders to prevent neighboring territory holders from entering. Most of the offspring who were not fathered by the territorial male were also not fathered by a neighboring territorial male. Neighboring males are being kept out of adjacent territories, thus prohibiting neighbors the opportunity of mating with a neighbor's resident females. Further evidence for a territorial male's success at preventing neighboring males from entering his territory is that the incidence of extra-pair paternity by a neighboring male is no different for a female whose territory is located on the periphery of a male's territory than for a female whose territory is centrally located within a male's territory. My second hypothesis, therefore, is not supported. Offspring of peripherally located females are not fathered by neighboring males more often than offspring of centrally located females. Neighboring males are not more successful at moving a short distance into another male's territory to mate with females than they are at moving a long distance although neighboring males do

mate with both centrally and peripherally located females. Neighboring males may be successful at moving into another male's territory to mate with a resident female, but a resident female may also briefly move into the territory of the neighboring male to mate. This movement of females into a neighboring male's territory may occur as she is returning to her territory following having moved to the base of a tree in order to oviposit her egg in the moist soil there.

Territorial males seem to detect covert males within their territories and the mating of their resident females with these males only occasionally. These covert males are fathering 21% of the offspring born to females residing within a male's territory. If territorial males are spending a majority of their time patrolling their territories and being successful at preventing neighbors from moving in, then the territorial males may be spending their patrol time patrolling the borders of their territory but may not be spending as much time patrolling within their territories. Thus, a male residing undetected within the territory of a resident male would be able to successfully mate with the females in residence there.

These non-territorial males are smaller than territorial males, sedentary, and inconspicuous. It is possible that non-territorial males are not residents, but move from territory to territory (i.e., float). However, given evidence that females encountering novel males will scatter so as to "judge new males from afar" (Greenberg & Noble 1944), these males are probably not transitory, but simply non-territorial residents. In being permanent residents, a non-territorial resident would be recognized as familiar by females with home ranges within the area and thus more likely to be successful at mating with them than a male who constantly moves about the habitat. Covert males mate with both centrally and peripherally located females. Given that I captured covert males throughout the area patrolled by the territorial male, it is likely that covert males are moving in order to encounter females as opposed to females moving to the location of the covert male.

In *A. carolinensis*, growth rates are asymptotic during development, such that larger lizards are older than smaller ones (Michaud 1990). Given that covert males are

significantly smaller than territorial males, these younger, covert males appear to be engaging in a temporary strategy for reproduction until they are old enough and hence, large enough, to contest a territory. If males were followed through multiple seasons, covert males should become territorial males late in their first breeding season or at the start of the subsequent breeding season.

Why do territorial males seemingly tolerate these younger males living within their territories and gaining access to resident females? A territory owner must first detect the covert male. *Anolis carolinensis* appear to recognize individuals. Males determine which females to approach for mating (Jenssen & Nunez 1998) and can recognize individual females (Orrell & Jenssen, in press), females shy away from unfamiliar males (Greenberg & Noble 1944), resident males almost immediately approach unfamiliar conspecific males on their territory (personal observation) and prefer to mate with unfamiliar females (Orrell & Jenssen, in press; in *A. sagrei*, Tokarz 1992). So it is not that a resident male would mistake a covert male for a female, it is that the territorial male, with patrolling his territory borders, locating and mating with resident females and eating when possible, may not be as likely to detect a male who is spending his time attempting to elude the territory owner.

To maximize fitness, a female should insure that the highest quality sperm fertilize her eggs. If a female mated only with her resident male and that male were sterile, then her fitness would be zero. It pays therefore, for a female to hedge her bets and mate with multiple males. By mating with multiple males, at the very least, a female insures viable sperm in her tract (Gibson & Jewell 1982). By mating with multiple males and having multiple sources of sperm in her tract, a female moves the competition from pre-copulation to post-copulation, from males competing over access to the female herself to sperm competing against other sperm. Cryptic female choice (sensu Thornhill 1983, Eberhard 1991) can be employed to determine which sperm fertilize the eggs. Because a female has the ability to store sperm, she is able to "size up" new males, whether these new males be resident males who have just been successful in a territory takeover or

covert males who have just moved onto the territory containing the female's home range prior to mating with them, while still having sperm available to fertilize her eggs.

In summary, I have shown that extra-pair paternity occurs in *A. carolinensis*. The fitness of territorial males decreases with the size of a male's territory. The incidence of polygamy by resident females is independent of the location of a female's home range within the male's territory. While neighboring territorial males do contribute to some instances of extra-pair paternity, the majority of offspring who are not fathered by the territorial male are fathered by covert males.

Appendix 1: Data set for Chapter 1

Key:
SVL: snout-vent length
Neighbor: male with a territory that borders that of the resident male of interest
X: indicates male to which offspring paternity was assigned
Female identifiers contain the letter of the male in whose territory she resides followed by a number indicating when she was captured. E.g. Female A2 was the second female captured from within the territory of resident male A.
Offspring identifiers contain the id of their mother followed by a number indicating when they were laid. E.g. Offspring A3.2 hatched from the second egg laid by female A3 following her capture.

Field season 2000

Territory A, vol.: 142.2 m^3			
Resident females and location within territory	Offspring of resident females	Territorial male A *SVL: 65 mm *mass: 5.9 g	Covert male Aa *SVL: 63 mm *mass: 5.4 g
A1 interior *SVL: 49 mm *mass: 2.4 g	A1.1	X	
	A1.2	X	
A2 peripheral *SVL: 50 mm *mass: 3.5 g	A2.1	X	
	A2.2		X
A3 interior *SVL: 49 mm *mass: 3.4 g	A3.1	X	
	A3.2	father unaccounted for	

Territory B, vol.: 124.7 m^3			
Resident females and location within territory	Offspring of resident females	Territorial male B SVL: 66 mm mass: 5.8 g	Covert male Ba SVL: 65 mm mass: 5.4 g
B2 interior SVL: 62 mm mass: 4.5 g	B2.2	father unaccounted for	

Territory C, vol.: 152.0 m^3			
Resident females and location within territory	Offspring of resident females	Covert male Ca SVL: 60 mm mass: 3.9 g	Covert male Cb SVL: 55 mm mass: 3.0 g
C1 peripheral SVL: 58 mm mass: 4.2 g	C1.2	X	
	C1-3	father unaccounted for	
	C1-4		X
	C1-5		X

Territory D, vol.: 105.4 m^3			
Resident females and location within territory	Offspring of resident females	Territorial male D SVL: 65 mm mass: 4.8 g	Covert male Da SVL: 61 mm mass: 5.5 g
D1 interior SVL: 55 mm mass: 3.3 g	D1.1	X	
D2 peripheral SVL: 55 mm mass: 4.5 g	D2.3	X	

Territory E, vol.: 315.7 m^3				
Resident females and location within territory	Offspring of resident females	Territorial male E SVL: 74 mm mass: 6.2 g	Covert male Ea SVL: 59 mm mass: 3.8 g	Neighbor: Territorial male F
E2 interior SVL: 55 mm mass: 3.6 g	E2.1		X	
	E2.2		X	
E4 interior SVL: 61 mm mass: 4.5 g	E4.1		X	
	E4.2		X	
	E4.3	father unaccounted for		

Territory F, vol.: 297.8 m^3						
Resident females and location within territory	Offspring of resident females	Territorial male F SVL: 61 mm mass: 5.0 g	Covert male Fa SVL: 53 mm mass: 2.8 g	Covert male Fb SVL: 55 mm mass: 2.5 g	Neighbor: Territorial male E	Neighbor: Territorial male G
F1 peripheral SVL: 50 mm mass: 2.4 g	F1.2	X				

Territory G, vol.: 147.2 m^3						
Resident females and location within territory	Offspring of resident females	Territorial male G SVL: 64 mm mass: 4.9 g	Covert male Ga SVL: 65 mm mass: 4.5 g	Covert male Gb SVL: 59 mm mass: 3.3 g	Neighbor: Territorial male Gn SVL: 62 mm mass: 4.7 g	Neighbor: Territorial male F
G1 interior SVL: 55 mm mass: 3.5 g	G1.1	father unaccounted for				
	G1.2	father unaccounted for				
G3 peripheral SVL: 57 mm mass: 3.0 g	G3.1				X	
	G3.2				X	

Territory K, vol.: 142.2 m^3			
Resident females and location within territory	Offspring of resident females	Territorial male K SVL: 62 mm mass: 5.1 g	Covert male Ka SVL: 57 mm mass: 3.7 g
K1 interior SVL: 50 mm mass: 2.8 g	K1.1	X	
	K1.2	X	
K2 peripheral SVL: 53 mm mass: 3.3 g	K2.1		X
	K2.2	X	

Territory L, vol.: 50.6 m^3			
Resident females and location within territory	Offspring of resident females	Territorial male L SVL: 67 mm mass: 5.9 g	Covert male La SVL: 59 mm mass: 3.8 g
L1 interior SVL: 52 mm mass: 3.1 g	L1.1		X

Territory M, vol.: 92.2 m^3			
Resident females and location within territory	Offspring of resident females	Territorial male M SVL: 70 mm mass: 6.0 g	Neighbor: Territorial male L
M1 peripheral SVL: 56 mm mass: 3.0 g	M1.1	X	
M2 interior SVL: 54 mm mass: 3.0 g	M2.1	X	
	M2.2	X	
M3 interior SVL: 57 mm mass: 3.2 g	M3.1	X	
	M3.2		X

Field season 1998

Territory B, vol.: 60.6 m^3			
Resident females and location within territory	Offspring of resident females	Territorial male B SVL: 60 mm mass: 6.5 g	Neighbor: Territorial male C
B1 interior SVL: 51 mm mass: 5.4 g	B1.2	X	
B4 interior SVL: 55 mm mass: 4.8 g	B4.1	X	
	B4.2	X	

Territory C, vol.: 150.6 m^3			
Resident females and location within territory	Offspring of resident females	Territorial male C SVL: 61 mm mass: 5.1 g	Neighbor: Territorial male B
C1 peripheral SVL: 50 mm mass: 4.5 g	C1.1		X
	C1.2		X

Territory L, vol.: 75.1 m^3			
Resident females and location within territory	Offspring of resident females	Territorial male L SVL: 62 mm mass: 7.1 g	Neighbor: Territorial male M
L1 interior SVL: 60 mm mass: 6.4 g	L1.1	X	
L2 interior SVL: 54 mm mass: 4.8 g	L2.1	X	
	L2.2	X	

Territory M, vol.: 139.5 m^3			
Resident females and location within territory	Offspring of resident females	Territorial male M SVL: 69 mm mass: 7.4 g	Neighbor: Territorial male L
M1 interior SVL: 54 mm mass: 4.7 g	M1.1	X	

Territory O, vol.: 106.5 m^3			
Resident females and location within territory	Offspring of resident females	Territorial male O SVL: 70 mm mass: 7.8 g	Neighbor: Territorial male Q SVL: 75 mm mass: 9.1 g
O1 interior SVL: 55 mm mass: 4.4 g	O1.1		X
	O1.2		X

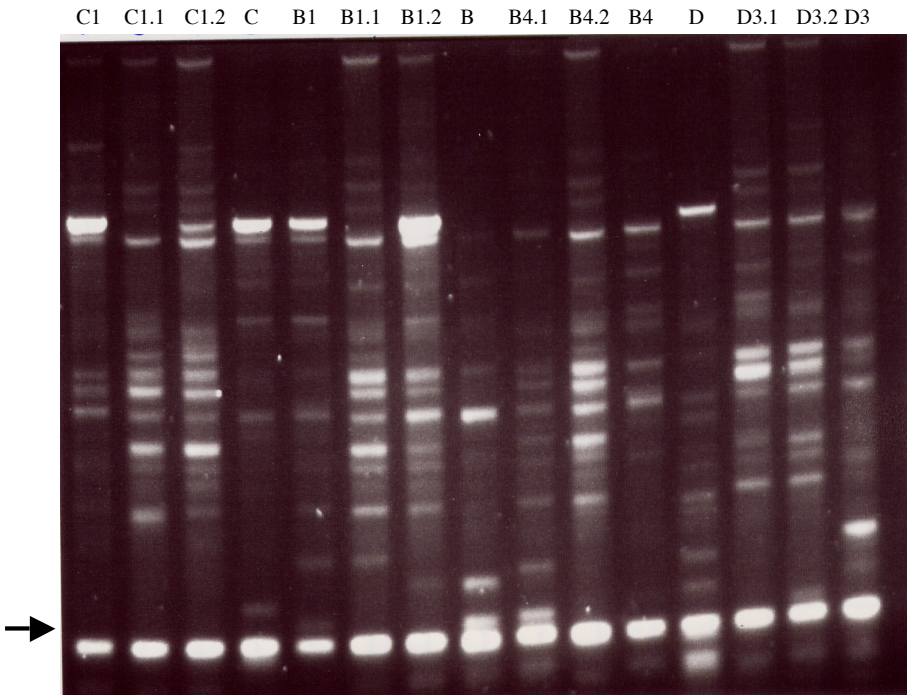
Territory R, vol.: 132.0 m^3			
Resident females and location within territory	Offspring of resident females	Territorial male R SVL: 65 mm mass: 5.5 g	Neighbor: Territorial male Q SVL: 75 mm mass: 9.1 g
R1 peripheral SVL: 55 mm mass: 4.4 g	R1.1	X	
	R1.2	X	
R3 interior SVL: 56 mm mass: 4.6 g	R3.1	X	
	R3.2	X	

*Territorial male (n=17)
SVL mean±SE = 65.8±1.09 mm
mass mean±SE = 6.04±0.29 g

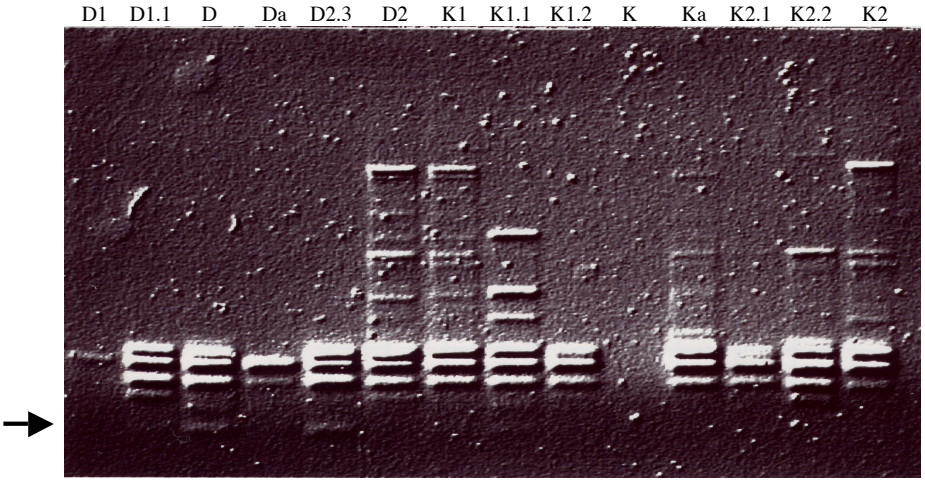
Covert male (n=12)
SVL mean±SE = 59.2±1.11 mm
mass mean±SE = 3.97±0.29 g

Resident female (n=27)
SVL mean±SE = 54.4±0.68 mm
mass mean±SE = 3.87±0.19 g

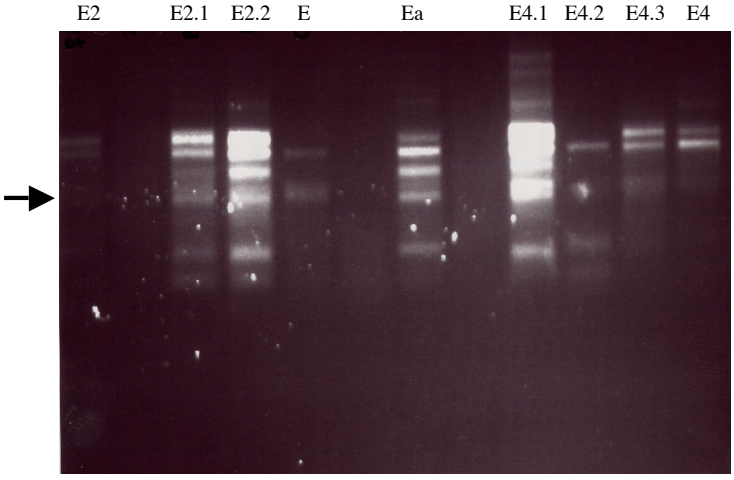
Appendix 2: Additional gel photos generated via RAPD-PCR showing assignment of paternity based on the presence of diagnostic bands (arrow). Each lane indicated by a single uppercase letter contains the profile of a resident male. Covert males are indicated by the uppercase letter of the resident male in whose territory they reside and a lowercase letter. Females are indicated by the uppercase letter of the male in whose territory they reside and a single number to indicate the order in which they were captured. Hatchlings are indicated by the letter and number of their mothers followed by a second number to distinguish them from their siblings. (e.g. C1.2 is the second hatchling born to female C1).



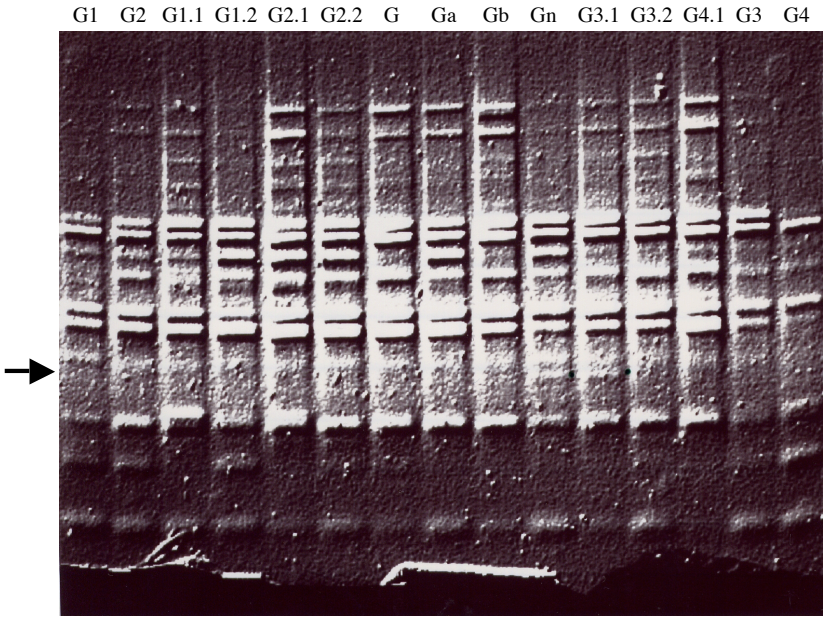
Based on the presence of the diagnostic band (arrow), paternity of hatchling B4.1 is assigned to male B. This gel also shows the repeatability of the banding patterns as it is a repeat of the RAPD-PCR shown in fig. 1.3 (primer OPA-16).



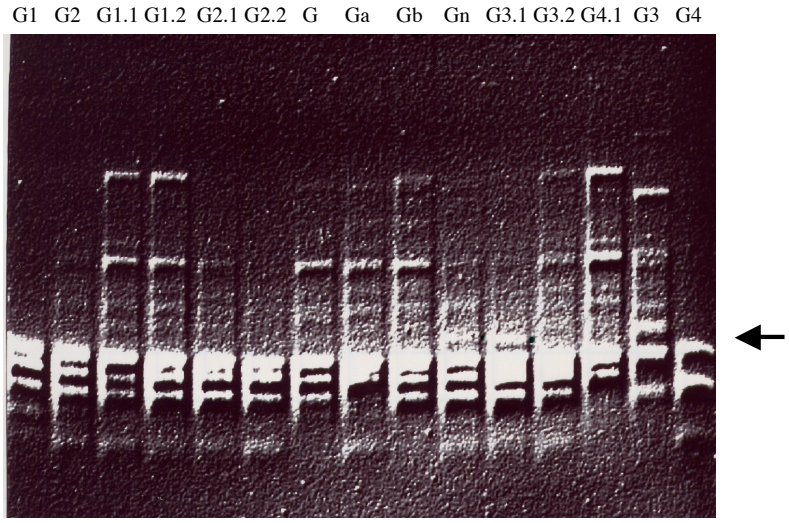
Based on the presence of the diagnostic band (arrow), paternity of hatchling D2.3 is assigned to male D (primer OPA-16).



Based on the presence of the diagnostic band (arrow), paternity of hatchlings E2.1 and E2.2 are assigned to male Ea (primer OPB-17).



Based on the presence of the diagnostic band (arrow), paternity of hatchling G3.1 is assigned to male Gn (primer OPB-17).



Based on the presence of the diagnostic band (arrow), paternity of hatchling G3.1 is assigned to male Gn (primer OPA-16).

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Table 1.1. Amplification patterns produced by primers used in RAPD-PCR of male *Anolis carolinensis*.

Pattern	Primer	Sequence
Polymorphic	OPA-07	GAAACGGGTG
	OPA-11	CAATCGCCGT
	OPA-12	TCGGCGATAG
	OPA-16	AGCCAGCGAA
	OPA-18	AGGTGACCGT
	OPA-19	CAAACGTCGG
	OPA-20	GTTGCGATCC
	OPB-03	CATCCCCCTG
	OPB-17	AGGGAACGAG
	OPC-02	GTGAGGCGTC
	OPC-04	CCGCATCTAC
	OPC-18	TGAGTGGGTG
	OPC-19	GTTGCCAGCC
	OPC-20	ACTTCGCCAC
Non Polymorphic	OPA-09	GGGTAACGCC
	OPA-13	CAGCACCCAC
	OPB-04	GGACTGGAGT
	OPB-05	TGCGCCCTTC
	OPB-06	TGCTCTGCCC
	OPB-07	GGTGACGCAG
	OPB-08	GTCCACACGG
	OPB-09	TGGGGGACTC
	OPB-10	CTGCTGGGAC
	OPB-12	CCTTGACGCA
	OPB-18	CCACAGCAGT
	OPC-01	TTCGAGCCAG
	OPC-05	GATGACCGCC
	OPC-16	CACACTCCAG

Low Amplification	OPA-03	AGTCAGCCAC
	OPA-14	TCTGTGCTGG
	OPA-15	TTCCGAACCC
	OPA-17	GACCGCTTGT
	OPB-01	GTTTCGCTCC
	OPB-11	GTAGACCCGT
	OPB-13	TTCCCCCGCT
	OPB-15	GGAGGGTGTT
	OPC-06	GAACGGACTC
	OPC-07	GTCCCGACGA
	OPC-08	TGGACCGGTG
	OPC-09	CTCACCGTCC
	OPC-10	TGTCTGGGTG
	OPC-11	AAAGCTGCGG
	OPC-12	TGTCATCCCC
	OPC-13	AAGCCTCGTC
	OPC-14	TGCGTGCTTG
	OPC-15	GACGGATCAG
No Amplification	OPA-01	CAGGCCCTTC
	OPA-02	TGCCGAGCTG
	OPA-04	AATCGGGCTG
	OPA-05	AGGGGTCTTG
	OPA-06	GGTCCCTGAC
	OPA-08	GTGACGTAGG
	OPA-10	GTGATCGCAG
	OPB-02	TGATCCCTGG
	OPB-14	TCCGCTCTGG
	OPB-16	TTTGCCCGGA
	OPB-19	ACCCCCGAAG
	OPB-20	GGACCCTTAC
	OPC-03	GGGGGTCTTT
	OPC-17	TTCCCCCCAG

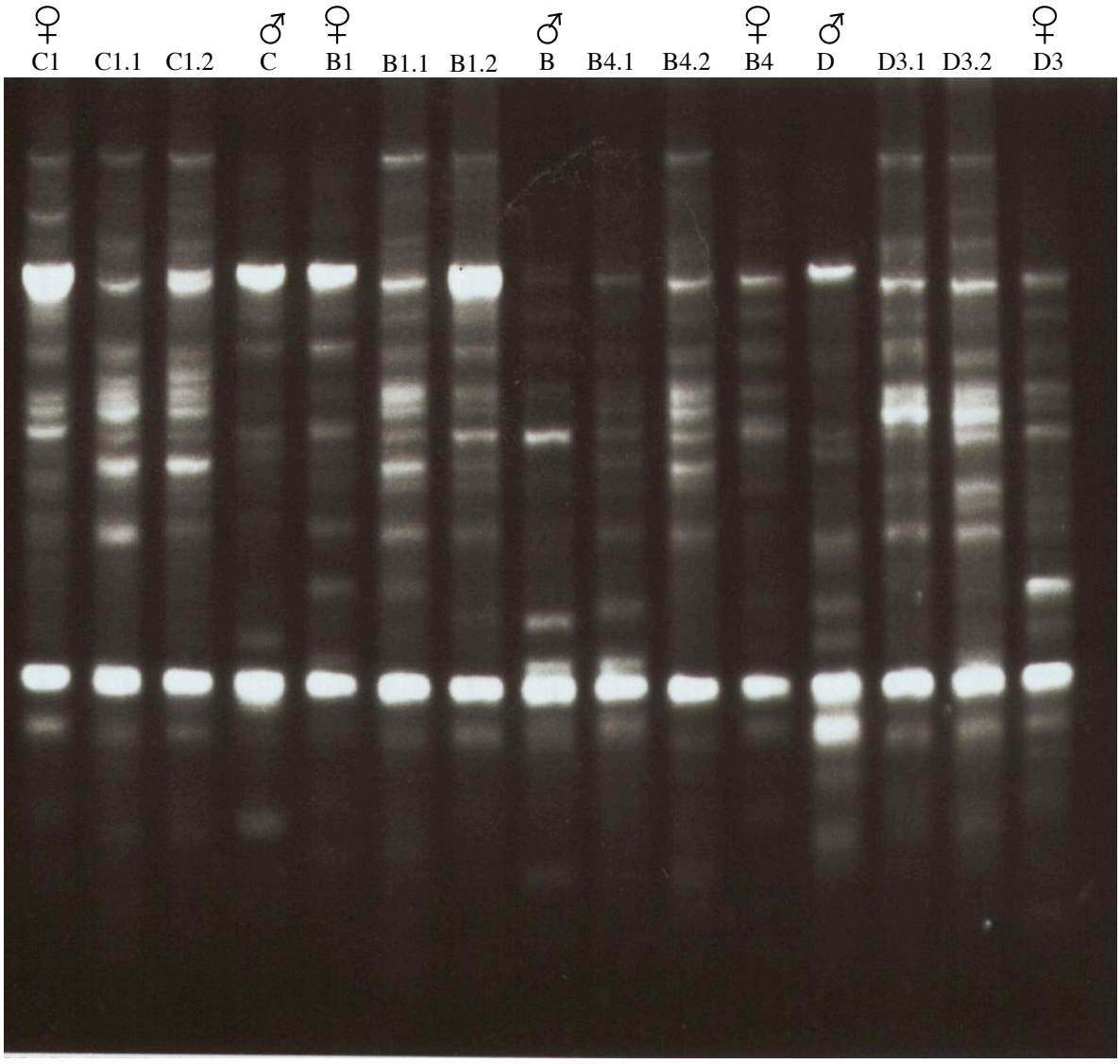

Figure 1.1. Profile generated via RAPD-PCR illustrating use of diagnostic bands (arrow) to assign paternity. The profile shows banding patterns generated by primer OPA-16 for lizards from territories B, C, and D from the 1998 field season. Males B, C and D are territorial males with territories adjacent to each other such that B held the center territory with C and D on either side of him. Each lane indicated with a male symbol followed by a letter is the profile of a resident male. Females residing within his territory are indicated with a female symbol followed by the same letter as their resident male and a number indicating the order in which she was captured (e.g. female C1 is the first female captured from her home range within the area patrolled by male C). Between the resident male and resident female lanes are the hatchlings born to that resident female (e.g. C1.2 is the second hatchling born to female C1. A diagnostic band is indicated by the arrow and allows paternity of hatchling B4.1 to be assigned to male B. The band is present in both hatchling B4.1 and male B, but not female B4 (mother of B4.1) or either neighboring male.

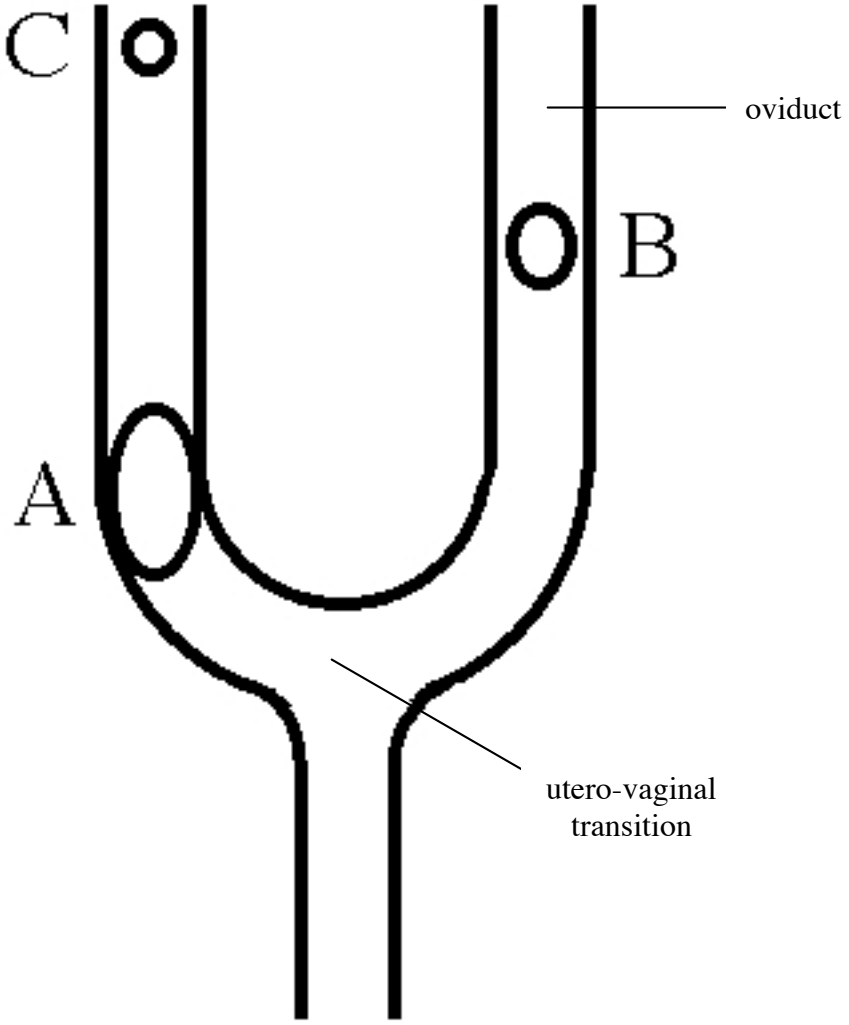

Figure 1.2. Diagram of the oviduct illustrating the location of three successive eggs, where A represents an egg fertilized ten days prior to capture, B represents an egg fertilized five days prior to capture, and C represents an egg fertilized at or near capture.

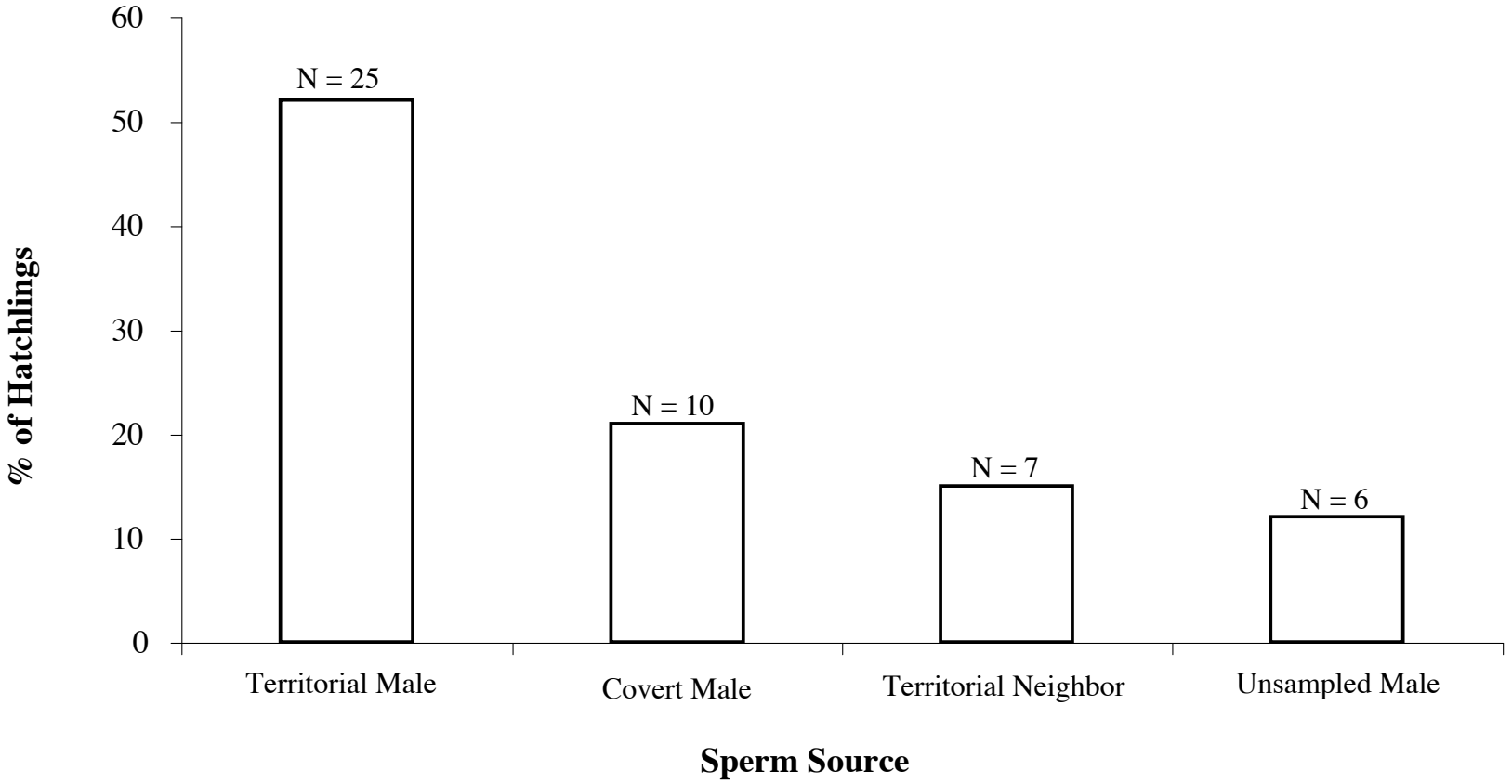

Figure 1.3. Source of paternity for 48 hatchling *Anolis carolinensis* based on results of RAPD-PCR analysis. Bars indicate the percentage of hatchlings fathered by each of four categories of males. Territorial males are those males who patrolled the territory containing the mother's territory. Covert males are those non-territorial males that reside within a resident male's territory. Territorial neighbors are those males who hold a territory adjacent to the territorial resident. Unsampled males are males that were not collected from the field, and so whether they are covert males or territorial neighbors is unknown, but they are not territorial residents and so represent additional non-resident male paternity.

Chapter 2

Sperm precedence in controlled matings of *Anolis carolinensis*

Abstract

Sperm precedence describes the nonrandom fertilization of a female's eggs. Three types of sperm precedence have been described. Sperm stratification describes a situation in which the position of sperm within the sperm storage organ of the female reproductive tract predicts which sperm are likely to fertilize the egg. Sperm loading describes sperm precedence that results simply due to the number of sperm introduced by a given male into the female tract. Sperm selection involves not only competition among males to mate with a female, but also competition among ejaculations. I tested the lizard, *Anolis carolinensis*, known to store sperm, for evidence of sperm precedence within controlled matings. Four females were sequentially paired with two of four males. Following mating of each female with both of the males, RAPD-PCR techniques were used to determine the paternity of the resulting hatchlings. Regardless of mating order, one male of the pair fathered all of the offspring. As fertilization was not random, sperm precedence does occur in *A. carolinensis*. Because one male fathered all of the offspring of a given female regardless of mating order, sperm precedence in the form of sperm stratification does not occur. However, from these results, I am unable to distinguish between sperm precedence in the forms of sperm loading and sperm selection.

Introduction

In species in which females mate with multiple males per ovulatory event or store viable sperm of multiple males within a breeding season, sperm precedence becomes an issue. Sperm precedence describes the nonrandom fertilization of a female's eggs. It is a form of sexual competition that continues following copulation (Andersson 1994). Sperm precedence can take the form of sperm stratification, sperm loading, or sperm selection (Simmons & Siva-Jothy 1998).

Sperm stratification describes a situation in which the position of sperm within the sperm storage organ of the female reproductive tract predicts which sperm are likely to fertilize the eggs (Simmons & Siva-Jothy 1998). Sperm storage organs or areas may be arranged such that the sperm of the first male fills the more proximal area of the storage organs (thus closer to the egg that will be released) and the second male's sperm will thus be relegated to being stored more distally. This situation would result in the sperm from the first male fertilizing the eggs (i.e. first male sperm precedence). Sperm storage organs may also be arranged such that the sperm of the second male is layered over the sperm of the first male. In these instances, the sperm of the second male would fertilize the eggs (i.e. second male sperm precedence). Sperm stratification, specifically where the sperm of the last male to mate with the female fertilizes the eggs, is the most common type of sperm precedence observed (DeVilliers & Hanrahan 1991).

Sperm loading describes sperm precedence that results simply due to the number of sperm introduced by a given male into the female tract (Simmons & Siva-Jothy 1998). In this mechanism, a male who was able to introduce more sperm into the female tract than another male would father the most offspring. By increasing the ejaculate size or extra-gonadal reserves, a male would increase the number of eggs fertilized. However, there is a trade-off. In simply allocating more sperm to a particular female, a male increases the risk of depleting his sperm store with a single female (Gage & Baker 1991).

Both sperm stratification and sperm loading appear to be a consequence of anatomical structure. In the case of sperm stratification, sperm precedence is based on the structure of the female sperm storage organs. In the case of sperm loading, sperm precedence is based on the size and productivity of the male testes and extra-gonadal reserves.

Sperm selection, however, describes the "extension of female choice at the genetic level" (Simmons & Siva-Jothy 1998). Sperm selection involves not only competition among males to mate with a female, but also competition within ejaculates (i.e., interejaculate competition, sensu Parker 1998) (Smith 1998).

Selection should favor males who can displace stored sperm in the female tract with their own to increase their paternity of available eggs. For species that exhibit mechanisms of sperm competition, male fitness is not necessarily proportional with number of mated females (Eberhard 1996). A male who is able to both introduce a large amount of sperm into the female tract and also prevent additional males from copulating with her would have a higher selective advantage over males who employ only one of the above strategies (Parker 1970).

Anolis carolinensis is a species in which sperm competition is likely for the following reasons. First, *A. carolinensis* exhibits a polygynous mating strategy. Despite a reported 1:1 adult sex ratio (Jenssen et al. 1995), Jenssen et al. (1995) and Nunez et al. (1997) have observed up to six resident females defended and mated by a single territorial male. While *A. carolinensis* males defend territories that contain multiple female home ranges, peripheral females may be overlapped by more than one male. Females tend to mate with the male(s) who overlaps their home range (Ruby 1984). This provides the opportunity for females to mate multiple times during each reproductive cycle. This potential for females to mate with more than one male would prevent any one male from being able to insure paternity of the offspring of his resident females. In polygynous species, success in competition over mates is crucial for the fitness of the male, so one would expect to see male strategies evolving to reduce the impact of extra-pair copulations (Birkhead & Moller 1992).

Second, the breeding season of *A. carolinensis* extends from mid-April through mid-August (Gordon 1956). During this four month breeding season, females continuously clutch a single egg every four to ten days (Andrews 1985). Thus, there is opportunity for a separate insemination of each of the 16-18 clutches.

Third, sperm storage is present in *A. carolinensis*. Fox (1963) initially reported the presence of sperm storage tubules in *A. carolinensis*. These tubules have been further described as being embedded in the wall of the utero-vaginal transition, having been formed by the folding and fusion of the oviducal epithelium. Sperm enter the sperm storage tubules between 2-6 h after insemination, and if these sperm fertilize an egg, they enter the infundibulum 6-24 h post mating (Conner & Crews 1980).

Given that female *A. carolinensis* have the opportunity to mate with multiple males per ovulatory cycle and have the ability to store sperm, sperm precedence is likely occurring. I hypothesized that fertilization was not random, but that some type of sperm precedence existed. To examine the role that females might play in promoting sperm competition, I conducted controlled matings in a laboratory setting in which I paired individual females first with one male and then with another male and then analyzed the paternity for the hatchlings born to the females to determine which sperm fertilized the eggs.

Methods

I tested whether mating order by males predicted paternity, where my assumption was that fertilization was not random, but that sperm precedence occurred. To perform the test, I ran a replicated sequence of matings by pairs of males in the following design.

I captured 16 adult female (mean SVL±SE = 50.2±1.22 mm) and eight adult male (mean SVL±SE = 66.1±1.77 mm) *A. carolinensis* from my study site on the Augusta Canal between 05-06 March 2000. Lizards were obtained prior to the start of the reproductive

season (mid-April through mid-August; Gordon 1956) to insure that females had no fresh sperm in their reproductive tracts.

Collected lizards were individually numbered on their bellies with a permanent marker and measured for body length, then transported to Virginia Tech where they were housed individually in cages measuring 55 x 27.5 x 60 cm. Lizards were fed crickets dusted with calcium powder and cages were misted to provide water daily. Lizards were kept on a 12:12 light:dark cycle (light cycle ran from 0800 to 2000 h) from 07-13 March 2000 and then the light cycle was increased to 14:10 light:dark (light cycle ran from 0600 to 2000 h) for the duration for the experiment (01 May 00 to 07 June 00) to mimic natural light conditions.

Nine days following capture, a blood sample (75-200 μl volume) was taken from the postorbital sinus of each male lizard. Blood was collected in heparinized microhematocrit tubes and then placed into 1.5 ml microcentrifuge tubes containing 200 μl 1X TNE buffer. Blood and buffer were pipetted up and down two times to mix and placed at –80 °C. RAPD profiles were obtained for all eight male lizards using the procedures described in Chapter 1 for DNA extraction and RAPD-PCR. Three profiles were obtained for each male using the primers OPA-16, OPB-17 and OPC-18 (Operon Technologies, Inc.). Profiles from all males were compared in order to determine which males could be molecularly differentiated from each other. A male lizard was paired with another male lizard if at least one male in the pair had a unique (diagnostic) band in its profile.

Mating trials were constructed by randomly assigning four females to each pair of males. Two females of each four were randomly chosen to be placed first with one male of the pair and then the other male and the remaining two females had this pairing order reversed (mating first with the male with whom the other two females would mate with second). Trials were constructed such that each female was run with only one pair of males, but each pair of males was placed in mating trials with more than one female (Table 2.1).

The experiment was designed so as to allow four mating trials to be run simultaneously. Four 19 L enclosures were set up adjacent to each other with partitions in between to prevent a lizard in one enclosure from seeing into another. The bottom surface of each enclosure was covered with 1-2 cm of mulch and each enclosure contained two wooden dowels that lizards used for perching. Enclosures were covered with a wire screen and a light fixture containing a Durotest bulb (full spectrum) was placed directly on top of the screen over all of the enclosures.

Trials were continuously videotaped and periodically observed from behind a blind. Videotapes were reviewed prior to beginning the next set of trials to verify the occurrence of copulations. Videotaping began just prior to introducing the lizards into the enclosures and average trial duration was 3.5 h. Controlled matings with *A. sagrei* indicated that matings are most likely to occur within the first hour following introduction of the male and female lizards (Tokarz, personal communication). If a copulation occurred, the female was placed into the trial cage with the second male of her pair following at least 24 h alone in her home cage. If mating did not occur during the trial, both lizards were returned to their home cages and that trial was repeated within the next three days (usually repeated the next day). Unsuccessful trials were sometimes not repeated the following day because priority went to females completing successful trials (i.e., a trial in which a copulation occurred). These females would then be placed with the second male of her pair even when it meant letting the female who had been engaged in trials with this second male go untested for the day. Once a female copulated with both males of her pair, no further manipulation of this female occurred; she was returned to her holding cage and monitored for newly laid eggs.

Females were palpated to determine if eggs were present in their tract prior to each trial. Although females were collected prior to the start of the reproductive season, they are capable of storing sperm (Fox 1963). So a female that was reproductive last season may have viable sperm in her tract. Because I was interested in only the paternity of the eggs following inseminations by both experimental males, I needed to be aware of when an egg(s) was present in the tract relative to where a female was in terms of completing the

mating trials. Two examples can be used to clarify this point. First, if prior to the start of the mating trial with the first male, a female was found to have an egg(s) in her tract, then I knew that that egg was fertilized with stored sperm and I would be unable to determine paternity for that hatchling. Second, if prior to mating with the second male, a female was found to have an egg(s) in her tract, then that egg was fertilized with either stored sperm or sperm from the first male of the experimental trials. Given that I wanted to investigate sperm precedence, I was interested in the paternity of the eggs that a female oviposits once she had sperm available from both experimental males to fertilize the eggs (in possible addition to stored sperm).

Females were provided with a dish containing moist soil in their home cages in which they oviposited their eggs. Dishes were checked daily for eggs. Upon finding an egg, I followed incubation procedures as described in Chapter 1. Once I did not detect any additional eggs in the female tract through palpation, blood samples were taken from the females following the same protocol that I used for the eight males. Males and females were then placed at –80 °C.

Seven females successfully completed the mating trials by copulating with both males of their pair. Of these seven females, four laid at least the egg that would have been fertilized while sperm from both males were present in their tract. The other three females ceased laying eggs prior to laying the egg of interest for this study. Three of these females had been assigned to the same pair of males for their mating trials, so in effect, my sample size was reduced to two pair of males: pair 1 who mated with female #1 and pair 2 who mated with females #2, 3 and 4.

At hatching, each hatchling was given an identification number consisting of its mother's identification number and a number to uniquely identify it from its siblings. Hatchlings were then placed at –80 °C. RAPD-PCR was used to determine the paternity of each hatchling. Protocols were followed as described in Chapter 1. Paternity was assigned using the presence of diagnostic bands.

Statistical analyses involving the binomial distribution were performed to determine if fertilization was random or if sperm precedence was occurring. A Wilcoxon rank sums test was used to compare male SVLs to determine if paired males were significantly different in size from each other.

Results

Evidence for non-random fertilization

Twenty eggs were laid and incubated to hatching when sperm from both males of the mating trials was present in the females' tracts. For all hatchlings for which paternity was assigned (n = 16), only one male of the pair that the female mated with fathered all of the offspring. Assuming that neither male of the pair was sterile, if fertilization was random, it would be expected that 1/2 of the eggs would be fertilized by one male of the pair and 1/2 by the other male of the pair. Of the hatchlings for which paternity was assigned, male #4 fathered four of four hatchlings (all from the same female) while male #3 fathered none and male #8 fathered 12 of 12 hatchlings (from three females) while male #7 fathered none. Hatchlings for which I was unable to assign paternity to either male of the mating trials (n = 4) were from the last eggs laid by a given female. It is possible that these eggs were fertilized with sperm stored from the previous season (Table 2.2).

Using the binomial distribution to test the hypothesis that fertilization is random, if eggs are independent and fertilization occurs at random, then the probability that 4 out of 4 hatchlings belong to male #4 and none to male #3 is $(4/4)\ (1/2)^4 = 0.06$ and the probability that 12 out of 12 hatchlings belong to male #8 and none to male #7 is $(12/12)\ (1/2)^{12} = 0.0002$. These probabilities are extremely small and so I conclude that fertilization did not occur at random. So, sperm precedence in *A. carolinensis* does not seem to be affected by mating order (i.e., neither first nor second male sperm precedence occurring).

Males within a pair were not significantly different in size. The SVL of male #4 SVL was 69 mm as compared to 65 mm of male #3 and 67 mm of male #8 as compared to 61 mm of male #7. A Wilcoxon rank sums test to test whether these lengths are significantly different from each other, indicates that the male who fathered the hatchlings is not significantly different in size from the male who did not father the hatchlings ($W = 1.16$, $df = 1$, $p = 0.25$).

Copulation duration

Average copulation duration ± SE for 22 matings was 18.7±2.61 min (range 2.0 to 45.0 min). In four instances, females copulated twice during a single trial. In the first instance, the duration of the first copulation was 26 min followed by a 15 min copulation 1 h 27 min later. In the second instance, the duration of the first copulation was 16 min followed by a 2 min copulation 2 h 3 min later. This same female then mated twice with the second male of her pair. In this third instance, the duration of the first copulation was 7 min followed by a 25 min copulation 29 min later. In the fourth instance, the duration of the first copulation was 16 min followed by a 4 min copulation 2 h 32 min later. Average copulation duration for only the first copulation for each female ($n = 11$), ± SE was 20.8±4.03 min. (range 5.0 min to 41.0 min).

Duration of viable stored sperm

Shelled and unshelled eggs were found in female cages prior to the start of the mating trials. Females ≤ 49mm SVL (N=10) oviposited only yolked eggs without shells. However, females ≥ 52 mm SVL (N=6) oviposited shelled eggs. No females sampled ranged between 50-51 mm SVL. Eggs that were unshelled but yolked were never buried in the dishes containing moist soil, but left unburied on the floor of the cage or stuck to the vegetation or wooden dowels in the cages. In contrast, shelled eggs were buried in the soil dishes. Shelled eggs were fertile. Fertility was confirmed either by incubating the egg until hatching or by manually opening the egg and looking for an embryo. The earliest fertile egg laid once females were in captivity was 31 March. No female initially laid an

unshelled egg followed by a shelled egg, but four females initially laid at least one shelled egg followed by at least one unshelled egg. One of the four females alternated between laying shelled and unshelled eggs for her first four laying events. This laying of unshelled eggs following shelled eggs may indicate that the sperm reserves (sperm carried over from the previous season) of the female had been depleted.

Discussion

Evidence for non-random fertilization

My results suggest that fertilization in *Anolis carolinensis* is not random, and that sperm precedence is not based on sperm stratification. Regardless of mating order, when a female mates with two males within the same estrus cycle, only one of the males fathers the offspring. However, distinguishing between sperm quality and sperm quantity in this study was not possible. Barring sterility of either male of the pair, it may be that in each of the two pair of males investigated, one male had sperm of higher quality than the other, which would suggest that sperm precedence was based on sperm selection. However, fertilization could simply be determined by sheer number of sperm, where the more sperm a male introduces into the female tract, the greater his chances for paternity (i.e., sperm loading). If one male produced more sperm than the other, he would presumably be able to introduce more sperm into the female tract during copulation. Size-based sperm precedence is present in another lizard species. Using genetic analysis to determine paternity, Lewis et al (2000) found that the largest male in an enclosure of males fertilized the most eggs in *Ameiva exsul*.

Copulation duration

Anolis carolinensis has a relatively long coitus (Table 2.3). Interestingly however, in both *A. aeneus* (Stamps 1975) and *A. sagrei* (Tokarz 1999), copulation duration has been shown to be dependent upon when in the breeding season it occurred. Copulation duration increases further into the breeding season and a comparision of copulation

durations from the first half of the breeding season and the second half of the breeding season shows the latter half copulations to be significantly longer than those early in the breeding season (Tokarz 1999).

Three purposes for prolonged copulation in reptiles have been proposed (Olsson & Madsen 1998): 1. more sperm could be transferred; 2. more secretions from the renal sex segment (RSS) could be transferred; 3. males could provide a physical barrier to additional matings by other males. Based on data collected by Blanchard & Blanchard (1942) and Devine (1975), long copulation time in reptiles is not used for sperm transfer. If we use *A. sagrei* as a model for anoles, sperm transfer occurs almost immediately after intromission (Tokarz 1999). It is unlikely then that the long copulation duration of *A. carolinensis* has to do with transferring sperm. Long copulation time may allow a male to increase the amount of nourishment he provides to his sperm through high RSS secretions (Olsson & Madsen 1998). Thus a male who copulates for a long period of time may be insuring that his sperm will stay viable in the female tract for a long period of time (Weil 1984). But unlike in snake sperm storage tubules in which the sperm are nourished, no nourishment is provided to the sperm once they have entered the storage tubules of *A. carolinensis* (Fox 1963). Long copulations as a barrier to additional matings by other males may be feasible if timing of insemination is important. Parker (1984) hypothesized that prolonged copulation can function as a type of sperm competition by reducing the probability that a female will be remated prior to ovipositing.

Repeated mating of a female lizard by a resident male can also be viewed as a strategy to dilute the sperm of competitors. These repeated matings may have evolved as a consequence of sperm competition (Devine 1984). *Anolis carolinensis*, however, does not seem to use this strategy although a discrepancy is present over the number of times that a male *A. carolinensis* will copulate per day. A captive *A. carolinensis* male mates, on average, two times per day (Greenberg & Noble 1944). However, evidence from field data (Jenssen et al. 1995 and Nunez et al. 1997) shows that a resident male will mate about once per day regardless of the number of females in his territory. In four of 22

instances, I observed a male to mate twice with the same female with the same testing period (i.e. the same day).

Duration of viable stored sperm

Female *A. carolinensis* also play a role in determining which sperm fertilize their eggs. Fox (1963) previously reported that females can store viable sperm in their tracts for up to seven months. My work extends this time of viable storage to at least ten months (females were laying fertile eggs in the laboratory in May 01 after not having mated since July 00). By mating with multiple males and storing sperm, a female guards against the possibility that her resident male is sterile (Conner & Crews 1980) or that she finds herself without a resident male for a period of time (perhaps during a shift in territorial ownership).

Sperm storage is most likely functioning to allow the female to lay fertile eggs in the absence of the resident male (Crews 1973) and to lengthen the period of fertile egg laying (Fox 1963). An interesting comparison can be made to an anole without sperm storage capabilities *(A. aeneus*). If copulation does not occur when a female *A. aeneus* has an intermediate sized follicle in her tract, she will resorb that follicle (Stamps 1975). On the contrary, egg resorption seemingly does not regularly occur in *A. carolinensis* given that females will lay unshelled, unfertilized eggs at the onset of the breeding season (Passek, this study).

By storing sperm throughout the breeding season, a female is able to remain reproductively active independent of male reproductive status during the early and late stages of the breeding season. By storing sperm from one breeding season to the next, a female is able to begin laying eggs while the males are still undergoing testicular recrudesence. This effectively removes female reproduction from under the control of behavioral cues from the males, as is commonly reported (Crews et al. 1974), to under the control of environmental cues. Females in their first reproductive season begin laying unshelled eggs and females in their second reproductive season begin laying fertilized

eggs in late March (Passek, this study) as males undergo testicular recrudesence (Jenssen et al. 2001).

In summary, sperm competition in *A. carolinensis* likely involves issues of sperm quantity as well as sperm quality. Long copulation duration may play a role in insuring sperm viability and sperm transport. Females remain receptive following copulation. Females are able to store viable sperm in their tracts for at least ten months. This insures that viable sperm will be present in her tract within and between reproductive seasons. Female reproduction is under the control of environmental cues and is independent from male behavior.

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Table 2.1 Pairing of male and female lizards for mating trials to investigate sperm precedence

Key: X = pairing of male and female lizard for a mating trial

	Trial 1				Trial 2			
	Female 1	Female 2	Female 3	Female 4	Female 1	Female 2	Female 3	Female 4
Male A	X	X					X	X
Male B			X	X	X	X		

Table 2.2 Paternity of hatchlings from controlled mating experiment to investigate sperm precedence

Key: SVL = snout-vent length
X = diagnostic band identified, paternity assigned to that male

Offspring of female S13 (SVL 55 mm) (3 days between matings)

Hatchling id	First male Male 4 SVL 69 mm	Second male Male 3 SVL 65 mm	Sperm present in female tract
13-1			Neither
13-3	X		Only male 4
13-4	X		
13-5	X		Both male 4 and male 3
13-6	X		
13-7	X		
13-8			
13-9	X		

Offspring of female S5 (SVL 54 mm) (5 days between matings)

Hatchling id	First male Male 8 SVL 67 mm	Second male Male 7 SVL 61 mm	Sperm present in female tract
5-1			Neither
5-2			
5-3	X		Both male 8 and male 7
5-4	X		

Offspring of female S6 (SVL 53 mm) (4 days between matings)

Hatchling id	First male Male 8 SVL 67 mm	Second male Male 7 SVL 61 mm	Sperm present in female tract
6-3			Neither
6-4			
6-5	X		Both male 8 and male 7
6-6	X		
6-7	X		
6-8	X		
6-9	X		
6-10			

Offspring of female S12 (SVL 54 mm) (1 day between matings)

Hatchling id	First male Male 7 SVL 61 mm	Second male Male 8 SVL 67 mm	Sperm present in female tract
12-3			Neither
12-4			
12-5		X	Both male 7 and male 8
12-6		X	
12-7		X	
12-8		X	
12-9		X	
12-10			
12-11			

Table 2.3. Average copulation durations of Anoline lizards

Species	Average copulation duration ± SE and location of observations	N	Notes	Reference
A. carolinensis	15±3 min (field)	6		Jenssen et al. 1995
	26.4±6.7 min (field)	8		Nunez et al. 1997
	9.22±0.89 min (lab)	100		Valenstein & Crews 1977
	18.7±12.2 min (lab)	22		Passek, this study
A. aneus	69.7 min (lab)	11	Type II copulations in which copulation was uninterrupted	Stamps 1975
A. caudalis	13.4±1.34 min (field)	7	lizards removed from home ranges and transferred to another location within their habitat	Jenssen 1996
A. garmani	≥10 min (field)	49	all copulations were already in progress when timing began	Trivers 1976
A. opalinus	1.31±0.5 min (field)	30	lizards removed from home ranges and transferred to another location within their habitat	Jenssen, unpub. data
A. sagrei	5.78±1.08 min (field)	21	intromission with left hemipenis	Tokarz 1999
	4.23±0.52 min (field)	25	intromission with right hemipenis	
A. websteri	≤1 sec (field)	13	engage in “cloacal kiss”	Jenssen 1996

Conclusions

In addition to the obvious joy that comes from the completion of the writing of my dissertation, I am excited that my work has generated new questions and avenues to pursue. There are three areas of research that are of interest to me for future study.

First, I am intrigued by the temporary secondary strategy in which smaller males engage in order to achieve some reproductive success prior to holding a territory. I would like to follow males for multiple seasons to obtain a measure of overall lifetime reproductive success.

My questions:
Do all males engage in the strategy of covert male followed by being a territory holder? Is there a difference in fitness associated with those males who were covert males as opposed to males who may have been simply floating between territories before holding a territory? How does the "shift in strategy" from covert male to territory holder occur and when? Do smaller males attempt to take over a territory in the middle of the breeding season or are males covert one season and then territory holders the next season? Do male hatchlings disperse from the area where they were born to avoid competition with their brothers?

Second, I don't think that there is an accurate measure of the frequency of territorial exchanges resulting from territory takeovers. This information would also contribute to our knowledge of the overall reproductive success of males. If takeovers are frequent, then the strategy of being a covert male may need to be viewed as not just a secondary strategy, but as a necessary way to obtain opportunities for reproduction.

My questions:
Who takes over a territory after removal of the resident male (e.g. after a non-combat related death). Does a neighboring resident male move in and absorb the territory or does a covert male move up in rank? If a neighbor does move over, what happens in the

interim—do covert males become less secretive? How long before resident females will mate with a new male if he is a neighbor compared to if he is a unfamiliar male? What happens to the ousted resident male?

Third, in compiling my data and reviewing the published data, I came across some interesting results regarding the relationship between copulation duration and breeding season time.

My questions: Does this relationship hold true for *A. carolinensis* and what is its adaptiveness?

My work stresses the importance of field-based observations coupled with very specific questions studied through laboratory-based manipulations. Too often, questions are answered by bringing animals into the laboratory and placing them under conditions that fail to represent what occurs in nature. We cannot remove an animal from its environment and expect to be able to correctly interpret its behavior nor should we make the mistake of failing to question theories constructed solely based on laboratory-derived observations. These mistakes have been made for many years with *A. carolinensis* and I am pleased to be contributing to information regarding its actual behavior.

I chose to work with *A. carolinensis* because in doing so I would become part of a group in which we were all working toward the same goal: gaining an understanding of the behavioral ecology of *A. carolinensis* by carefully observing it under natural conditions and using novel techniques and tools in biology to help answer our questions. I believe that I, along with my labmates and our advisor, have been successful in doing so.

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Sperm competition as a mechanism for sexual selection in *Anolis carolinensis*. Ph.D. dissertation research conducted at Virginia Tech from 1996 to 2002.

Kin recognition abilities in hatchling American alligators, *Alligator mississippiensis* Daudin. Master's thesis project conducted at Central Michigan University from 1993-1996.

Thermal influence on defensive behaviors in the Eastern garter snake, *Thamnophis sirtalis*. Project conducted at the Central Michigan University Biological Station on Beaver Island, MI, during the Summer of 1994.

Foraging behavior of whiptail lizards, *Cnemidophorus sexlineatus*, and fence lizards, *Sceloporus woodi*. Project conducted at Archbold Biological Station in Lake Placid, FL, during the Summer of 1993.

Morphological determinants of diet in whiptail lizards, *Cnemidophorus uniparens*. Project conducted at the Southwestern Research Station of the American Museum of Natural History in Portal, AZ, during the Summer of 1992.

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Extra-pair paternity within the female-defense polygyny of the lizard, *Anolis carolinensis*: Evidence of alternative mating strategies. Defense seminar on 07 May 2002, Biology Department, Virginia Tech, Blacksburg, VA.

Sex ratio variation in the lizard, *Anolis carolinensis*. Seminar on 02 April 2001 as part of the Virginia Tech Biology Department's Ecology, Evolution and Systematics Seminar Series in Blacksburg, VA.

Sperm competition as a mechanism for sexual selection in the lizard, *Anolis carolinensis*. Seminar on 25 May 2000 at the Annual Meetings of the Virginia Academy of Science in Radford, Va. Awarded "Best Student Paper in the Biology Section."

Plausibility of sperm competition in *Anolis carolinensis*. Seminar on 27 March 1999 at the 1999 Virginia Tech-University of Tennessee *Anolis* Research Symposium in Knoxville, TN.

Kin recognition in hatchling American alligators, *Alligator mississippiensis* Daudin. Seminar on 01 March 1996 at the Annual Meetings of the Michigan Academy of Science in Alma, MI.

Kin recognition abilities in hatchling American alligators, *Alligator mississippiensis* Daudin. Seminar on 18 April 1995 to the Biology Department of Central Michigan University in Mt. Pleasant, MI.

Thermal influence on defensive behaviors of the Eastern garter snake, *Thamnophis sirtalis*. Seminar on 11 March 1995 at the Annual Meetings of the Michigan Academy of Science in Big Rapids, MI.

The effects of symmetrical and asymmetrical septal lesions on left-right response differentiation in the rat. Seminar in April 1992 at the 26th Annual New York State Undergraduate Psychology Conference in Binghamton, NY.

An analysis of tree size selectivity in the foraging of beavers at Tifft Farm: evidence for energy optimizing. Seminar on 5 March 1992 to the staff of the Tifft Farm Nature Preserve of the Buffalo Museum of Science in Buffalo, NY.

Kin recognition abilities in hatchling American alligators, *Alligator mississippiensis* Daudin. Poster presentation at the Annual Student Research and Creative Endeavors Exhibition, Spring 1996, Central Michigan University, Mt. Pleasant, MI.

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