

## SPERM VIABILITY IN THE REPRODUCTIVE TRACT OF FEMALES IN A POPULATION OF *SCELOPORUS MUCRONATUS* EXHIBITING ASYNCHRONOUS REPRODUCTION

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**ABSTRACT.**—Asynchronous reproduction is a common phenomenon in high-elevation populations of lizards from Central México. Sperm storage in the reproductive tract of females is the mechanism for making oocyte fertilization possible. Our study addresses questions related to functional oviductal sperm storage of females mating on different dates throughout the reproductive season. A population of *Sceloporus mucronatus* with copulation in the summer and ovulation in the fall was chosen for this experiment. Eleven females that copulated in the field during June and 13 females that copulated in captivity during August were maintained in the laboratory until parturition. The number of pregnant females and the litter sizes produced in each experimental group were indicative of the viability of the stored sperm. Sperm stored in the reproductive tract of females were able to fertilize eggs after 4 months. No significant differences were found in the number of pregnant females between the 2 experimental groups nor in the litter sizes that they produced. We found that the amount of time sperm were held in the female reproductive tract (ca. 3 months) had no effect on the capacity of sperm to fertilize eggs. Histological examination of 8 oviducts collected before the mating season eliminated the possibility of sperm storage from one year to the next. In this system, sperm retention could have evolved as a response mechanism to deal with the asynchrony between sexes in the reproductive cycles. However, we cannot rule out alternative hypotheses.

*Key words:* *Sceloporus mucronatus*, *asynchronous reproduction*, *sperm retention*, *sperm viability*.

Lizard species exhibit wide variation in their reproductive cycles. In tropical latitudes at moderate elevations, viviparous lizards usually exhibit synchronous reproductive cycles (i.e., similar reproductive timing in both sexes). However, at higher altitudes and latitudes, also in tropical regions, some species show asynchronous reproductive cycles (i.e., different reproductive timing between the sexes), with males reaching peak activity before females (Méndez–de la Cruz et al. 1988, 1994, 1999, Estrada-Flores et al. 1990, Villagrán–Santa Cruz et al. 1992, Jones et al. 1997, Méndez–de la Cruz and Villagrán–Santa Cruz 1998, Ramírez-Bautista et al. 2002).

In the case of lizard species with asynchronous reproductive cycles, sperm retention by females is required because mating occurs when males exhibit their peak testicular activity, about 3 months before ovulation (Méndez–de la Cruz et al. 1988, 1999, Villagrán–Santa Cruz et al. 1992). Thus, male reproductive success

depends, to some extent, on the viability of sperm retained inside the female until fertilization occurs. It has been reported that in some reptile species, females store sperm in the oviducts, which allows them to produce fertile eggs well after copulation (Girling 2002, Kast 2007, Olsson et al. 2007). In squamates, sperm retention by females is highly developed, and spermatozoa are stored in the vaginal region, either in the lumen, crypts in the vaginal folds, or in short ciliated tubules formed from epithelial evaginations (reviewed in Blackburn 1998, Girling 2002, Sever and Hamlett 2002).

For lizards, wide variation in the duration of sperm retention in the reproductive tracts of females has been documented (reviewed in Birkhead and Møller 1993). These data are based on histological examination of sperm occurring in the oviduct during nonovulating periods and these sperm, therefore, cannot be used for egg fertilization (Murphy-Walker and

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Haley 1996, Sever and Hopkins 2004). In some species, including several from the genus *Sceloporus*, the asynchrony between the sexes in their reproductive cycles strongly suggests the occurrence of sperm retention (Smyth and Smith 1968, Guillette and Casas-Andreu 1980, Guillette and Sullivan 1985, Villagrán-Santa Cruz et al. 1992). Some experimental studies (none focusing on *Sceloporus*) have shown that even after long periods of isolation from males, captive females can still produce viable clutches (Murphy-Walker and Haley 1996, Chun-Fu et al. 2004, Yamamoto and Ota 2006, Kast 2007). Nonetheless, evidence demonstrating the time that sperm remain viable inside females and useful for egg fertilization is scarce.

In this study, we attempt to test for the presence of retained viable sperm in females of *S. mucronatus* that inhabit a montane environment. In this locality, both the peak of male reproductive activity and mating occur 3–4 months before female ovulation (Méndez-de la Cruz et al. 1988, Estrada-Flores et al. 1990). We tried to determine (1) the viability of sperm stored by females, (2) whether the capability of stored sperm to fertilize eggs differs between females inseminated early in the season and those inseminated later, (3) whether the number of offspring produced differs between females that mate at the beginning of the reproductive season and those that mate at its end, and (4) whether sperm stored after mating in the summer is used only for egg fertilization in the fall or whether storage extends until the following reproductive season.

## METHODS

### Study System

*Sceloporus mucronatus* is a lizard species endemic to central México. It inhabits diverse environments usually above 2000 m, from pine forests to xeric regions. It exhibits diurnal and saxicolous habits as well as viviparity and a marked sexual dimorphism, in which males are bigger than females (Toledo 2005). Both sexes reach maturity at 63 mm snout-vent length (SVL) and show seasonal reproductive activity. At our study site, males and females differ in their peaks of gonadal activity (i.e., asynchrony in their reproductive cycles; Méndez-de la Cruz et al. 1988, Estrada-Flores et al. 1990). Males reach their maximal testicular activity during the summer (June–August),

when their behavior is characterized by frequent displays and male-to-male aggressions. Territories are established during these months when mating occurs (Ortega-León unpublished data). Females reach their peak of ovarian activity in the fall, when fertilization should take place. Gestation occurs all throughout the winter, with parturitions in the spring and litter size varying between 2 and 12 young (Méndez-de la Cruz et al. 1988, Estrada-Flores et al. 1990).

Our study population inhabits Valle de la Cantimplora, Parque Nacional “El Ajusco” (19°12'N and 99°16'W), south of México City at 3375 m elevation. Dominant vegetation is composed of pines and grasses, and there are abundant volcanic rocks, which provide numerous crevices that serve as refuges for these lizards. Mean annual temperature is 7.3 °C, with a minimum of 0 °C during the winter. Precipitation varies widely within the year, with August being the wettest month (293.4 mm) and May the driest (9.7 mm; García 1973).

### Sperm Viability Experiment

To test for differences in the viability of the stored sperm between females mating early versus those mating late in the reproductive season, we used 24 females divided into 2 experimental groups that differed in the mating date. We chose females larger than the reported size at maturity ( $\bar{x}$  = 73.08 mm SVL, range 67.0–79.0 mm SVL; Méndez-de la Cruz et al. 1988) to guarantee that we were considering only sexually mature females. The 1st group (early mating females), composed of 11 females, was collected at the onset of the mating season (12 June 2005). We assumed that these females had mated already on the basis of either recent marks made by males during the mating event or residual seminal fluid in the female's vent. While a mating event does not necessarily imply successful fertilization (Hunter et al. 1993, Olsson and Shine 1997, Uller and Olsson 2005), we used these criteria to select a group of females which potentially could already have sperm in their oviducts. Thus, these females were useful for our attempt to document viability of sperm deposited early in the reproductive season (and therefore stored until ovulation), as well as for evaluating whether these sperm have a diminished capacity for fertilizing eggs compared to sperm deposited right before ovulation. These females

were kept together in a 5-m<sup>2</sup> metal mesh enclosure at the Jardín Botánico of the Instituto de Biología, Universidad Nacional Autónoma de México, in an open area with rocks, vegetation, and environmental conditions similar to those existing under natural conditions. This enclosure was provisioned with refuge crevices and basking areas. We fed the lizards once a week with an abundance of crickets. Water was sometimes provided, depending on the frequency of rainfall during the experiment.

The 2nd group (late-mating females), composed of 13 females, was collected in March 2005, before the onset of the mating season. These females were kept in the laboratory in plastic boxes (59 × 43 × 31-cm) with rocks for cover. The day-night cycle (10 hours light, 14 hours dark) was simulated with artificial light. Boxes were sprayed with water every day to keep humidity constant. Temperature varied between 10 and 18 °C. Food (crickets and beetle larvae) and water were provided *ad libitum*. These females were maintained under these conditions until the late phases of the mating season. On 8 August 2005 we moved them to outdoor enclosures similar to those described above (metal mesh cages) that also included adult males. These males were between 86 and 98 mm SVL and were collected from the same population as the females during late July. We included 2 females and 1 male per enclosure. Allocation of females to males was randomized, as was the location of individuals in the cages. Males stayed with females until early September, when males were removed from the enclosures and released back into the field.

We removed females from the enclosures in early December and moved them to the laboratory, where they were maintained in the same conditions as described above until parturition (April–May). At time of birth we recorded the number of young (either dead or alive) produced per female and measured the size (SVL) of the females that gave birth. Afterwards, all individuals were released at the study site. All our experimental protocols followed the guide for the care and use of laboratory animals (Institute of Laboratory Animal Resources, National Research Council 1996).

We estimated retention time of viable sperm, assuming a gestation period of 6 months (Méndez-de la Cruz et al. 1988, Villagrán–Santa Cruz et al. 2005). We considered the day when females of the early mating group were col-

lected and the day when females of the late mating group were joined with the males as feasible mating dates for the 2 groups, respectively. We approximated the date of fertilization by subtracting 6 months (i.e., the gestation period) from the birth date. We then estimated the approximate number of days that the sperm were retained by the female from the difference between fertilization date and feasible mating date.

#### Oviductal Histology

In order to evaluate the possibility of sperm storage from one year to the next, we examined both oviducts of 8 adult females (72–89 mm SVL)—4 pregnant and 4 nonpregnant. These females were collected at the Ajusco population between March and May of the year (2004) prior to our sperm viability experiment. We used traditional histological protocols for preparation of the oviduct tissue (e.g., dehydrated in alcohol graduals, cleared in xylene, embedded in paraplast, sectioned at 7 μm thickness and stained with Hematoxylin-Eosin; Humason 1979). We examined oviducts by light microscopy. Previous examinations of the oviducts of *S. mucronatus* throughout its active reproductive season showed that in this species, sperm storage occurs only in the utero-vaginal transition area or in the vagina (Serano et al. unpublished data). Therefore, histological examination in our study was restricted to these sections of the reproductive tract.

#### Statistical Analyses

We used a *G*-test of independence to analyze differences in the frequency of pregnant females per treatment. Wilcoxon's tests were conducted to compare litter sizes between treatments (Zar 1999). Because fecundity in reptiles is usually related to female size (Dunham et al. 1988, Clobert et al. 1998, Shine 2005), we corrected for size effect by means of linear regression between female SVL and litter size. Comparison of litter sizes was conducted using the residuals of such a regression. All statistical analyses were conducted using JMP 6.0 (SAS Institute, Inc., Cary, NC).

## RESULTS

### Sperm Viability Experiment

Births occurred between 12 April and 20 May, and the average retention time of viable

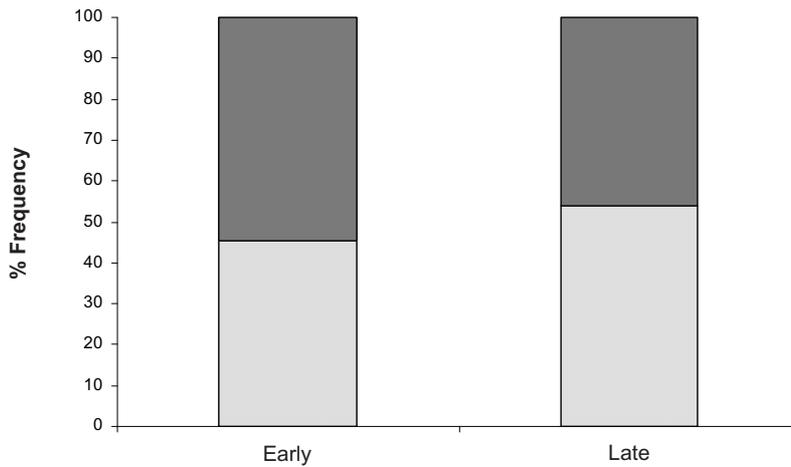


Fig. 1. Relative percent frequencies of pregnant (light gray) and nonpregnant (dark gray) females in our 2 experimental groups. "Early" indicates those females that mated early in the reproductive season (June). "Late" indicates those females that mated late in the season (August). No significant differences were found between groups.

sperm was 104 days, with a maximum of 140 days. In both experimental groups, we had both pregnant and nonpregnant females. Of the 11 early mating females, 5 (45.5%) became pregnant (Fig. 1). In the late-mating group, 7 of 13 females (53.9%) produced offspring (Fig. 1). No significant difference in the proportion of pregnant females was detected between groups ( $G = 0.17$ ,  $df = 1$ ,  $P = 0.68$ ). These relative frequencies of pregnant and nonpregnant females are relatively common in *S. mucronatus*. In fact, in unfavorable years females do not become pregnant because of their decrease in body condition (Méndez-de la Cruz et al. 1992, Rodríguez-Romero and Méndez-de la Cruz 2004). Females that mated at the beginning of the reproductive season produced 3.6 young on average ( $s = 0.89$ ), whereas those that mated at the end of the season produced a mean of 4.1 young ( $s = 1.07$ ). We found a significant positive relationship between female size and litter size (litter size = 0.16 female SVL - 7.46,  $R^2 = 0.49$ ,  $P = 0.01$ ; Fig. 2A). When the effect of female size was removed, we did not find differences in the litter sizes between treatment groups (Wilcoxon's test:  $\chi^2 = 3.21$ ,  $df = 1$ ,  $P = 0.07$ ; Fig. 2B).

#### Oviductal Histology

Histological examination revealed that sperm were not present in the reproductive tracts of either pregnant or nonpregnant females col-

lected between March and May of 2004. That is, before the mating season and during late pregnancy (when embryos are present in the uterus), sperm are not stored in the vagina (anterior or posterior) or in the uterovaginal transition area (Fig. 3).

## DISCUSSION

### Sperm Viability

Viable sperm can be maintained in female reproductive tracts for approximately 4 months. Moreover, we found that sperm deposited at any time during the mating season (June–August) are able to fertilize eggs, as females became pregnant in both the early mating group and the late-mating group. This is the first study to provide experimental evidence of this sperm storage phenomenon in the genus *Sceloporus*, the most diverse group of North American lizards (Sites et al. 1992). Previous studies on sceloporine lizards with asynchronous reproductive cycles have suggested that sperm storage might be the physiological mechanism that allows reproductive asynchrony; however, none provided experimental evidence (Méndez-de la Cruz et al. 1988, Villagrán-Santa Cruz et al. 1992).

The histological examination of female oviducts, in which sperm were not present, collected prior to the mating season also revealed that retention of viable sperm only occurs for relatively short periods of time. Thus, we

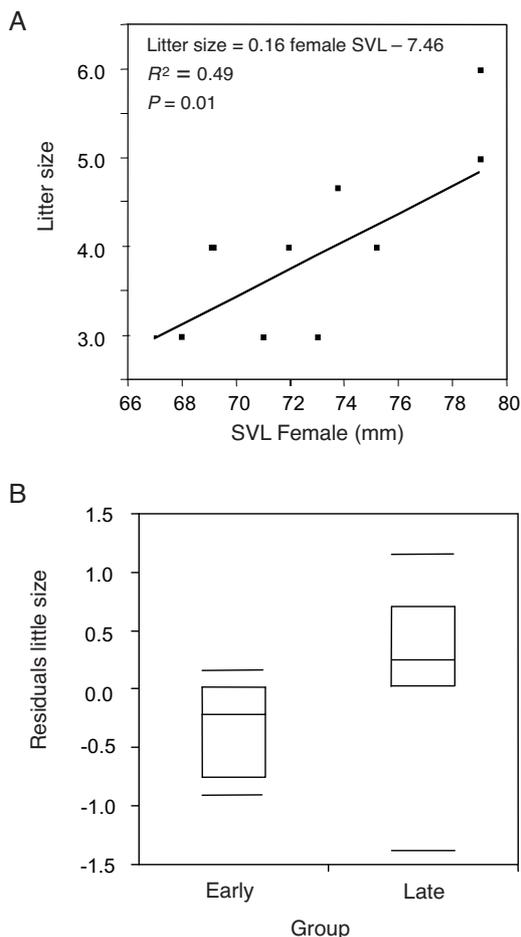


Fig. 2. (A) Linear regression scatterplot of female size (SVL) against litter size. There is a significant relationship between the 2 variables. (B) Box-plots (2 experimental groups) comparing the residuals of the regression between SVL and litter size of 2 experimental groups. "Early" indicates those females that mated early in the reproductive season (June). "Late" indicates those females that mated late in the season (August). Lines within boxes represent medians, box ends represent the 25th and 75th percentiles, and lines above and below the boxes represent the 10th and 90th percentiles.

conclude that females of *S. mucronatus* exhibit oviductal sperm retention only before fertilization and not from one year to the next. Similarly, there is no evidence of prolonged sperm storage (i.e., longer than one year) in other sceloporine lizards with asynchronous reproductive cycles (Méndez-de la Cruz et al. 1988, Villagrán-Santa Cruz et al. 1992, Méndez-de la Cruz and Villagrán-Santa Cruz 1998). In this system, sperm retention could have evolved as a response mechanism to deal with the asyn-

chrony between sexes in the reproductive cycles. Alternatively, the physiological possibility of sperm storage could have been one of the factors that facilitated the evolution of reproductive asynchrony in *S. mucronatus*.

#### Fertilization Capacity

We were interested in possible differences in the success of the sperm, in terms of the number of eggs fertilized, between females inseminated early in the season and those inseminated later. It seemed reasonable that sperm stored for a shorter period might have a higher capacity for fertilization, thus producing more young; however, we did not find differences in fertilization capacities of sperm stored early and late in the mating season. Females that mated early (June) and late (August) in the reproductive season produced similar litter sizes. In other words, sperm remain viable during the whole reproductive season and are able to fertilize eggs when females ovulate in the fall.

Nevertheless, we recognize that the observed number of offspring produced by our experimental females could have been confounded by factors other than early or late insemination. For example, housing conditions differed between experimental groups (i.e., plastic boxes in the laboratory versus outdoor enclosures), which could have resulted in differences in the body condition of females during pregnancy. This in turn could have affected the litter sizes produced. In addition, the quantity and quality of the sperm with which females were inseminated, along with postcopulatory cryptic female choice, might have interacted to produce the litter sizes that we observed (Olsson et al. 1996, Olsson and Shine 1997). Therefore, our findings with respect to the number of young produced by those females mating early versus those mating late should be interpreted cautiously.

#### Future Research

Different hypotheses have been proposed to explain the evolution of the capacity for sperm storage by females of reptile species. For species with asynchronous reproductive cycles, such as *Sceloporus mucronatus*, it has been proposed that sperm storage is an obligate process because a physiological restriction forces the peak of testicular activity to occur before ovulation (Marion 1982, Litch 1984, Guillette

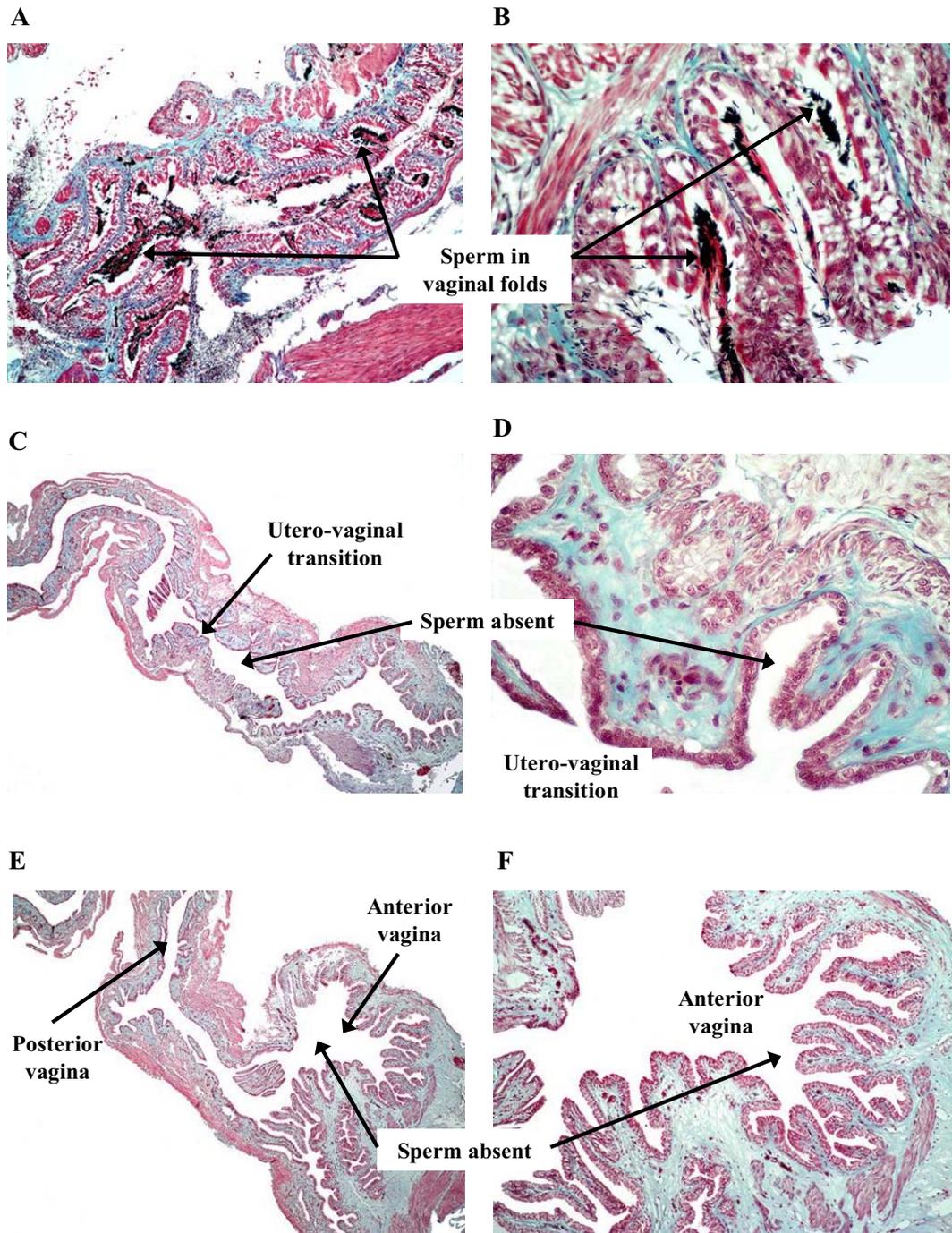


Fig. 3. Sections through the vagina and uterovaginal transition area of *Sceloporus mucronatus*. Vaginal folds with sperm retention ([A] 10X and [B] 40X) in comparison to microphotography of the same areas without sperm storage (C–F); (C) Uterovaginal transition area showing lack of sperm, 4X; (D) Detail of uterovaginal transition area, 40X; (E) Vaginal area without sperm retention, 4X; (F) Detail of vagina, 10X.

and Sullivan 1985, Méndez-de la Cruz et al. 1988). However, we cannot discard alternative explanatory hypotheses. For instance, selection could have acted upon the time when births take place, forcing them to occur under the most favorable conditions for offspring survival (Méndez-de la Cruz et al. 1995). If gestation is constrained to a specific period, then mating and subsequent ovulation have also been indirectly governed by the same selective pressure. If this scenario is true, and male reproductive activity was not affected, then sperm storage could have evolved as a “solution.”

Alternatively, in the context of sexual selection, the separation between copulation and fertilization might be beneficial for females, as the time interval between these 2 events allows for postcopulatory selection mechanisms to operate (Birkhead and Møller 1993). Females might gain substantial benefits by producing more offspring from the most competitive sperm or by modifying their choice by using only the sperm from the most competitive male (Olsson and Madssen 1998, Birkhead and Pizzari 2002, Olsson et al. 2007).

Little is known about the mating system of *S. mucronatus*, and even less is known about possible postcopulatory sexual selection mechanisms in this species. We know that the mating season is relatively long (June–August) and, according to behavioral observations at the study site, females interact with several males during the reproductive season (Ortega-León unpublished data). Together these 2 facts suggest the possibility that females mate multiple times with different males during a single reproductive season (polyandry), which appears to be common in lizard species (Olsson and Madsen 1998, Uller and Olsson 2008). Therefore, studies like ours can provide the empirical basis to assess the possibility of multiple paternity and postcopulatory competition mechanisms, which in turn should affect the reproductive success of males (Birkhead and Møller 1998, Birkhead and Pizzari 2002, Uller and Olsson 2008).

*Sceloporus mucronatus* also has populations with synchronous reproductive cycles, allowing for comparative studies on the capacity of sperm storage between localities with and without physiological restrictions. If females from synchronous populations also show sperm retention, this would provide evidence for an

alternative explanation for the origin of sperm retention in this system: it could have arisen as a sexually selected trait that allows females to mate with multiple males. This phenomenon might benefit females by improving the genetic quality of their offspring (Zeh and Zeh 1996, Jennions and Petrie 2000, Neff and Pitcher 2005, Fisher et al. 2006, Calsbeek et al. 2007). *Sceloporus mucronatus* from our study site, as well as other species that exhibit both asynchronous and synchronous populations, are ideal models for the study of the origin of sperm retention and postcopulatory selection mechanisms.

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