DECLINE IN FUNCTIONAL SHORT-TERM SPERM STORAGE AND REPRODUCTION IN TWO OVIPAROUS SCELOPORINE LIZARDS FROM FLORIDA, USA

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Abstract.—Oviductal sperm storage occurs in most squamate lineages, as well as in *Tuatara*. It is hypothesized to confer fitness benefits by increasing opportunities for reproductive success. These opportunities are specific to each selective landscape, but all are constrained by an underlying requisite phenotype, the duration that sperm can be stored and remain viable. Functional sperm storage durations are generally estimated by field observations of reproductive ecology or the presence of sperm in the oviduct, with few direct tests of sperm viability over time or reproductive events. In sceloporine lizards of the family Phrynosomatidae, functional sperm storage has been examined in many viviparous species with asynchronous reproduction, but oviparous species with synchronous reproduction have not been examined. We assessed functional sperm storage in two oviparous sceloporine lizards with synchronous reproduction, the Eastern Fence Lizard (*Sceloporus undulatus*) and the Florida Scrub Lizard (*S. woodi*). Over a 4-mo period of isolation, we found reproductive success of gravid females declined in both species (3.9% per week in *S. undulatus* and 6.7% in *S. woodi*), although moderate levels of fertility and hatching success were observed for up to 12 weeks in *S. undulatus* and 14 weeks in *S. woodi*. Our data suggest, for these populations, that functional sperm storage occurs across the reproductive season, but is unlikely to continue into the next year.

Key Words.—clutch size; egg number; fertilization rate; hatching success; oviduct; squamates; Sceloporus woodi; Sceloporus undulatus

INTRODUCTION

The ability of females to store sperm in the genital tract, and thus delay fertilization, is a common trait among both vertebrates and invertebrates (Parker 1970; Howarth 1974; Birkhead and Moller 1998; Sever and Brizzi 1998). Indeed, sperm storage has been observed in many reptilian lineages including avian reptiles (Sasanami et al. 2013), chelonians, crocodilians, squamates (Cuellar 1966a; Girling 2002; Sever and Hamlett 2002; Eckstut et al. 2009), and tuataras (Dawbin 1962). The ability to store sperm and maintain its viability confers many potential advantages. Sperm storage allows extended reproductive periods or asynchronous reproductive cycles between males and females (Schuett 1992; Gist and Fischer 1993; Birkhead and Moller 1998; Girling 2002), allowing populations to capitalize on favorable environmental conditions for parents or offspring. It can increase fitness of individuals in populations with low encounter rates among potential mates if sperm can be stored for multiple broods or clutches (Birkhead and Moller 1998). In a similar fashion, sperm storage may facilitate colonization by requiring only a single female storing sperm to disperse to new territory and subsequently produce multiple fertile clutches (Conner and Crews 1980; Sever and Brizzi 1998). By reducing copulation frequency, sperm storage may minimize the risk of predation in species with mating behaviors that increase vulnerability to predators (Conner and Crews 1980). When females store sperm, they allow the possibility for sperm competition and multiple paternity (Schuett 1992; Gist and Fischer 1993; Birkhead and Moller 1998; Girling 2002). In lizards, even short-term sperm storage can result in males producing multiple clutches of offspring from a single fertilization event and facilitate female choice, sperm competition, and multiple paternity (Olsson and Madsen 1998; Olsson et al. 2009). Short-term sperm storage can also affect the sex ratio of clutches over time (Olsson et al. 2007).

Seminal receptacles (sperm storage tubules, pockets, folds, and uterine crypts) are sites for sperm residence inside the oviduct, and help sperm survive for a period post copulation (Sever and Hamlett 2002). In squamates, seminal receptacles are generally found in the vagina (non-glandular uterus) or anterior oviducts (posterior uterine tube and infundibulum), with number and placement varying greatly (Cuellar 1966a; Girling 2002; Sever and Hamlett 2002; Eckstut et al. 2009; Siegel et al. 2015). The presence and morphology of seminal receptacles has been documented for many *Sceloporus*



FIGURE 1. Female Eastern Fence Lizard, *Sceloporus undulatus* (left), and male Florida Scrub Lizard, *Sceloporus woodi* (right), in Florida, USA. (Photographed by Travis R. Robbins).

lizard species, such as the Southern Bunchgrass Lizard (S. aeneus; Guillette and Jones 1985), Trans Volcanic Bunchgrass Lizard (S. bicanthalis; Villagran-Santa Cruz et al. 2017), Mesquite Lizard (S. grammicus; Villagran-Santa Cruz et al. 1992), Yarrow's Spiny Lizard (S. jarrovi; Goldberg 1970), Central Cleft Lizard (S. mucronatus; Ortega-Leon et al. 2009), Eastern Fence Lizard (S. undulatus; Cuellar 1966a), and Florida Scrub Lizard (S. woodi; Palmer et al. 1993). In S. undulatus and S. woodi, the only oviparous sceloporines that have been examined, there are a high number of folds, crypts and/or sperm storage tubules in the middle and anterior portion of the vagina where sperm are stored (Cuellar 1966a; Palmer et al. 1993; Eckstut et al. 2009). These histological studies confirm that sperm storage occurs, but do not assess the duration of sperm viability (but see Ortega-Leon et al. 2009 for duration of sperm viability in S. mucronatus).

Duration of functional sperm storage has been examined in few squamates, with ranges from months in some lizards to years in some snakes (although some instances of reproduction by isolated females may instead be parthenogenesis; Fox 1977) and is unknown for amphisbaenians (Halpert et al. 1982; Blackburn 1998; Girling 2002; Sever and Hamlett 2002; Eckstut et al. 2009). In sceloporine lizards, and Phrynosomatidae in general, functional sperm storage duration ranges from 2-6 mo (Crisp 1964a,b; Cuellar 1966b; Pianka and Parker 1972; Guillette and Casaas-Andreu 1980; Mendez de la Cruz 1989), but these estimates are based primarily on field observations of asynchronous reproductive cycles or the presence of sperm in the oviduct (but see Ortega-Leon et al. 2009). A more direct method to assess functional sperm storage is to measure sperm viability over time. Stored sperm should deteriorate with age or be eliminated as eggs pass through the oviduct (Saint-Girons 1975; Murphy-Walker and Haley 1996; Olsson et al. 2007). How long after insemination sperm begin to deteriorate and the rates

at which deterioration occurs is currently unresolved in *Sceloporus* and most squamates in general.

Here, we examine functional sperm storage in two oviparous lizard species (*Sceloporus undulatus* and *S. woodi*; Fig. 1) by assessing reproductive success of individuals isolated in the lab over multiple clutch events. We measured reproductive success by fertilization rates and subsequent hatching rates. Our goals were to confirm whether or not functional sperm storage occurs, provide a baseline measure of sperm storage duration, and estimate rates of functional decline in sperm viability in relation to time since copulation.

MATERIALS AND METHODS

Study site.—We collected gravid female *S. undulatus* and *S. woodi* in March-September 2004 and 2005 (total n = 17) at sites in Florida, USA. The *S. undulatus* collection sites were Ocala National Forest, Marion County (sandhill habitat; 29°02'18"N, 81°33'35"W; n = 8) and Balm Boyette Preserve, Hillsborough County (27°45'60"N, 82°15'07"W; n = 2). The *S. woodi* sites were Ocala National Forest (scrub habitat; 29°06'29"N, 81°48'34"W; n = 4) and Avon Park Air Force Range, Highlands County (27°37'07"N, 81°15'20"W; n = 3). The latitudinal range among these sites is approximately 1.5°.

Husbandry and data collection.—Immediately after capture, we palpated lizards to confirm gravidity and we uniquely toe clipped each lizard for identification (ID; Waichman 1992). We recorded lizard ID and date of capture and transported all lizards in a cooler to the laboratory at the University of South Florida in Tampa. We housed lizards individually in plastic containers ($34.5 \times 21 \times 12.5$ cm, length \times width \times height) with sand substrate for nesting. To maintain moisture and temperature gradients, we added water to one side of the container daily and heated the other side via heat lamps. Temperatures averaged 31° C during the daytime portion of a 12/12 h light/dark cycle. We added freshwater ad libitum and we added crickets, dusted with vitamin powder, every other day. We checked containers daily for oviposition by examining the bottom of the container for eggs and we made visual checks of lizard gravidity. When clutches were found, we recorded the date of oviposition, number of eggs laid, and the number fertilized. Fertilization was confirmed by the presence of an area vasculosa (i.e., pink spot) on one side of the egg (Cuellar 1966b; Knotek et al. 2017). We kept eggs (n = 216) from each clutch (n = 21 S. undulatus, n = 216)16 S. woodi) together and we buried them completely in vermiculite that was premixed with water (water potential of -450 kPa) and placed inside a 120 ml glass jar. To minimize moisture loss, we sealed each jar with plastic wrap and placed jars in an incubator set at a constant 28° C. We replaced vermiculite for each clutch after 25 d of incubation. Daily, we checked clutches for hatchlings, which we removed and housed as part of another experiment. We checked eggs that had not hatched after 50 d of incubation to confirm egg mortality and/or lack of fertilization.

Data analysis.-We assessed interovipositional duration, number of eggs per clutch, overall reproductive success, fertility, and hatching success. We tested all data for normality using the Shapiro-Wilk test and for homogeneity of variances using Levene's test. When parametric assumptions were not met, we transformed data. We assessed interovipositional duration (weeks) and number of eggs per clutch for species effects using Analysis of Variance (ANOVA) with species as the main factor. We examined overall reproductive success as the percentage of eggs produced per clutch that successfully hatched and we assessed effects of post-isolation duration (weeks after capture) using Linear Regression. Because we could not confirm the exact dates of copulation or the initial fertilization event, we recognize that our use of post-isolation duration is likely an underestimate of sperm storage duration. We ran homogeneity of slopes tests to examine species effects on rates of change. We assessed fertility as the percentage of fertile eggs per clutch and examined it in relation to post-isolation duration, successive clutch events, and cumulative egg depositions, as well as species effects.

We used Linear Regression analysis for effects of post-isolation duration and cumulative egg depositions and examined the slope for rates of change. We ran homogeneity of slopes tests to examine species effects on rates of change. We assessed the effects of successive clutch events using ANOVA with the difference in percentage fertility between second and first clutch as the dependent variable and species as factor. Thus, the rate of change in relation to successive clutch events was the average difference. Significance of the rate was attributed if zero was not bounded by the 95% confidence intervals. We also examined the potential effects of date of capture and time in captivity. We tested for effects of each on the fertility of the first clutches with regression analyses within species. Hatching success was calculated as the percentage hatching out of fertilized eggs. We assessed hatching success using linear regression analysis for effects of post-isolation duration and homogeneity of slopes test to examine species effects on rates of change. We considered sperm storage functional if any eggs were fertilized within a clutch. We analyzed all data with SPSS, Version 26.0 (IBM Corp., Armonk, New York, New York, USA) using $\alpha = 0.05$.

RESULTS

Lizards isolated in the laboratory exhibited different average times between oviposition events ($F_{1.18} = 8.594$, P = 0.009). Interovipositional duration for *Sceloporus undulatus* averaged 6.13 ± 0.60 (standard deviation) weeks and for *S. woodi* averaged 4.62 ± 1.49 weeks (range, 5.4–7.6 and 2.4–6.6 weeks, respectively). The number of eggs per clutch was also different between species ($F_{1.35} = 17.78$, P < 0.001) with *S. undulatus* producing an average of 6.7 ± 1.4 eggs and *S. woodi* producing 4.8 ± 1.3 eggs per clutch (range, 4–10 and 2–7 eggs, respectively; Fig. 2).

Reproductive success declined in both species as duration of isolation increased (Fig. 2). The decline in overall reproductive success was 3.9% per week in S. undulatus ($F_{1.19} = 5.038$, $r^2 = 0.21$, P = 0.037) and 6.7% per week in S. woodi ($F_{1,14} = 5.890, r^2 = 0.30, P =$ 0.029). These rates of decline were different between species ($F_{2.34} = 10.02, P < 0.001$). We found fertile eggs for up to 12 weeks for S. undulatus and 14 weeks for S. woodi (Fig. 3). We estimated a decline in fertility of 2.5% per week in S. undulatus and 1.0% per week in S. woodi as duration of isolation increased (Fig. 3). Fertility, however, was not significantly related to duration of isolation for either species ($F_{1,19} = 2.581$, $P = 0.125; F_{1.14} = 0.095, P = 0.763$, respectively), nor were rates of change per week different between species ($F_{234} = 1.352$, P = 0.272). Rates of change in fertility in relation to metrics other than time, such as across clutch and across cumulative egg depositions, also were not significantly different between species $(F_{1,18} = 0.335, P = 0.570; F_{2,34} = 0.482, P = 0.622,$ respectively) or within species (see below). Across clutches, fertility in S. undulatus increased an average of $2.0 \pm 13.6\%$ (standard error) per clutch event and in S. woodi decreased an average of $9.8 \pm 15.1\%$ per clutch event. Across cumulative egg depositions, fertility in S. undulatus decreased 1.7% per egg deposited and in S.



FIGURE 2. Reproductive success in relation to weeks post-isolation for female *Sceloporus* lizards over multiple clutches. Eggs per clutch represents the number of eggs deposited by each female lizard. Percentage reproductive success was calculated per clutch from the number of deposited eggs that successfully hatched. The rates of change in reproductive success were estimated by the slopes of the regression line (dashed lines). The regression equation associated with reproductive success for *S. undulatus* was Percentage Reproductive Success = $-3.9 \times$ Weeks Post-isolation + 100, and for *S. woodi* was Percentage Reproductive Success = $-6.7 \times$ Weeks Post-isolation + 84.

woodi increased 1.0% per egg deposited, although these changes were not significant ($F_{1,19} = 1.395$, P = 0.252; $F_{1,14} = 0.135$, P = 0.719, respectively). Fertility of first clutches was not influenced by date of capture or time in captivity in either *S. undulatus* ($F_{1,8} = 0.014$, P = 0.909; $F_{1,8} = 2.303$, P = 0.168, respectively) or *S. woodi* ($F_{1,5} = 0.451$, P = 0.532; $F_{1,5} = 0.858$, P = 0.397, respectively).

Hatching success was observed in fertile eggs for up to 12 weeks in *S. undulatus* and 14 weeks in *S. woodi* (Fig. 3). Rates of decline in hatching success tended to be greater than those in fertility, but were still not significant ($F_{1,18} = 1.220$, P = 0.284; $F_{1,11} = 4.629$, P = 0.055, respectively). Rates of change in hatching success were significantly different between species ($F_{2,30} = 7.535$, P = 0.002), however, with *S. undulatus* exhibiting a 2.2% decrease per week and *S. woodi* exhibiting a 6.6% decrease per week (Fig. 3).

DISCUSSION

We provide evidence of a functional decline in stored sperm in two oviparous sceloporine lizard species. After holding gravid female lizards of *Sceloporus woodi* and *S. undulatus* in isolation for 4 mo, overall reproductive success decreased, but neither underlying component, egg fertility or hatching success, was significant on its own. The decline in weekly reproductive success was greater in *S. woodi* (6.7%) than *S. undulatus* (3.9%), as was the relative influence of hatching success on the overall decline. Fertility and hatching success declined at relatively similar rates in *S. undulatus* (2.5% and 2.2% per week, respectively), while fertility declined little relative to hatching success in *S. woodi* (1.0% and 6.6% per week, respectively). Despite the overall decline in functionality, many individuals exhibited high fertility up to the end of our isolation period, including some with a third clutch. The functional sperm storage durations we observed are consistent with other oviparous Phrynosomatid lizards. The measured decline in sperm viability suggests that, as a phenotype, functional sperm storage duration may be an important constraint on reproductive strategies and thus species-specific life histories.

Functionality of stored sperm is hypothesized to decrease over time for two reasons. The vaginal epithelium, which consists of a high number of folds and/or sperm storage tubules in S. undulatus and S. woodi (Cueller 1966a; Saint-Girons 1975; Palmer et al. 1993), must greatly distend to facilitate oviposition. Thus, during oviposition there is a possibility of losing or damaging the sperm stored in seminal receptacles of the vagina (Cuellar 1966a; Bushman et al. 1985). Sperm could also deteriorate or die over time because of a lack of nutritional maintenance (Adams and Cooper 1988; Olsson et al. 2009), but we did not examine oviductal sperm viability or secretions specifically. If stored sperm is depleted (beyond the fertilization event itself) with every egg that passes through the oviduct, then individuals with larger or more frequent clutches should experience shorter functional sperm storage duration.



FIGURE 3. Sperm viability assessed as percentage of eggs that were fertilized and fertilized eggs that hatched in relation to weeks postisolation for female *Sceloporus* lizards over multiple clutches. Percentage fertility was calculated per clutch relative to the number of eggs deposited. Percentage hatching success was calculated per clutch out of the number of fertile eggs. The rates of change were estimated by the slopes of the regression line (dashed lines).

Our data do not support this hypothesis because S. woodi experienced shorter functional sperm storage duration but had smaller clutches than S. undulatus. Our attempt to assess directly the fertility rate changes related to clutch events and cumulative egg depositions resulted in high variances that did not further elucidate support or lack thereof regarding the hypothesis of sperm depletion by oviductal distension. Egg size relative to body size (or oviduct size) may also influence this relationship, because larger values may require greater distention and perturbation of sperm receptacles, but we did not collect data on cloaca, vagina, or oviduct sizes. Other potential factors related to oviductal sperm depletion rates are the number of seminal receptacles, and thus stored sperm, in the respective oviducts and amount of sperm transferred by the male or males. Polygamous mating strategies could affect multiple aspects of sperm storage beyond the amount of sperm collected by the female by introducing variation caused by male-specific sperm viability, which could even cause variation in functional sperm storage duration within female lizards over a single breeding season. Regardless of the possible covariables, our data suggest the relationship is more complicated than simply the number of clutches or number of eggs deposited, at least between species.

As some level of fertility continued through the entire study, sperm storage may continue longer than the proposed 12–14 weeks post-isolation. Unfortunately, we did not have lizards long enough to obtain data on further in-season or next season clutch viability, and these data would be necessary to confirm a maximum

functional sperm storage duration. We can estimate an average maximum duration, however, based on the linear regression formula associated with overall reproductive success for each species. The maximum estimated functional sperm storage duration in our S. undulatus populations would be 25 weeks, and in our S. woodi populations would be 12.5 weeks. Copulations do not likely occur after July in these populations (Jackson and Teleford 1974; Marion 1982). Therefore, our estimates of maximum duration suggest that it is unlikely that either species stores functional sperm over winter or between mating seasons, although we cannot rule out different functional sperm storage durations in distant northern populations of S. undulatus that experience fewer clutches, shorter reproductive seasons, and longer winters. We also cannot rule out captivityinduced stress causing decreased reproductive output in the lab that would not be observed in the wild and may differ between species.

The scenario of functional sperm storage over single reproductive seasons may be common among oviparous lizard species that exhibit synchronous reproductive cycles, such as *S. undulatus* and *S. woodi*. Although few have been examined, Phrynosomatid lizards with synchronous reproductive cycles generally store functional sperm for 2–4 mo (Adams and Cooper 1988; Eckstut et al. 2009). In Common Side-blotched Lizards (*Uta stansburiana*), functional sperm storage was observed for 12 weeks (81 d), with the first two clutches produced being at least partially fertile, but not the third (Cuellar 1966b). In Ornate Tree Lizards (*Urosaurus*)

ornatus), functional sperm storage occurred over a 12week period and across three clutches, but a decline in viability was apparent (Villaverde and Zucker 1998). In Keeled Earless Lizards (*Holbrookia propinqua*), the number of stored sperm decreased significantly during an 11-week period (78 d), but fertility was not examined (Adams and Cooper 1988).

Beyond our study, functional duration of sperm storage has only been examined directly in one other sceloporine lizard, *Sceloporus mucronatus*, a viviparous species that exhibits asynchronous male and female reproductive cycles. Functional sperm storage occurred over a 12-week period in *S. mucronatus* without a decline in fertility, but it did not extend over winter into the next breeding season (Mendez de la Cruz et al. 1988; Ortega-Leon et al. 2009). Functional sperm storage has been inferred in many other viviparous *Sceloporus* species, although rates of change in sperm viability have not been assessed.

Viviparous sceloporine lizards that store sperm exhibit a variety of reproductive strategies. Sceloporus mucronatus exhibits delayed ovulation after storing sperm for 8-12 weeks and subsequent slow embryonic development over winter (about 24 weeks; Mendez de la Cruz et al 1988). Sceloporus jarrovi mates in late fall (September-December), ovulation and fertilization shift slightly later (November-December), and embryonic development is suspended over winter for 12 weeks at the blastoderm stage (Goldberg 1971). Reproductive cycles suggest functional sperm storage may be most pronounced between September-December, which would be about 10 weeks, like many Phrynosomatids. Female S. jarrovi also store sperm in uterine crypts until as late as April (about 20 weeks; Goldberg 1970), but viability of sperm stored over winter has not been determined. In high-altitude populations, Northern Mesquite Lizards (S. grammicus microlepidotus) mate in the fall, presumably store sperm over winter, and give birth the following spring (Guillette and Casas-Andreu 1980). Other S. grammicus populations mate in the summer, store sperm for 12 weeks, and ovulate in the fall (Mendez-de la Cruz 1989; Villagran-Santa Cruz et al. 1992). Sceloporus aeneus stores sperm all year long, but reproduction occurs continuously as does male production of sperm. Functional duration of stored sperm has not been determined in S. aeneus (Guillette and Jones 1985; Villagran-Santa Cruz et al. 2017).

With the exception of *S. aeneus*, sperm storage allows for asynchronous reproduction in these viviparous sceloporines. Although other hypotheses may provide evolutionary reasons for sperm storage in these viviparous species (sperm competition, multiple paternity, reducing predation risk, cryptic female choice, or obligatory colonizing), the most parsimonious hypothesis is the allowance of asynchronous reproduction. Greater

relative fitness may be associated with individuals able to shift reproductive cycles to capitalize on favorable environmental conditions for adult energy acquisition or embryonic or juvenile development (Schuett 1992; Gist and Fischer 1993; Birkhead and Moller 1998; Girling 2002). Reproductive cycles in S. undulatus and S. woodi are not asynchronous, however, suggesting other ecological reasons for functional sperm storage (unless vestigial or nonadaptive) in these species. We cannot rule out any of the other potential hypotheses but note that S. woodi has a life span of 13 mo on average (McCoy et al. 2004) and exists in meta-populations (Hokit et al. 2001), a scenario in which sperm storing females with dispersing behavior may incur a fitness benefit associated with relatively frequent colonization events.

In our sceloporines, we found functional sperm storage for 12-14 weeks although a decline in functional sperm was observed over time. Whether the direct cause of declined sperm storage functionality is number of clutch events, number of eggs oviposited, or sperm viability itself is not resolved and warrants further investigation. Functional sperm storage and delayed fertilization occur in almost all vertebrate classes (Howarth 1974). It is likely that functional sperm storage occurs in most squamates, but the duration and associated adaptive significance, or lack thereof, will differ on a case by case basis. Certainly, the fitness landscapes vary considerably across sperm storing species and so to do the ecological reasons for this phenotype. Resolving these issues will require many more studies on functional sperm storage and its fitness benefits.

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