

Testing the ability to store sperm: an experimental manipulation of mating opportunities in the common wall lizard, *Podarcis muralis*

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Abstract. Female common wall lizards (*Podarcis muralis*) typically reproduce annually and lay more than one clutch per season. In this study, we tested whether females store sperm between clutches and between years by manipulating mating opportunities of females through appropriate experiments. Our results revealed that females are definitely unable to store sperm for medium or long-term, suggesting they necessary need to repeatedly mate with males to fertilize their eggs. Finally, by comparing our results to other similar multi-clutched species, we conclude that sperm storage probably does not constitute a selective advantage for species with a promiscuous reproductive system based on multiple mating in populations with high densities.

Keywords. Multiple mating, sperm storage, sexual selection, unfertilized eggs, common wall lizard.

INTRODUCTION

In reptiles, multiple mating and sperm storage frequently occur (Sever and Hamlett, 2002), as documented for chelonians (Johnston et al., 2006), ophidians (Schuett, 1992) and saurians (Villaverde and Zucker, 1998; Olsson et al., 2007), even though the organs devoted to sperm storage are relatively undifferentiated. Moreover, sperm storage in reptiles shows a considerable variation in time duration, ranging from hours to months or even years (Schuett and Gillingham, 1986; Pearse et al., 2001; Olsson et al., 2007). The evolutionary causes of sperm storage are debated. In a review of multiple paternity in reptiles, Uller and Olsson (2008) have suggested that the main cause for sperm retention is selection for sperm longevity resulting from sperm competition when the turnover of

female reproductive cycles is quick. Alternatively, sperm storage may also contribute to cryptic female choice, enabling female control of paternity and retention of optimal sperm across reproductive cycles (Olsson and Madsen, 1998; Uller and Olsson, 2008). Noteworthy, these reasons are not mutually exclusive and can occur simultaneously within a species.

Multiple mating and female sperm storage could be promoted by sexual selection as they increase offspring diversity (genetic bet-hedging Hp, Watson, 1991; see also Yasui, 1998 for a review) and elicit cryptic female choice or sperm competition, thereby increasing the probability that eggs are fertilized by sperm from high-quality males (Olsson and Shine, 1997; Yasui, 1997). Opportunity for cryptic female choice could have driven the evolution of sperm storage especially when females are able to control paternity through the retention of sperm from high quality males over breeding cycles (Olsson and Madsen, 1998).

Apart from sexual selection, long-term sperm storage can evolve to delay the time between copulation and fertilization, when deferred emergence increases offspring survival (Birkhead and Møller, 1998; Villaverde and Zucker, 1998). The advantages of long time sperm storing are also particularly evident in species having few mating opportunities, due to the sexual segregation of both sexes in time and space (Ruckstuhl and Neuhaus, 2005), male sperm limitation (Preston et al., 2003), or asynchrony among spermatogenesis, mating, and ovulation (Schuett, 1992). For example, females of several snake species evolved long time sperm storage as a response to the lack of synchrony among spermatogenesis, mating, and ovulation (Fox, 1952; Saint Girons, 1982; Schuett, 1992).

Distinguishing between the different hypotheses explaining multiple mating and sperm storage represents one of the most interesting topic of mating systems, but it is also a hard question to be solved. This is because observational data collected during field researches frequently agree with different hypothesis. A first approach to address these issues is to determine whether a species is actually able to store sperm or not and this can be achieved only through experiments that manipulate mating opportunities for females (by reducing or increasing the number of copulations) as well as the timing of sperm storage (by shortening or lengthening the time between copulations), thus recording the effects on the production of fertilized eggs.

Due to their biological and reproductive traits, lizards represent an ideal model to investigate these subjects since, for example, most species are promiscuous, and lack post-hatching parental care (Madsen et al., 1992; Olsson et al., 1996, 2007; Uller and Olsson, 2008). The common wall lizard (*Podarcis muralis*, Laurenti 1768) is a small (snout-vent length, SVL, 45-75 mm) lizard occurring in southern and central Europe (Arnold and Burton, 1985). Both males and females mate multiply during the same breeding season, and females produce on average two clutches per year (Barbault and Mou, 1986; Brana and Ji, 2007). The breeding season lasts from mid-March to late July (Corti and Lo Cascio, 1999), although significant differences in the onset of mating can arise as a consequence of climate variation not only at macro-geographic scale, but even among different sites at local scale. Recently, Oppliger et al. (2007) showed that multiple paternity occurred in at least 87% of the clutches, thus suggesting possible short-term effects of sperm storage (Uller and Olsson, 2008). However, to date the ability of this species to store sperm has not been demonstrated nor for medium-term nor for

long-term periods. Therefore, it would be really interesting to verify if females are actually able to store sperm, and above all whether sperm is stored to fertilize two successive clutches laid by the same female in the same breeding season, or whether sperm is stored over successive breeding seasons.

In this study we manipulated the mating opportunities of females in order to investigate if they are able to a) use sperm stored before the first clutch to fertilize the eggs of the second clutch without new copulations (hereafter medium-term sperm storage), and b) use sperm stored before last clutch in a breeding season to fertilize eggs in the first clutch of the next season without new copulations (hereafter long-term sperm storage).

MATERIAL AND METHODS

Medium-term sperm storage experiment

In spring 2007 (April to May), we captured by noosing 83 pregnant females in 6 sites in the surroundings of Pavia (Lombardy, Northern Italy). Pregnancy was assessed by the presence of mating scars, inflicted by the male on the female belly at copulation (Strijbosch et al., 1980). Females were transferred to our laboratory (Department of Animal Biology, University of Pavia) within 2 hours from capture, and housed individually in outdoor plastic jars (60 × 50 × 50 cm) containing soil mixed with small stones as substrate and a single brick as shelter. We provided female with 3 mealworms, *Tenebrio molitor* larvae, each day, and water *ad libitum*. Each cage was also supplied a small tank (10 × 10 × 5 cm) filled by wet sand and partially covered by a tile. Every morning tanks were carefully checked for egg presence and sprayed with water to keep sand wet. All lizards were maintained in captivity until egg laying, which occurred for 68 females within 15 days on average from collection. Mean clutch size was 4.6 (range: 2-8). We randomly selected 20 lizards that had laid their first fertilized clutch to investigate if they were able to lay a second fertile clutch without new copulations. The other 63 females were returned to their capture sites.

Long-term sperm storage experiment

During February and March 2008, we noosed 20 sexually mature virgin females in 5 sites located in the town of Pavia and in its surroundings. Virgin condition was inferred by the lack of male ventral scars, meaning that females had not mated yet in the current season. After captured, females were housed in our laboratory within 2 hours from capture. Contrary to previous experiment, females were held indoor under a natural light-dark cycle in individual transparent plastic jars (20 × 30 × 20 cm) provided with a newspaper sheet as substratum, a water tank and a shelter, and fed with 3 mealworms each day. Water was provided *ad libitum*. Plastic jars were placed under an UV-B lamp (18 W) in order to provide the daily UV requirements for calcium and vitamin D fixation, and an incandescent lamp (25 W) for heating. UV lamps were switched on for 3 h a day (from 10.00 AM to 13.00 PM), while incandescent lamps were set alight for 6 h a day (from 11.00 AM to 17.00 PM). Each cage was provided with a small tank (10 × 10 × 5 cm) filled by wet sand for egg laying. The aim of this second experiment was to check if females were able to fertilize eggs using the sperm eventually preserved by the copulations obtained in the previous breeding season. Finally, females that had laid an infertile clutch were paired with a male in order to check if they were actually able to lay fertile eggs. The other females were returned to their capture sites.

RESULTS

Medium-term sperm storage experiment

Nine females did not lay eggs at all, while eleven produced clutches including only not fertilized eggs, i.e. eggs with flabby consistency, greyish colour and irregular shape (Table 1). By contrast, fertilized and viable eggs appear white coloured with a tight and soft shell. As commonly occurring in most species of lizards, only fertilized eggs showed a clearly visible germinal disk that can be distinguished by the colour (orange to purple) inside the still moist egg shell. In addition, sterile eggs are usually smaller and not as firm as fertile ones (Köhler, 2004; see Fig. 1). Mean clutch size was 4.4 (range: 3-6), while the mean time elapsed between first and second clutch was 31 ± 4 days (range: 16-53). All lizards were released in their capture site at the end of the experiment.

Long-term sperm storage experiment

On the whole, 17 out 20 females laid a clutch, while the other three did not lay any egg at all (Table 1). Mean clutch size was 3.6 (range: 2-5), but none involved fertilized eggs. The mean time elapsed between capture and laying was 49 ± 2 days (range: 36-59). However, after having been paired with a male, 18 out 20 females laid clutches including fertilized eggs (Table 1).

Table 1. Number of the first and second clutches laid by common wall lizard females during mating manipulation experiments. Clutches are distinguished according to the viability of eggs laid.

	Female treatment	1 st clutch			Female treatment	2 nd clutch		
		fertile	infertile	not laid		fertile	infertile	not laid
Medium-term experiment	mated in wild	20	0	0	not mated	0	11	9
Long-term experiment	not mated	0	17	3	mated in captivity	18	0	2

DISCUSSION

Our experiments clearly showed that common wall lizard females do not store sperm for medium or long-term. Indeed, we have demonstrated that females who had laid a fertile first clutch are unable to produce a second one without mating again. This result clearly indicates that females are not able to keep the sperm collected during the period preceding the first deposition for enough time to fertilize the eggs of the second laying. This conclusion is reinforced by the fact that 11 females have anyway laid eggs, although unfertilized. These findings suggest that females of common wall lizard could produce eggs that

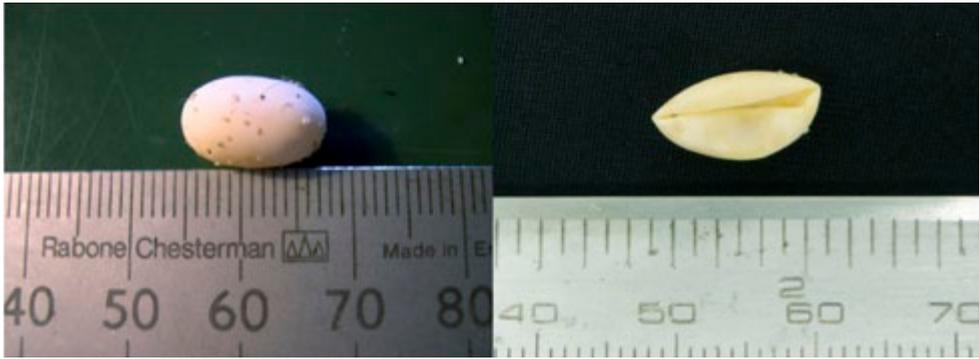


Fig. 1. Difference between a fertile (left) and a sterile egg (right) of *Podarcis muralis*.

must be laid at the end of the ovarian cycle, irrespective of male fertilization. We conclude that this reproductive strategy depends on the high density this species is found in natural populations, which makes unlikely that females do not find males for mating. In this scenario, females are sure to fertilize their eggs by at least one male and the existence of a sperm storage system in the medium-term would not provide them any substantial selective advantage.

The second experiment demonstrated the complete inability of females to store sperm between two consecutive breeding seasons. Indeed, none of the 20 virgin females captured at the beginning of the breeding season was able to lay fertile eggs. Nevertheless, as we found in the previous experiment, most females (17 out of 20) laid an infertile clutch, reinforcing our hypothesis that females must necessarily lay the eggs after a limited time period, apart from their fertilization by a reproductive male. We can presume that in this species female have not evolved any particular sperm storage organs.

We can exclude that the experimental conditions had affected eggs deposition by females, since in 2007 most of the 83 collected females were able to lay a good clutch after a period of captivity, and during 2008 experiment 90% of females (18 out of 20) that have laid a bad first clutch, were able to lay a good clutch after having been paired with a male. Therefore, the observed deposition of bad clutches could be actually due to the lack of a previous eggs fertilization.

Comparing our results with other similar multi-clutched squamate species, we detected the existence of medium and/or long-term sperm storage mechanisms in many snakes and some lizards. For example, several female adders store sperm for weeks or even months, as an obligatory component of their reproductive cycle, with maintenance of fertilization capacity (Schuett, 1992; Isogawa and Kato, 1995; Almeida-Santos and Salomão, 1997). In lizards, the ability to store sperm has been proved in some species (for example, *Hemiergis peronii*: Smyth and Smith, 1968; *Psammophilus dorsalis*: Srinivas et al., 1995; *Urosaurus ornatus*: Villaverde and Zucker, 1998; *Niveoscincus ocellatus*: Wapstra et al., 1999; *Uta stansburiana*: Zamudio and Sinervo, 2000; *Calotes versicolor*: Shanbhag, 2003; *Anolis sagrei*: Calsbeek et al., 2007; *Ctenophorus pictus*: Olsson et al., 2007), interpreting it as necessary reproductive choice when the mating season does not match the time of egg production, as occur in species with autumn mating and spring vitellogenesis and ovu-

lation (Uller et al., 2010); oviductal sperm storage strategy has been evolved in tropical lizards that lay eggs in multiple clutches possibly to eliminate repeated mating and reduce risk of predation (Shanbhag, 2003). In all these cases the evolution of a sperm storage system seems to have been driven by natural selection, although we cannot exclude the possibility of a selective advantage involving the facilitation of female choice and sperm competition at least for some of those species (Olsson and Madsen, 1998).

By contrast, our results clearly show that, at least for that concerning northern Italian lowland populations, female common wall lizards do not store sperm between clutches nor for the same breeding season nor for two successive breeding seasons. It's presumable that sperm storage does not constitute a selective advantage for such species characterized by promiscuous reproductive systems based on multiple mating in populations characterized by high densities, since females are certain to fertilize their eggs during each subsequent ovarian cycle, thus making sperm storage unnecessary.

Interesting enough, a previous study revealed the presence of multiple paternity within the same clutch in a highland population of common wall lizard (Oppliger et al., 2007). In the light of our findings, we can reasonably conclude that multiple paternity in *P. muralis* must be better explained by multiple matings occurring during each ovarian cycle, rather than by sperm storage across subsequent clutches.

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REFERENCES

- Almeida-Santos, S.M., Salomão, M.G. (1997): Long-term sperm storage in the Neotropical rattlesnake *Crotalus durissus terrificus* (Viperidae: Crotalinae). *Jpn. J. Herpetol.* **17**: 46-52.
- Arnold, E.N., Burton, J.A. (1980): *A Field Guide to Reptiles and Amphibians of Britain and Europe*. Collins, London.
- Barbault, R., Mou, Y.P. (1986): A population analysis of the common wall lizard *Podarcis muralis* in Southern France. In: Roček, Z., Ed., *Studies in Herpetology*. Charles University Press, Prague, pp. 513-518.
- Birkhead, T.R., Møller, A.P. (1998): *Sperm competition and sexual selection*. Academic Press, London.
- Braña, F., Ji, X. (2007): The selective basis for increased egg retention: early incubation temperature determines hatchling phenotype in wall lizards (*Podarcis muralis*). *Biol. J. Linn. Soc.* **92**: 441-447.

- Calsbeek, R., Bonneaud, C., Prabhu S., Manoukis N., Smith T.B. (2007): Multiple paternity and sperm storage lead to increased genetic diversity in Anolis lizards. *Evol. Ecol. Res.* **9**: 495-503.
- Corti, C., Lo Cascio, P. (1999): I lacertidi italiani. L'Epos Edizioni, Palermo.
- Fox, W. (1952): Seasonal variation in the male reproductive system of Pacific coast garter snakes. *J. Morph.* **90**: 481-553.
- Isogawa, K., Kato, M. (1995): Mating season of the Japanese mamushi, *Agkistrodon blomhoffii blomhoffii* (Viperidae: Crotalinae), in Southern Kyushu, Japan: relation with female ovarian development. *Jpn. J. Herpetol.* **16**: 42-48.
- Johnston, E.E., Rand, M.S., Zweifel, S.G. (2006): Detection of multiple paternity and sperm storage in a captive colony of the central Asian tortoise *Testudo horsfieldii*. *Can. J. Zool.* **84**: 520-526.
- Köhler, G. (2004): Incubation of Reptile eggs: basics, guidelines, experiences. Krieger Publishing Company, Malabar, Florida.
- Madsen, T., Shine, R., Loman, J., Hakansson, T. (1992): Why do female adders copulate so frequently? *Nature* **335**: 440-441.
- Olsson, M., Madsen, T. (1998): Sexual selection and sperm competition in Reptiles. In: Birkhaed, T.R., Møller, A.P., Eds, Sperm competition and sexual selection. Academic Press, London, pp 503-577.
- Olsson, M., Shine, R. (1997): Advantages of multiple mating to females: a test of the infertility hypothesis using lizards. *Evolution* **51**: 1684-1688.
- Olsson, M., Schwartz, T., Uller, T., Healey, M. (2007): Sons are made from old stores: sperm storage effects on sex ratio in a lizard. *Biol. Lett.* **3**: 491-493.
- Olsson, M., Shine, R., Madsen, T., Gullberg, A., Tegelström, H. (1996): Sperm selection by females. *Nature* **383**: 585.
- Oppliger, A., Degen, L., Bouteillier-Reuter, C., John-Alder, H.B. (2007): Promiscuity and high level of multiple paternity in common wall lizards (*Podarcis muralis*): data from microsatellite markers. *Amphibia-Reptilia* **28**: 3301-3303.
- Pearse, D.E., Janzen, F.J., Avise, J.C. (2001): Genetic markers substantiate long-term storage and utilization of sperm by female painted turtles. *Heredity* **86**: 378-384.
- Preston, B.T., Stevenson, I.R., Pemberton, J.M., Coltman, D.W., Wilson, K. (2003): Overt and covert competition in a promiscuous mammal: the importance of weaponry and testes size to male reproductive success. *Proc. R. Soc. Ser. B.* **270**: 633-640.
- Ruckstuhl, K.E., Neuhaus, P. (2005): Sexual segregation in vertebrates: ecology of the two sexes. University Press, Cambridge.
- Saint Girons, H. (1982): Reproductive cycles of male snakes and their relationships with climate and female reproductive cycle. *Herpetologica* **38**: 5-16.
- Schuett, G.W., Gillingham, J.C. (1986): Sperm storage and multiple paternity in the copperhead, *Agkistrodon contortrix*. *Copeia* **1986**: 807-811.
- Schuett, G.W. (1992): Is long-term sperm storage an important component of the reproductive biology of temperate pitvipers? In: Campbell, J.A., Brodie jr, E.D., Eds, Biology of the Pitvipers. Selva, Texas, pp 169-184.
- Sever, D.M., Hamlett, W.C. (2002): Female sperm storage in reptiles. *J. Exp. Zool.* **292**: 187-199.
- Shanbhag, B.A. (2003): Reproductive strategies in the lizard, *Calotes versicolor*. *Curr. Sci.* **84**: 646-652.

- Smyth, M., Smith, M.J. (1968): Obligatory sperm storage in the Skink *Hemiergis peronii*. *Science* **161**: 575-576.
- Srinivas, S.R., Hegde, S.N., Sarkar, H.B.D., Shivanandappa, T. (1995): Sperm storage in the oviduct of the tropical rock lizard, *Psammophilus dorsalis*. *J. Morph.* **224**: 293-301.
- Strijbosch, H., Bonnemayer, J.J.A.M., Dietvorst, P.J.M. (1980): The Northernmost population of *Podarcis muralis* (Lacertilia, Lacertidae). *Amphibia-Reptilia* **1**: 161-172.
- Uller, T., Olsson, M. (2008): Multiple paternity in reptiles: pattern and processes. *Mol. Ecol.* **17**: 2566-2580.
- Uller, T., Stuart-Fox, D., Olsson, M. (2010): Evolution of primary sexual characters in Reptiles. In: Leonard, J., Cordoba-Aguilar, A., Eds, *The Evolution of Primary Sexual Characters in Animals*. Oxford University Press, Oxford, pp 425-452.
- Villaverde, G.A., Zucker, N. (1998): Sperm storage resulting in viable offspring in the tree lizard *Urosaurus ornatus* (Sauria: Phrynosomatidae). *Southwest. Nat.* **43**: 92-95.
- Wapstra, E., Swain, R., Jones, S.M., O'Reilly, J. (1999): Geographic and annual variation in reproductive cycles in the Tasmanian spotted snow skink, *Niveoscincus ocellatus* (Squamata: Scincidae). *Austral. J. Zool.* **47**: 539-550.
- Watson, P. J. (1991): Multiple paternity as genetic bet-hedging in female sierra dome spiders, *Linyphia litigosa* (Linyphiidae). *Anim. Behav.* **41**: 343-360.
- Yasui, Y. (1997): A "good-sperm" model can explain the evolution of costly multiple mating by females. *Am. Nat.* **149**: 573-584.
- Yasui, Y. (1998): The 'genetic benefits' of multiple mating reconsidered. *Trends Ecol. Evol.* **13**: 246-250.
- Zamudio, K., Sinervo, B. (2000): Polygyny, mate-guarding, and post-humous fertilizations as alternative mating strategies. *Proc. Natl. Acad. Sci. USA* **97**: 14427-14432.