

Preliminary note on the copulatory plugs found in *Elaphe quatuorlineata* (Bonnaterre, 1790) (Reptilia Serpentes)

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ABSTRACT

During a study on the reproduction of *Elaphe quatuorlineata* (Bonnaterre, 1790) (Reptilia Serpentes) bifid clear formations were occasionally observed being expelled by gravid females along with false feces (without the figurative component). These occurrences were typically noted approximately two weeks prior to ovodeposition, often in conjunction with the pre-ovodeposition molt. Through comparative bibliographic research, these structures were identified as copulatory spines or plugs produced by males during copulation. Their expulsion, occurring about two weeks prior to ovodeposition, was likely caused by the pressure exerted by the eggs along the oviducts.

KEY WORDS

Copulatory plugs; *Elaphe quatuorlineata*; monogamy.

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INTRODUCTION

During a study on biology of *Elaphe quatuorlineata* (Bonnaterre, 1790) from Italian (Castel-porziano, Apulia) and Greek (various Aegean islands) localities, bifid clear formations were occasionally observed being expelled by gravid females along with false feces, that is, excrement from the urinary and digestive tracts without the figurative component and greenish hue due to the presence of bile pigments (gravid females do not feed). These occurrences were typically noted approximately two weeks prior to ovodeposition, often in conjunction with the pre-ovodeposition molt. The regularity of this occurrence indicated successful fertilization of the eggs. As the observation of these bifid formations was incidental and not the primary focus of the research, similar structures may have gone unnoticed at other times in the species' repro-

ductive cycle. Comparative bibliographic research identified these structures as copulatory spines or plugs produced by males during copulation.

MATERIAL AND METHODS

Reptile reproduction is generally more challenging to study compared to that of most amphibians. Reptiles typically do not gather in large reproductive aggregations, nor display their intentions as vocally as anurans do. As a result, it is much more difficult for herpetologists to be in the right place at the right time to observe reptile mating (Goin & Goin, 1971). In these cases, information collected from the captive specimens is the only available source.

As confirmation, this note was prompted by the incidental discovery of copulatory plugs in some fe-

male specimens of *Elaphe quatuorlineata*, temporarily kept in captivity. These observations are part of an ongoing study aimed at better understanding the reproductive behavior of the species. The specimens studied, along with their geographic origins, are indicated in Table 1; upon completion of the study, the specimens were released back to their respective finding locations.

For histological examination, the samples were fixed in 70° alcohol and then embedded in paraffin; sections of 5 µm thickness were routinely stained with Hematoxylin-Eosin and Masson's Trichrome, for observation under the optical microscope.

RESULTS AND CONSIDERATIONS

The copulatory spines we observed are unpaired organs that form in the distal (uterine) part of the oviducts. Snake oviducts vary in size; this is why the spines are asymmetrically bifid (Fig. 1). In four-lined snakes, they are quite large, proportional to the species' size. Histological examination at the *M. Aleandri* Experimental Zooprophyllactic Institute of Lazio and Tuscany revealed that the spines contain spermatozoa.

Histologically, the samples appeared to be made up of numerous tubular structures, with a large cen-

tral lumen (Fig. 2) or folded over themselves to form cord-like structures. Between the tubules, aggregates of spermatozoa were evident, of variable size; occasionally, they were also found in small quantities inside the tubular lumen. The wall of the tubules was composed of four different layers (Fig. 3): a serous layer on the outside, consisting of a single layer of flattened cells; a thick intermediate muscular tunic, formed by a longitudinally arranged muscle layer and a circularly arranged one; a sub-mucosal tunic, consisting mainly of loose connective tissue, in which vascular structures were evident; a mucous membrane on the inside. The latter was characterized by numerous layers of epithelial cells, which sometimes showed vacuolization of the cytoplasm and intercellular edema (Fig. 4) and which tended to exfoliate towards the lumen of the tubule, accumulating and sometimes causing occlusion of the lumen itself.

These structures have been reported in various taxa, including nematodes, crustaceans, arachnids, insects, reptiles, and mammals. Herman (1994), Moreira & Birkhead (2003, 2004) and Moreira et al. (2006, 2007) discussed these structures in lizards. Barros et al. (2017) reported the phenomenon in a Crotalid snake. We are not aware of records of copulatory spines in European snakes. In New World serpents, particular emphasis has been placed on the sexual behavior of the eastern garter-snake, *Thamnophis sirtalis* (Linnaeus, 1758), from which the following observations are derived.

In snakes, sperm is produced in the testes and transported through the vas deferens; it is believed that the copulatory plug is formed by the action of the renal sexual segment, via seminal fluid transported through the ureter. In fact, males with ureter ligation did not produce copulatory plugs, causing sperm to leak from the female cloaca immediately after copulation (Friesen et al., 2013). This seminal fluid appears to be homologous to that produced by the seminal vesicles and prostate in mammals (Friesen et al., 2020). In *Vipera berus* the "plug effect" is achieved by contraction of the uterus (Andr n et al., 1997). According to Shine et al. (2000) the plug material is produced rapidly (two minutes) at the conclusion of copulation, after a long period (eight minutes) of sperm transfer. The duration of plug retention inside the females can vary from two days to two weeks, depending on the ambient temperature; after this period, it seems that the females

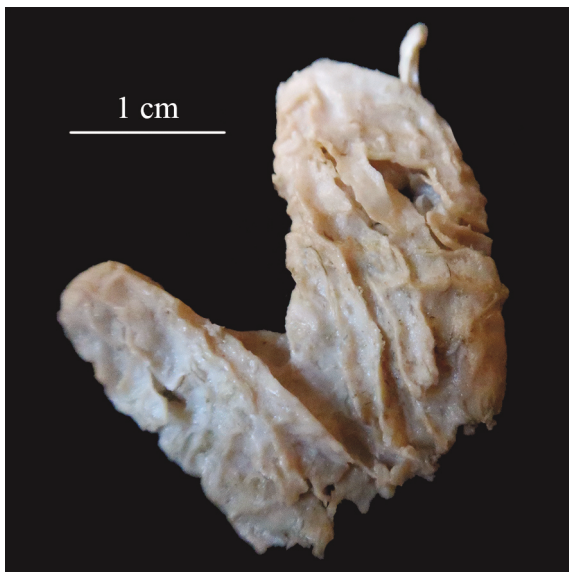


Figure 1. Copulatory spine expelled from a female specimen of *Elaphe quatuorlineata* (Bonnaterre, 1790) found in Castelporziano, Rome, Italy (specimen 19, Table 1).

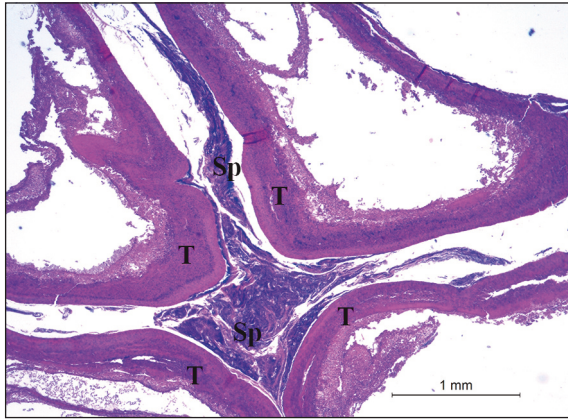


Figure 2. Histological section. The sample appears to be made up of numerous tubular structures (T), with a large central lumen; between the tubules, aggregates of spermatozoa are evident (Sp). Hematoxylin-Eosin Stain, 2.5x.

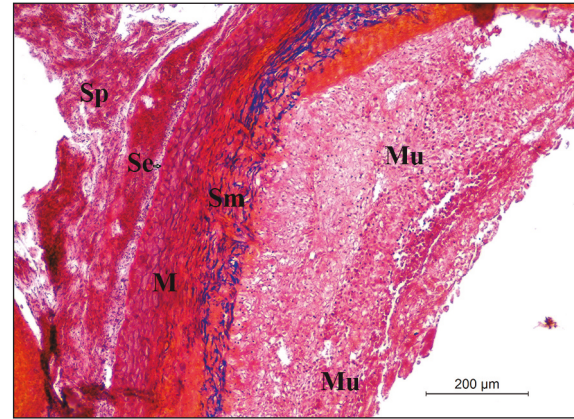


Figure 3. Histological section of the wall of the tubules. Se - Serous layer; M - Muscular tunic; Sm - Submucosal tunic; Mu - Mucous membrane. Outside the serous layer, aggregates of spermatozoa are observed (Sp). Masson's Trichrome Stain, 10x.

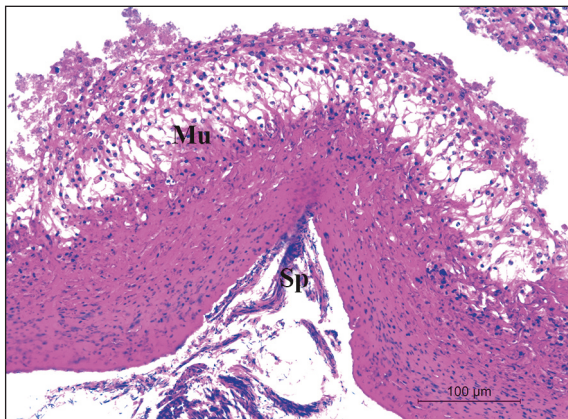


Figure 4. Histological section of the wall of the tubules. The mucous membrane (Mu) is characterized by numerous layers of epithelial cells, which sometimes showed vacuolization of the cytoplasm and intercellular edema. On the outside of the wall large aggregates of spermatozoa are observed (Sp). Hematoxylin-Eosin Stain, 20x.

themselves are able to dissolve or expel the plugs through specific enzymes (Devine, 1984). According to Shine et al. (2000) the plugs remain in place for less than 72 hours. Histological analysis revealed sperm distributed throughout the copulatory plug, suggesting that the plug may function as a spermatheca: the protein matrix holds the sperm, which is gradually released while the plug remains in place (Friesen et al., 2013). In this way, the ejaculated proteins can influence fertilization through various functions, including pH balance, sperm pro-

tection and nourishment, alteration of sperm physiology, and the induction of behavioral changes in females (Mangels et al., 2016).

In *Thamnophis sirtalis*, copulatory plugs become solid and firmly attached to the cloacal walls within 15 minutes of copulation, making it hard to imagine them being displaced by the hemipenis of a rival male (Shine et al., 2000). The same authors attempted to dislodge the plugs using a force of 300 g but were unsuccessful. From our observations, in *Elaphe quatuorlineata*, repeated matings, especially with different males under experimental conditions, can result in the expulsion of copulatory plugs (specimens 4, 8, 11 and 12, Table 1). This expulsion, which usually happens about two weeks before ovodeposition, is likely due to the pressure force exerted by the eggs along the oviducts (Table 1).

Functional meaning of the copulatory plug

The various hypotheses regarding the functional significance of the copulatory plug are briefly summarized:

1. Physically block the female from remating (Devine, 1975; Friesen et al., 2013). It should be noted, however, that remating by females with a copulatory spine can occur (Shine et al., 2000), even with two spines (Friesen et al., 2020).

2. Provide pheromone signals that indicate decreased female receptivity, thus discouraging rival males from courting (Devine, 1977). However,

these signals likely stem from the fluids associated with copulation rather than the presence of the spine itself (Shine et al., 2000).

3. Allow a gradual release of sperm within the female reproductive tract, effectively functioning as a spermatophore (Friesen et al., 2013).

4. Reduce sperm loss from the female's reproductive tract after copulation (Shine et al., 2000; Friesen et al., 2013).

The production of copulatory plugs could represent an adaptation that evolved in species sub-

jected to dense aggregations, such as *Thamnophis sirtalis* [where up to 62 males have been counted for a single female (Shine et al., 2001)]. In these intraspecific aggregations, there is strong selection pressure against prolonged copulation, with a selective advantage for males that can quickly transfer sperm (Friesen et al., 2013). These snakes, in fact, exhibit relatively brief copulation times, and the production of the copulatory plug helps minimize sperm competition. On the other hand, it appears that males modulate their ejaculates according to the intensity of sperm competition; in the lizard

	<i>Elaphe quatuorlineata</i> subspecies	Location, date found	Moult	Copulatory plug	Ovodeposition	Copulatory plug-ovodeposition
1	<i>quatuorlineata</i> (Bonnaterre, 1790)	Italy, Castelporziano, 1/7/1973	7/7/1973	4/7/1973	17/7/1973	13 days
2	<i>muenteri</i> (Bedriaga, 1881)	Greece, Mykonos, 8/5/1984	14/7/1984	13/7/1984	27-29/7/1984	19-21 days
3	<i>muenteri</i> (Bedriaga, 1881) "concolor" phenotype	Greece, Amorgos, 10/5/1987	28/6/1988	28/6/1988	14/7/1988	16 days
4	<i>quatuorlineata</i> (Bonnaterre, 1790)	Greece, Tinos, 18/5/1989	30/6/1989	5/6/1989	16/7/1989	41 days
5	<i>muenteri</i> (Bedriaga, 1881)	Greece, Amorgos, 24/5/1987	12/6/1990	12/6/1990	26/6/1990	14 days
6	<i>muenteri</i> (Bedriaga, 1881)	Greece, Naxos, 12/5/1990	13/7/1990	13/7/1990	25/7/1990	12 days
7	<i>muenteri</i> (Bedriaga, 1881)	Greece, Amorgos, 24/5/1987	16/6/1991	16/6/1991	1/7/1991	15 days
8	<i>parensis</i> Cattaneo, 1999	Greece, Paros, 21/5/1991	9/7/1991	24/6/1991 9/7/1991	24/7/1991	30 days 15 days
9	<i>parensis</i> Cattaneo, 1999	Greece, Paros, 21/5/1991	1/6/1992	1/6/1992	16/6/1992	15 days
10	<i>muenteri</i> (Bedriaga, 1881)	Greece, Amorgos, 24/5/1987	8/6/1992	8/6/1992	24/6/1992	16 days
11	<i>quatuorlinata</i> (Bonnaterre, 1790)	Greece, Skiathos, 21/5/1994	12/7/1994	5/6/1994	26/7/1994	51 days
12	<i>scyrensis</i> Cattaneo, 1998	Greece, Skyros, 19/5/1993	9/6/1995	30/5/1995	30/6/1995	31 days
13	<i>scyrensis</i> Cattaneo, 1998	Greece, Skyros, 12/5/1993	10/6/1997	14/6/1997	2/7/1997	18 days
14	<i>quatuorlineata</i> (Bonnaterre, 1790)	Italy, Apulia, 4/9/1998	27/5/2002	27/5/2002	14/6/2002	18 days
15	<i>scyrensis</i> Cattaneo, 1998	Greece, Skyros, 13/5/1993	4/6/2002	4/6/2002	20/6/2002	16 days
16	<i>muenteri</i> (Bedriaga, 1881)	Greece, Naxos, 18-21/8/1993	6/6/2002	6/8/2002	20/6/2002	12 days
17	<i>quatuorlineata</i> (Bonnaterre, 1790)	Italy, Castelporziano, 27/5/2021	28/6/2021	18/6/2021	10/7/2021	22 days
18	<i>quatuorlineata</i> (Bonnaterre, 1790)	Italy, Castelporziano, 29/6/2023	5/7/2023	5/7/2023	18/7/2023	13 days
19	<i>quatuorlineata</i> (Bonnaterre, 1790)	Italy, Castelporziano, 11/7/2024	11-12/7/2024	13/7/2024	23/7/2024	10 days

Table 1. Time correlations between the expulsion of the copulatory spine and some reproductive parameters of specimens belonging to the various subspecies of *Elaphe quatuorlineata*. All Greek locations indicate islands of the Aegean Sea.

Ctenophorus fordi (Storr, 1965), males copulate for longer and deposit more sperm when they copulate after observing the female mating with another male (Olsson, 2001); the male sand lizard *Lacerta agilis* Linnaeus, 1758 uses olfactory cues to adjust the duration of copulation based on the relatedness of his rival to the female (second males transfer larger ejaculates if the first male is more closely related to the female, as male-female relatedness negatively influences a male's probability of paternity) (Olsson et al., 2004).

In a recent study (Cattaneo, 2022) *Elaphe quatuorlineata* was described as monogamous; in fact, it appears that even monogamous species produce copulatory plugs [this is the case, for example, of rodents of the genus *Peromyscus* (Ribble, 1991)]. Obviously, in such cases, the first two hypotheses on the functional significance of copulatory plugs should not be considered. At this point, it is worth mentioning some observations concerning the reproductive biology of *Elaphe quatuorlineata*, which may be related to copulatory plug production in this species.

It has been observed that in the wild, females of *E. quatuorlineata* consistently choose the same male to reproduce, receiving him with a bite on the head at least under experimental conditions (see Cattaneo, 2022). Among the ophidic species known to us, male *E. quatuorlineata* expel the greatest amount of sperm during the effort to evacuate both true and false excrement. In these cases, white, gelatinous clumps can often be seen on the feces, very likely attributable to sperm. Once emitted, the sperm quickly solidifies in the air and adheres strongly to the substrate (all of these characteristics could contribute to the formation of copulatory spines). Copulations can last several hours and occur every 10–20 days throughout the reproductive period. During mating, there may be blood loss from the female's cloaca, and the partners may adopt a posture resembling an inverted caduceus; their envelopment is facilitated by a clear and very sticky fluid likely emitted by the renal sexual segment. For more information on the reproductive behavior of *E. quatuorlineata* see Cattaneo (2022).

In conclusion, based on limited data, it appears that the copulatory spines produced by *Elaphe quatuorlineata*, a monogamous species, may function to store and distribute sperm, as well as reduce sperm loss from the cloaca (see hypotheses 3 and 4

regarding the functional meaning of copulatory plugs). Due to the progressive pressure exerted by the continuously developing eggs, the spine is expelled about two weeks before ovodeposition, facilitating the egg laying. Further, focused investigations are needed to fully understand the functional significance of these copulatory spines in *Elaphe quatuorlineata*. In any case, counteracting multiple paternity should be excluded.

REFERENCES

- Andrén C., Nilson G., Höggren M. & Tegelström H., 1997. Reproductive strategies and sperm competition in the adder, *Vipera berus*. In: Thorpe R.S., Wüster W. & Malhotra A. (Eds.), *Venomous snakes: Ecology, evolution and snakebite*, Symp. zool. Soc. Lond., Oxford University Press, 70, pp. 129–141.
- Barros V.A., Rojas C.A. & Almeida-Santos S.M., 2017. Mating plugs and male sperm storage in *Bothrops cotiara* (Serpentes, Viperidae). *Herpetological Journal*, 27: 115–119.
- Cattaneo A., 2022. Filopatria, monogamia e stretta sintopia con *Hierophis viridiflavus* da parte di *Elaphe quatuorlineata* nella Tenuta di Castelporziano, con particolare riferimento all'area di Torpaterno (Reptilia: Colubridae). *Rendiconti Accad. Naz. Scienze detta dei XL, Memorie e Rendiconti di Chimica, Fisica, Matematica e Scienze Naturali*, 3 (serie VI): 197–213.
- Devine M.C., 1975. Copulatory plugs in snakes: enforced chastity. *Science*, 187: 844–845. <https://doi.org/10.1126/science.1114329>
- Devine M.C., 1977. Copulatory plugs, restricted mating opportunities and reproductive competition among male garter snakes. *Nature*, 267: 345–346.
- Devine M.C., 1984. Potential sperm competition in reptiles: behavioral and physiological consequences. In: *Sperm Competition and the Evolution of Animal Mating Systems*, Smith R.L. (Ed.), Academic Press, Orlando, pp. 509–521.
- Friesen C.R., Shine R., Krohmer R.W. & Mason R.T., 2013. Not just a chastity belt: the functional significance of mating plugs in garter snakes, revisited. *Biological Journal of the Linnean Society*, 109: 893–907.
- Friesen C.R., Kahrl A.F. & Olsson M., 2020. Sperm competition in squamate reptiles. *Philosophical Transactions of the Royal Society B*, 375: 20200079. <https://doi.org/10.1098/rstb.2020.0079>
- Goin C.J. & Goin O.B., 1971. *Introduction to Herpetology*. W.H. Freeman and Company, San Francisco, XI + 353 pp.

- Herman A., 1994. First record of mating plugs in lizards. *Amphibia-Reptilia*, 15: 89–93.
- Mangels R., Tsung K., Kwan K. & Dean M.D., 2016. Copulatory plugs inhibit the reproductive success of rival males. *Journal of Evolutionary Biology*, 29: 2289–2296.
<https://doi.org/10.1111/jeb.12956>
- Moreira P.L. & Birkhead T.R., 2003. Copulatory plugs in the Iberian rock lizard do not prevent insemination by rival males. *Functional Ecology*, 17: 796–802.
- Moreira P.L. & Birkhead T.R., 2004. Copulatory plug displacement and prolonged copulation in the Iberian rock lizard (*Lacerta monticola*). *Behavioral Ecology and Sociobiology*, 56: 290–297.
<https://doi.org/10.1007/s00265-004-0786-5>
- Moreira P.L., Lopez P. & Martín J., 2006. Femoral secretions and copulatory plugs convey chemical information about male identity and dominance status in Iberian rock lizards (*Lacerta monticola*). *Behavioral Ecology and Sociobiology*, 60: 166–174.
<https://doi.org/10.1007/s00265-005-0153-1>
- Moreira P.L., Nunes V.L., Martín J. & Paulo O.S., 2007. Copulatory plugs do not assure high first male fertilisation success: Sperm displacement in a lizard. *Behavioral Ecology and Sociobiology*, 62: 281–288.
<https://doi.org/10.1007/s00265-007-0463-6>
- Olsson M., 2001. “Voyeurism” prolongs copulation in the dragon lizard *Ctenophorus fordi*. *Behavioral Ecology and Sociobiology*, 50: 378–381.
<https://doi.org/10.1007/s002650100373>
- Olsson M., Madsen T., Ujvari B. & Wapstra E., 2004. Fecundity and MHC affects ejaculation tactics and paternity bias in sand lizards. *Evolution*, 58: 906–909.
<https://doi.org/10.1111/j.0014-3820.2004.tb00423.x>
- Ribble D.O., 1991. The monogamous mating system of *Peromyscus californicus* as revealed by DNA fingerprinting. *Behavioral Ecology and Sociobiology*, 29: 161–166.
- Shine R., Elphick M.J., Harlow P.S., Moore I.T., LeMaster M.P. & Mason R.T., 2001. Movements, mating, and dispersal of red-sided gartersnakes (*Thamnophis sirtalis parietalis*) from a communal den in Manitoba. *Copeia*, 1: 82–91.
- Shine R., Olsson M.M. & Mason R.T., 2000. Chastity belts in gartersnakes: the functional significance of mating plugs. *Biological Journal of the Linnean Society*, 70: 377–390.
<https://doi.org/10.1006/bijl.1999.0427>