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OBTAINING OVIPAROUS GRASS SNAKE, *NATRIX NATRIX* (SERPENTES, COLUBRIDAE), EMBRYOS AT EARLY DEVELOPMENTAL STAGES BY CAESAREAN SECTION

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Obtaining Oviparous Grass Snake, Natrix natrix (Serpentes, Colubridae), Embryos at Early Developmental Stages by Caesarean Section. Sheverdyukova, H. V., Merzlikin, I. R. — There is a specific feature in the developmental biology of oviparous snakes: embryos in the eggs, which were just laid, have already undergone significant development. This fact makes it significantly complicated to obtain data on organs' development at early stages of embryogenesis. In addition, the fertilization time and the duration of snake pregnancy in the wild are unknown. In order to obtain the embryos of an oviparous grass snake *Natrix natrix* (Linnaeus, 1758) at successive developmental stages with minimal harm to gravid females we used caesarean section. The past known experience of performing caesarean section in snakes and anesthesia in reptiles were used. All the embryos were taken from the upper oviduct of a female simultaneously; in this way we eliminated the influence of medications on embryos' development. The described method is valuable when it is necessary to obtain snake embryos and to preserve the life of the female and, possibly, its reproductive ability.
Key words: development, egg retention, embryogenesis, oviposition, snake.

Introduction

The study of embryogenesis of morphological features in different species is still relevant. The data on embryogenesis are of great interest for those who perform comparative analysis of the formation of morphological characteristics in different species, and also for establishing of phylogenetic connections (Jeffery et al., 2002; Organ et al., 2015; Richardson, 1995; Schlosser, 2001). It is a daunting task to obtain series of embryos at successive developmental stages to study the formation of a certain trait. Thus, in gravid reptiles, which caught in natural habitats, the exact fertilization dates as well as the developmental stage of the embryos inside the female are unknown (Billett et al., 1985; Velhagen and Savitzky, 1998). Although, there are data on the developmental rate of snake embryos within a female (Gomez et al., 2008; Holtzman and Halpern, 1989;

Zehr, 1962), we are not able to determine how quickly embryos develop in females under specific conditions.

As for oviparous snake species, it might seem that one can easily collect the needed number of eggs and select embryos at successive developmental stages. However, one feature of the snake developmental biology makes this task difficult: embryos develop in utero for about one-third of the total period of embryogenesis (Shine, 1983). So, at the time of oviposition the embryos are already at advanced stages of development (De La Panouse and Pellier, 1973; Fukada, 1956).

The entire period of embryogenesis is traditionally divided into developmental stages, based on morphological features of embryos. There are several tables of developmental stages for different snake species, both oviparous and viviparous (Boback et al., 2012; Boughner et al., 2007; Hubert and Dufaure, 1968; Jackson, 2002; Khannoon and Evans, 2014; Korneva, 1969; Tokita and Watanabe, 2019; Zehr, 1962). The developmental stages are determined by formation of one or another external morphological feature: for example, number of somites, formation of nostrils, number of body coils, presence of eyelid, eye pigmentation, presence of scales, etc. As a rule, the tables developed for oviparous species cover only the developmental stages of embryos after oviposition. While the tables for viviparous species may contain data on embryo development from the zygote.

The duration of snake embryogenesis may vary considerably in different species. It may depend on external factors such as temperature (Deeming and Ferguson, 1991; Hubert, 1985; Ji and Du, 2001; Lorioux et al., 2012; Vinegar, 1973; Zehr, 1962) or specific developmental properties of different species. Despite of the difference in embryonic rates, snake embryos undergo successive stages in the formation of morphological features. The latter are the same in all the studied species, whether the embryos develop inside the egg or in female's body. It is also difficult to compare the developmental stages of various snake species based on a single table, since there are some heterochronies in the formation of individual features in some species (Boback et al., 2012; Jackson, 2002; Khannoon and Zahradnicek, 2017). Either way, various researchers often sought to compare their data on developmental stages with existing tables of development. Traditionally, the developmental stages of snake embryos are compared with the table of stages of normal development proposed for *Thamnophis sirtalis sirtalis* (Colubridae) by Zehr (1962).

All the oviparous snake species whose embryos have been studied before, lay eggs with embryos being at different developmental stages: between 22 and 28 according to the table by Zehr (1962). According to Shine (1983), at the moment of oviposition embryos of various snake species are at the developmental stage 31–34 by Hubert and Dufaure (1968) (approximately 23–27 by Zehr). *Psammophis sibilans* (Khannoon and Zahradnicek, 2017) lays eggs with embryos at developmental stage 21–22, *Elaphe quadrivirgata* — 23 (Matsubara et al., 2014), *Natrix tessellata* — 27 (Korneva, 1969). Jackson (2002) argues that *Naja kaouthia* embryos after oviposition correspond to the developmental stage 25; Boughner and co-authors (2007) state that *Python sebae* embryos are at stage 26. Embryos of *Naja h. haje* after oviposition correspond to developmental stage 26 (Khannoon and Evans, 2014). Boback and co-authors (2012) determine that *Boaedon (Lamprophis) fuliginosus* lays eggs with embryos at Zehr stage 26. As a result of this feature of snake embryogenesis, the early developmental stages are overlooked, and many interesting and important data remain unknown. Therefore, those who research the development of a certain organ or organ system face the difficult task of obtaining embryos at earlier, necessarily successive developmental stages.

It becomes clear, to obtain pre-ovipositional or intra-uterine embryos of both oviparousand viviparous species, we have to resort to surgery (Savitzky et al., 2012). Researchers approached this task differently. The following procedures have been performed:

1. A series of caesarean sections

- in oviparous and ovoviviparous species of the family Colubridae (Clark, 1937) — several caesarean sections were performed on one female with an interval of at least 3 days. The embryo was taken and surgical staples were applied. Information on the survival of females is absent;
- in viviparous *Thamnophis sirtalis* (Zehr, 1962) — several caesarean sections were performed on one female with intervals of 1 to 7 days. Out of 242 operations, 15 were lethal.
- series of Thamnophiines embryos at different developmental stages were obtained surgically through a procedure similar to that of Clark (1937) (Velhagen and Savitzky, 1998). Several surgeries were performed on the same females. The embryos and eggs were removed with oviduct section.

2. Several embryos were taken from the ovoviviparous species of genus *Thamnophis* (Holtzman and Halpern, 1989), followed by their incubation in a special medium in vitro until the necessary stage. The maximum period of embryonic maintenance under such conditions was 35 days in one embryo. Information on females' survival is absent. There is also the experience of growing embryos of viviparous snakes in culture: *Vipera berus* developed over 24 hours (Billett et al., 1985) and *Vipera aspis* also developed for two weeks in culture (Hubert, 1985).

In all experiments mentioned above, the authors emphasized that embryos, taken during repeated operations and newborns from females that survived surgery, were morphologically normal and healthy, and therefore concluded that anesthesia and operational stress did not affect the embryo development.

3. In the oviparous species *Elaphe quadrivirgata* (Matsubara et al., 2014), two oviducts with eggs and ovarian arteries were completely removed and cultivated ex vivo in a special medium. The authors were able to maintain embryonic development in the eggs for at least 39 hours. This method was called the sausage style culture (SSC). Information on the females' survival is absent.

4. To obtain the embryos at different developmental stages in viviparous *Gloydius blomhoffii* the authors used decapitation of females (Tokita and Watanabe, 2019).

We set the task to obtain grass snake, *Natrix natrix* Linnaeus 1758, embryos at successive developmental stages until oviposition by means of caesarean section. The main task was to obtain healthy embryos, to exclude the medicinal effect on embryo development and cause minimal harm to gravid females.

The grass snake *N. natrix* is a widespread and common nonvenomous oviparous European species. All these characteristics make it a convenient object for morphological studies. In Ukraine the mating season of *N. natrix* begins in May. Oviposition time is extended from mid-June to early August. Females lay 6 to 30 eggs. Juveniles appear more often in late August (Tarashchuk, 1959). As in other snake species, embryo retention is also noted in *N. natrix*. According to our data, in all just oviposed clutches, embryos are at the developmental stage 27 according to the table of the stages of normal development by Zehr (1962) (Kovtun and Sheverdyukova, 2015). There is a table of embryo development of *N. natrix* that includes the period of embryogenesis after oviposition (Rupik, 2002), but there is still no table of normal development for this species, covering all stages of development — from the zygote to hatching.

Material and methods

Our experiment was conducted in 2011. Nine gravid females were caught in natural habitats in the middle of June in Sumy region, Ukraine. We placed them in specially equipped terraria with water, shelter and wet moss. The temperature was maintained at 29 ± 2 °C. In such conditions, all the females were kept until the natural oviposition and the beginning of feeding. The period lasted about a month. After the experiment all the females were released into their natural habitats in the middle of July. The experiment was performed in accordance with relevant institutional and national guidelines.

During the operations, modified Clark's method (Clark, 1937) was used. The anesthesia of females was held in accordance with protocol, designed especially for reptiles (Vasiliev and Timerina, 2000).

The photos of embryos (figs 1 and 2) were taken with a stereo microscope Leica M156 C equipped with Leica DFC450 C digital camera and SW Kit. The figure 3 was taken using Konica Minolta Dimage Z10 camera.

Results

As already mentioned in the introduction, it is rather difficult to determine the gestational age of a female caught in the wild. We noticed that a few days (14–17) before oviposition, *N. natrix* females refused to eat. We assumed that this is exactly the stage when the eggs reached the right size. So the number of days of anorexia became the main criterion for the selection of females for operations. Females were selected for the operations between the 9th and 15th days of anorexia.

During the operation the modified technique of anesthesia developed especially for reptiles was used. Aminazine (0.5 mg/kg) was injected intramuscularly into gravid females 40–60 minutes before the anesthesia for preliminary preparation. It allowed reducing the dose of the anesthesia by 2–3 times. Such preparation for anesthesia facilitates and accelerates the recovery period after operation. Atropine sulfate (0.04 mg/kg) was used to inhibit the vagosympathetic reactions. It was injected intramuscularly 10–15 min before the anesthesia. We used ketamine (30 mg/kg) as an anesthetic. Ketamine was injected starting with the lowest dose; if anesthesia did not work after 30 minutes, we added 2 mg step by step until complete anesthesia was reached. The depth of anesthesia was determined by complete tail sedation (oppression of reflexes and loss of muscle tone). Cardiamin (0.02 ml/kg) was used intramuscularly to reverse anesthesia. To avoid the rapid elimination of medications from the female, all drugs were injected into the anterior third of the body. To prevent infection, the antibiotic lincomycin (10 mg/kg) was injected for 5 days after the operation.

We used modified Clark's method of caesarean section, developed specifically for snakes. The main task was to make operations as sparing as possible not only to save females' lives, but also their ability to reproduce in the future. Therefore, we conducted only one operation on one female and opened only one (upper right) oviduct. All eggs were taken from the oviduct in order to have sufficient quantity of embryos and to avoid influence of the medications.

After the narcotization, a longitudinal incision was made slightly lateral to the abdominal midline at the level of the second from the most cranial egg. Clark made the

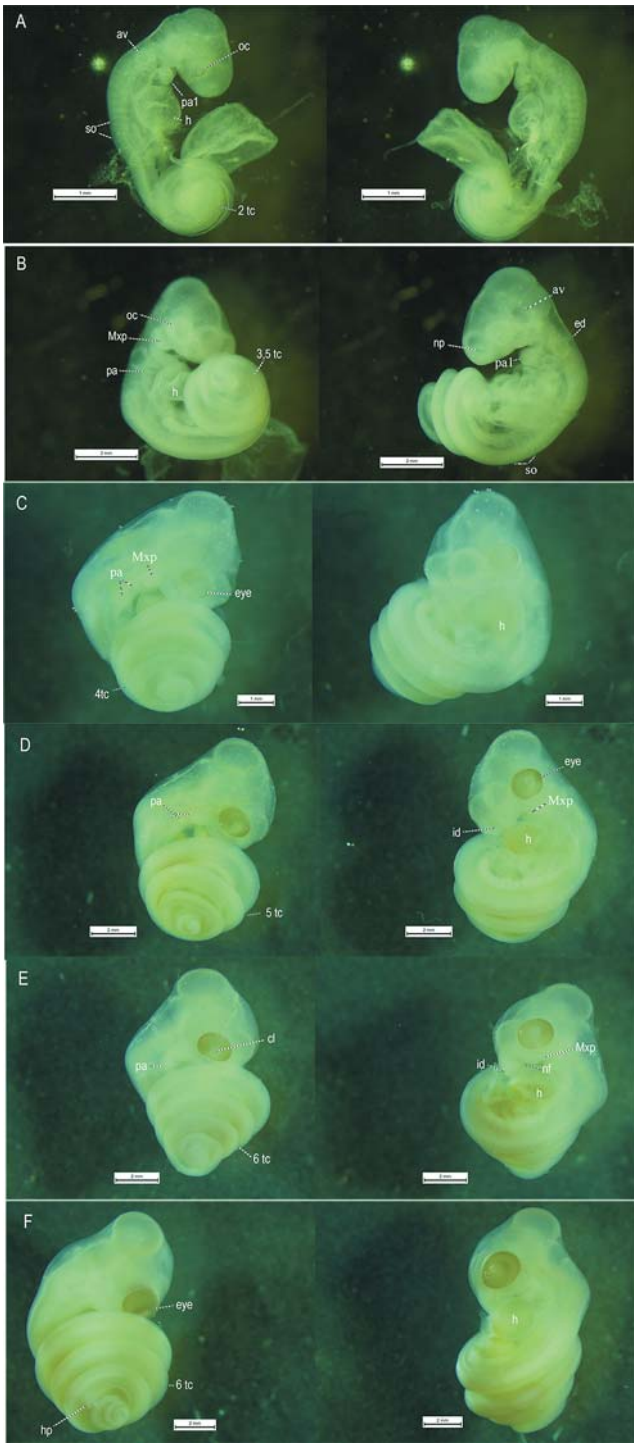

Fig. 1. *N. natrix* embryos at developmental stages A. 20. B. 22. C. 24. D. 25. E. 26. F. 27: av —auditory vesicle; cl —crystalline lens; ed — endolymphatic duct; h — hart; hp — hemipenes; id — internasal depression; Mxp — maxillary process; nf — nasal fold; np — nasal pit; oc — optic cup; pa — pharyngeal arch; so — somite; tc — trunk coils.

incision at the level of the first cranial egg, but according to our experience in this case the lung may be extruded. In order not to damage the very thin lung tissue, which may lead to the female's death, we performed the incision further caudad. After the oviduct was released from the peritoneum, a small incision was made on its median line, through which the eggs were taken out. Because the tissue of the oviduct is too thin and tender, it was not sewn. The second oviduct remained untouched. The incision was sewn with surgical threads: one stitch at the level of each scute. The seam was treated with an antiseptic. To minimize the negative impact on animals' bodies, we did not perform repeated surgeries, as it was done by previous authors.

Nine caesarean sections were performed. As a result we obtained embryos at developmental stages 20, 22, 24, 25, 26, 27 according to the table of Zehr (1962) (fig. 1). Using this method we can also obtain embryos at earlier developmental stages, considering that embryos develop most rapidly at the beginning of embryogenesis. Embryos of intermediate developmental stages were also obtained. Such stages are marked with "+". For example, the developmental stage 22+ is the intermediate stage between developmental stages 22 and 23. The results of operations are described in table 1. It is also interesting to note that in some cases, simultaneously delivered embryos from one female differed in the rate of development (in females 1 and 4, see table 1) (fig. 2).

Subsequently, the females oviposited from the untouched oviduct 6–12 days after the operations. Most of the clutches, which were laid after a longer period of

time, were externally normal. In one case, we performed an operation on the eve of natural oviposition. The obtained embryos were at development stage 27, but were all dead. In this case, the operation did not accelerate or delay oviposition, but affected the embryos. The female began to lay the remaining inside eggs 0.5 days after the surgery. It could not oviposit them itself, so we squeezed the eggs out; they turned out to be deprived of a shell membrane.

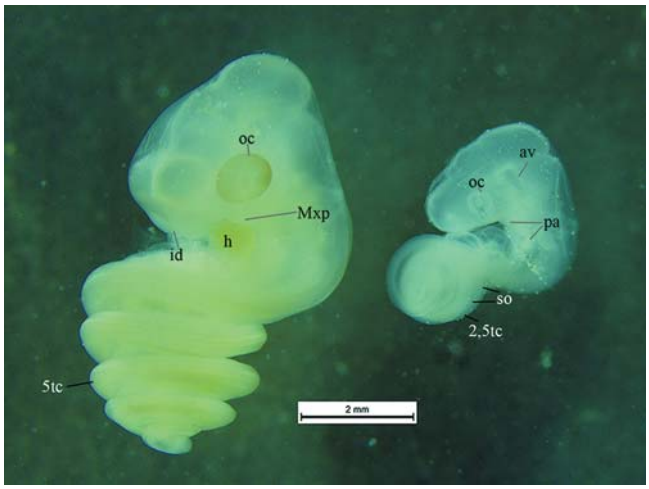

Fig. 2. *N. natrix* embryos at developmental stages 20 and 25+ from one clutch.

After oviposition and post-operative rehabilitation, when the incisions were healed and the females began to feed actively, they were released at their sites of capture. A year later, one gravid female with a scar after a caesarean section was caught (fig. 3). This indicates the success of our experiment, as a result of which the females survived.

Discussion

We were able to successfully conduct several caesarean sections in order to obtain *N. natrix* embryos at successive developmental stages. At the same time, minimal harm was done to gravid females, and drugs did not affect the development of the obtained embryos.

In two cases, we observed a different rate of development in embryos delivered simultaneously from one female. Such a difference in the rate of development in some cases was also described by Matsubara et al. (2014): the difference in the development of embryos reached up to 10 somites in one clutch. The reasons for this non-simultaneity of development are not precisely known. As well as it is not known, whether the embryos lagged behind in development would catch up with the other embryos, or whether the developmental delay was associated with the pathology of development that would cause the death of the embryos. Thus, in such operations, the removal of only one embryo, as previously described (Clark, 1937; Holtzman and Halpern, 1989; Matsubara et al., 2014; Zehr, 1962) may misrepresent the developmental stages of all embryos inside one female.

Fig. 3. Scar on the abdomen of the *N. natrix* female, caught one year after caesarean section.

The early developmental stages of embryos are rapid. Gomez et al. (2008) found that the rate of somite formation in snakes before oviposition (at 30 °C) is one somite per 71–105 min. According to these authors there are about 91 hours (3.5 days) between stages 23 and 25. Developmental stages 25 and 27 are separated by no more than 3 days (at 23–27 °C) (Zehr, 1962). *In vitro* there are 3 days between the developmental stages 24 and 26 and 2 days between stages 26 and 28 (at

Table 1. Details of the performed caesarean sections

Female	Days of anorexia	Number of eggs delivered from the upper oviduct	Developmental stage of embryos (Zehr, 1962)	Number of days after the section, when natural oviposition happened	Number of oviposited eggs	Embryos in naturally oviposited eggs
1	11	14+1 embryo was dead before the section	22 (21+ – 1,22+ – 2)			
2	min12	19	24			
3	12	5	25	12	9	all embryos dead
4	14	6	25+ – 5 (20 –1)	8	6	two dead embryos
5	min 13	8 (all embryos are dead)	27	0,5	9	all embryos dead
6	?	7	25	6	3	all embryos healthy
7	11	12	26	6	6 (+1 egg left in the female)	all embryos healthy
8	9	6	20	12	4	all embryos healthy
9	15	6+1 embryo was dead before the section	25	6	6	one dead embryo

27 ± 4 °C) (Holtzman and Halpern, 1989). We kept *N. natrix* females at 29 ± 2 °C during the whole experiment. In this situation, when embryos are taken out, for example, at developmental stage 26, it is expected that the female will lay eggs naturally in 1–2 days after the operation and the embryos will be at developmental stage 27. However, we observed an interesting phenomenon: if the operation was carried out with embryos at development stage 25 or 26, then the females laid eggs with healthy embryos at developmental stage 27 six days after the operation (females 6, 7 and 9 in table 1). The embryos developed normally inside the eggs, were superficially healthy and were taken at needed stages and fixed. Thus, after the chemical and mechanical stress, applied on the female during the operation, the development of embryos was suspended in some incomprehensible way, and, probably, resumed when female's body recovered. The mechanism of this phenomenon was not clear. Perhaps, one of the used drugs affected the development. In one case (female 4 in table 1), oviposition was delayed up to eight days, but the number of dead embryos also increased. In the case of one operation with embryos at the developmental stage 25 (female 3 in table 1), oviposition occurred only on the twelfth day, but all the embryos were dead. Probably, in this case, there was an individual overdose of some medicines, which resulted in the embryo death, the female needed more time to recover, and the need to lay eggs on time disappeared; therefore, oviposition occurred after a longer period.

Year later, the capture of a gravid female indicated that the operated females were not only able to survive, but also retain their reproductive ability. We deliberately performed surgeries on one oviduct only, knowing that its tissues are unlikely to be recovered in the future. The second oviduct remained intact. Our experience shows that in some cases (see table 1) all the eggs were situated in one oviduct, the second one was empty. Why this happens in nature is not known. We assume that in operated females all the eggs will be formed in one oviduct. If females laid the remaining eggs themselves even in a few days after surgeries, they will probably be able to do it in future.

Conclusion

The described method of obtaining snake embryos at successive developmental stages can be successfully used to obtain embryos not affected by drugs in various oviparous snake species. Depending on the goals of morphological studies, the caesarean section can also be used to obtain embryos at earlier developmental stages, considering that development at earlier stages is faster. This method is especially valuable when it is necessary to obtain embryos of rare species and save female's life, and possibly its reproductive ability.

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