



# Reproductive characteristics, diet composition and fat reserves of nose-horned vipers (*Vipera ammodytes*)

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Long-term ecological studies are usually both time-consuming and costly, particularly when conducted on species with low detectability, such as vipers. An alternative, non-expensive method for obtaining detailed information about numerous important ecological traits, e.g. size at maturity, reproductive output, diet composition and amount of body reserves, are dissections of museum specimens. We dissected 237 museum specimens (125 males and 112 females of all age classes) of the nose-horned vipers *Vipera ammodytes* from the central and western regions of the Balkan Peninsula. Their reproductive and digestive systems were examined, and fat stores estimated. Relative testes volumes were significantly higher in adults than in subadults. Also, we found seasonal variation of testes volumes in adult males. Females became mature at around 43.8 cm snout-to-vent length (SVL); 38.1 % of adult females were pregnant (i.e. had developed eggs/embryos, suggesting bi- or even triennial reproduction frequency). The average number of developed eggs/embryos was 9.1 (range 5–17). As expected, there was a positive correlation between maternal body size (SVL) and the number of embryos. The nose-horned vipers fed predominately on lizards (64.7 %) and mammals (31.9 %); only a few remains of different prey were found (birds, snakes and centipedes). Analyses of inter-sexual differences showed that males more frequently consumed lizards than mammals, while in females both types of prey were equally present. A subtle ontogenetic change in diet was recorded, with a shift from lizards towards mammals. Adult individuals had more fat reserves than subadults, but there were no inter-sexual differences, and gravid females had similar amounts of fat reserves as non-gravid individuals. The data about reproductive output and dietary specialisation demonstrate the vulnerability of the species, generally regarded as “quite common” and non-threatened. This study might help in the establishment of future conservation studies, and management of the impacts of anthropogenic factors on populations of *V. ammodytes* in the central and western Balkans.

**Keywords:** testes volumes, follicles and embryos, prey spectrum, fat bodies, herpetological collection, Viperidae

## INTRODUCTION

For many common snake species, there are no data on population status, diet and reproduction, or these data are out of date. Such data are essential for assessment of conservation status and threats. Venomous snakes such as e.g. nose-horned vipers (*Vipera ammodytes*) are often not the subject of systematic studies, and are under great pressure due to intentional killing, hunting for anti-venom production (Jelić et al., 2013) and the illegal pet trade (Filippi & Luiselli, 2000). However, in some countries in the Balkans, *V. ammodytes* is not protected.

*Vipera ammodytes* is a medium-sized venomous snake with a relatively wide distribution, from Italy and Austria through the Balkans to the Caucasus (Heckes et al., 2005; but see Freitas et al., 2020). Although inhabiting elevations from 0 m to > 2,500 m, it is usually found in hills and low mountains, at 400–800 m (Crnobrnja-Isailović & Haxhiu, 1997; Jelić et al., 2013). The taxonomy, biogeography, and morphological and genetic

diversification of *V. ammodytes* are well documented (Tomović, 2006; Ursenbacher et al., 2008; Tomović et al., 2010; Freitas et al., 2020), as well as its activity patterns (Crnobrnja-Isailović et al., 2007). However, information regarding its diet is meagre (Beškov, 1977; Dushkov, 1978; Saint Girons, 1980; Bea et al., 1992; Luiselli, 1996) compared to its congeners (e.g. Saint Girons, 1980; Bea & Braña, 1988; Luiselli & Agrimi, 1991; Bea et al., 1992; Luiselli et al., 1995; Brito, 2004; Santos et al., 2007, 2008). Members of the Viperidae family are almost exclusively sit-and-wait predators (Bea et al., 1992; Santos et al., 2008; Burbrink & Crother, 2011).

Data about reproduction of *V. ammodytes* is mainly derived from captive individuals (Gulden, 1988; Kutrup, 2000; Kariş & Göçmen, 2016; but see Beškov, 1977; Dushkov, 1978; Luiselli & Zuffi, 2002). Like other viviparous snakes, *V. ammodytes* is considered a capital breeder (Bonnet et al., 1998). Individuals of this species mate during April – May, and the young are born in late August – September (Luiselli & Zuffi, 2002; Tomović,

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2015). In different parts of its distribution range females reproduce annually or bi-annually (Luiselli & Zuffi, 2002; Tomović et al., 2015). Litter size varies from four to 20 (Beškov & Nanev, 2002; Luiselli & Zuffi, 2002).

Given the fact that long-term field-based studies are logistically challenging, i.e. they are time-consuming (e.g. capture-mark-recapture population studies) and/or quite expensive (e.g. telemetry), dissections of museum specimens provide an uncomplicated alternative for obtaining detailed information about important ecological traits such as size/age at maturity, reproductive output, diet composition and body reserves (Shine et al., 2014). Although Luiselli & Amori (2016) made an important point that studies of snake diet made on museum specimens may have several drawbacks, such studies are inexpensive and avoid ethical and conservation problems related to collecting and killing of animals (Shine et al., 2014).

In this study we examined museum specimens of *V. ammodytes* from the central and western regions of the Balkan Peninsula to address the following questions: 1) At what body size does the species attain sexual maturity? 2) Are there differences in relative testes volumes between age groups and seasons? 3) What is the average number of offspring produced? 4) Is there a correlation between maternal body size and litter size? 5) Are there inter-sexual differences and ontogenetic shifts in diet composition? 6) Do seasonal differences in dietary preference exist? 7) Do fat reserves differ between sexes, age groups and seasons?

## MATERIAL & METHODS

The herpetological collection of the Institute for Biological Research “Siniša Stanković”, University of Belgrade, contains 460 specimens of *Vipera ammodytes* from the central and western parts of the Balkan Peninsula (Slovenia, Croatia, Bosnia and Herzegovina, Montenegro, North Macedonia and Serbia, as well as few individuals from Bulgaria), collected between 1957 and 2007 (Džukić et al., 2017). According to the current taxonomic revision of Freitas et al. (2020), our samples belong to *V. ammodytes* sensu stricto.

Of the stored specimens from the Institute “Siniša Stanković”, many could not be fully processed. In small individuals, we did not analyse reproductive organs, while many additional specimens were very old and in poor condition. For this study, we selected 125 males and 112 females that were non-systematically collected from February (males) and March (females) to September, i.e. during the active season. For 70 animals (29.5 %) location and/or the date of capture was not available (the “unknown date” group onwards) and they were excluded from some analyses.

Specimens were classified into the adult category if their snout-to-vent length (SVL) was larger than 43.8 cm for both sexes. This corresponded to a minimum of 49.3 cm total length (TotL) for males (the smallest male recorded mating in nature: Dushkov, 1978), and to 49.0 cm (TotL) for females (our results; the smallest female with embryos). Animals of < 30 cm TotL were classified

as juveniles (following Luiselli (1996)). In our case, these corresponded to 26.7 and 26.3 cm SVL, for males and females, respectively. The individuals of intermediate body sizes were regarded as subadults. We measured body width (BW at mid-body), SVL, and tail length (TL) of the animals (with a tape measure and digital callipers); from the latter two, we calculated TotL.

In males (N = 117) we measured the dimensions of testes (length and width; with digital callipers, precision 0.01 mm). In females (N = 99), we measured the sizes of follicles/eggs and divided them into two groups: undeveloped follicles (< 10 mm of length) and developed eggs/embryos (> 10.0 mm) (Trauth et al., 1994; Shine et al., 2014). We counted all undeveloped follicles between 2 mm and 10 mm in the left and right ovary+oviduct. Length and width of embryos (not extending), were measured. Volumes of the entire body (for males), testes (TV) and developed eggs/embryos (> 10 mm, EV) were estimated from the SVL, length (L) of testes and developed eggs/embryos and width of the body (BW), and of testes and developed eggs/embryos (W). We used the equation for the volume of an ellipsoid:  $EV = 4/3 \times \pi \times (L/2) \times (W/2)^2$  (Pleguezuelos & Feriche, 1999). As the testes volumes are correlated with body volumes, in some analyses we calculated relative values, considering the body (excluding tail) of the snake as a cylinder.

Gut content was checked in 125 males and 112 females. Due to the bad preservation quality of a large part of the sample, prey items were only identified to class level and categorised into the following prey types: mammal, lizard, bird, snake, and centipede. The presence of food remains was also separated according to their position in the stomach or intestine. Due to the small sample size for juveniles, in analyses of diet composition, we pooled them with subadults (i.e. immatures). In analyses of seasonal differences of diet composition, data were pooled into two seasons: spring (February to June) and summer (July to September). In 116 males and 96 females, the amount of the stored fat (fat reserves) was visually assessed following Pleguezuelos & Feriche (1999): 0 – no traces of fat; 1 – small traces of fat among the intestine loops; 2 – fat bodies cover less than half of the intestinal surface; 3 – fat bodies cover more than half of the intestinal surface; 4 – a continuous fat layer in the ventral zone of the abdominal cavity. Bearing in mind the effects of ethanol on the stored material (Vervust et al., 2009), we did not weigh any of the sampled animals or their internal organs.

Before any analyses, we tested the data for normality of distribution. The differences in relative testes volume between left and right testes in subadults and adults, the volume of developed eggs/embryos between left and right ovary+oviduct, and the number of undeveloped follicles and developed eggs/embryos between left and right ovary+oviduct for subadults and adults were analysed with the Wilcoxon Matched Pairs test. The differences between subadults and adults in relative testes volumes were analysed with the Mann-Whitney U test. Monthly variation (from February to September) in right testes volume (residual values of absolute testes volumes against SVL, both values log<sub>10</sub>-transformed) in

**Table 1.** Dimensions of testes in millimetres (average  $\pm$  standard deviation, min–max), and the results of Wilcoxon Matched Pairs tests for differences in relative testes volumes between left and right testes within subadult and adult males of nose-horned vipers. LTL = left testes length, RTL = right testes length, LTW = left testes width, RTW = right testes width, RTV = relative testes volumes.

Age category	LTL	RTL	LTW	RTW	RTV
Subadults	24.6 $\pm$ 6.65 (14.6–37.7) N = 27	32.1 $\pm$ 8.57 (17.2–52.4) N = 26	2.9 $\pm$ 0.92 (0.9–4.8) N = 27	2.4 $\pm$ 0.89 (1.2–4.6) N = 26	Z = 0.19 p = 0.849
	34.5 $\pm$ 9.26 (17.5–60.7) N = 78	47.7 $\pm$ 10.69 (24.3–72.8) N = 80	4.3 $\pm$ 0.99 (2.0–6.0) N = 78	4.0 $\pm$ 1.10 (1.8–6.5) N = 80	Z = 3.49 p = 0.001

adult males was analysed by One-Way ANOVA test and Post-hoc (Fisher LSD) tests. The differences in volumes of the developed eggs/embryos among months were tested with the Kruskal-Wallis test. Linear regression was used to test the relationship between maternal body size (SVL) and the number of developed eggs/embryos; both values were log<sub>10</sub>-transformed.

Differences in diet composition between sexes, age groups (immatures and adults) and two seasons (spring and summer) were tested by  $\chi^2$  tests and contingency tables. Differences in the amount of fat reserves between sexes, subadults and adults (sexes separated), gravid and non-gravid females, as well as among months were analysed with non-parametric statistics (Mann-Whitney U test and Kruskal-Wallis test). Statistical analyses were performed with Statistica v. 10.0.

## RESULTS

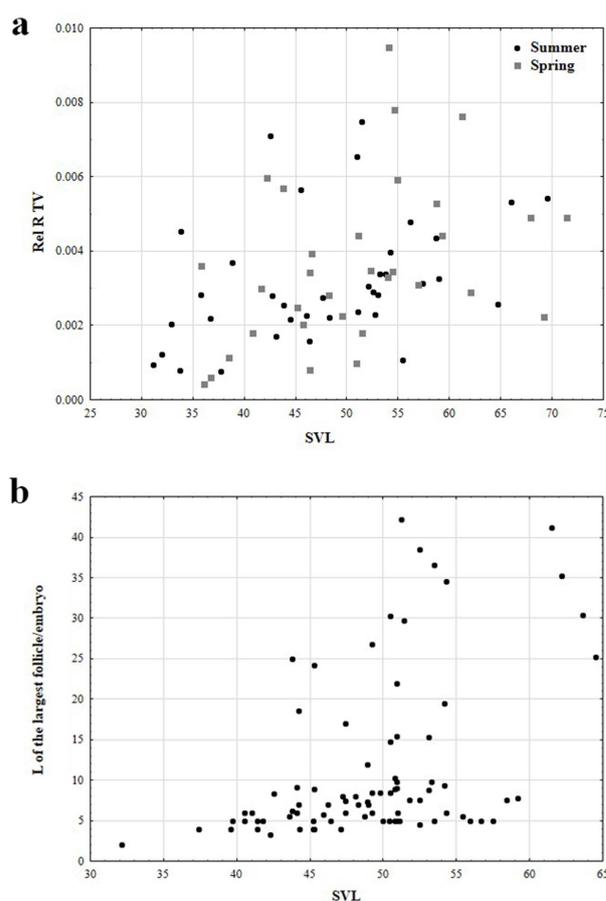
### Reproductive characteristics

#### Males

The relationship between the relative volume of the right testes against SVL showed that enlarged testes could be found only in individuals larger than 43 cm SVL (Fig. 1a). Mann-Whitney U tests showed that relative testes volumes were significantly higher in adult than in subadult males for the right organs ( $Z = 2.85$ ,  $p = 0.004$ ), but not for the left ( $Z = 1.63$ ,  $p = 0.102$ ) testes. In both subadult and adult males, the right testes were longer than the left (Table 1). Results of Wilcoxon Matched Pairs tests showed differences between left and right relative testes volumes only in adults (Table 1). We found seasonal variation in residual values of absolute right testes volumes in adult males: testis growth began in March–April, peaked in May, dropped during the summer, and then increased again in September (Fig. 2). Results of One-Way ANOVA showed that this variation was not significant ( $F_{1,48} = 1.12$ ,  $p = 0.367$ ). However, the results of post-hoc (Fisher LSD) tests showed that residual values of right testis volumes from June to August were significantly lower than in September (Table 2).

#### Females

We plotted the largest developed eggs/embryos length against the SVL of females to obtain female body size at maturity: enlarged follicles (> 10 mm) were observed only in individuals larger than 43 cm SVL (Fig. 1b), supporting



**Figure 1.** Graphic representation of body sizes at which enlarged testes (in males) or follicles/embryos (in females) occur: **a)** males – relative right testes volume against the body size (SVL); and **b)** females – length of the largest developed eggs/embryos against the body size (SVL)

that females achieve sexual maturity at this size. These females had between six and 39 follicles in both ovaries (14.0  $\pm$  6.42 on average, 6.4  $\pm$  2.83 and 7.6  $\pm$  3.99 in left and right ovaries, respectively). In 75 adult females, we also found undeveloped follicles of different sizes (Table 3). Adults had between four and 35 undeveloped follicles in both ovaries (16.8  $\pm$  6.50 on average, 7.8  $\pm$  3.18 and 9.1  $\pm$  3.96 in left and right ovaries, respectively). Wilcoxon Matched Pairs tests showed significant differences in the number of undeveloped follicles between left and right ovaries only in adult females ( $Z = 3.20$ ,  $p = 0.001$ ).

**Table 2.** Results of Post-hoc (Fisher LSD) tests of residual values of right testes volumes in adult males of nose-horned vipers among months. \*  $p < 0.05$ .

Month	February	March	April	May	June	July	August	September
<b>February</b>								
<b>March</b>	0.393							
<b>April</b>	0.430	0.827						
<b>May</b>	0.217	0.741	0.424					
<b>June</b>	0.536	0.628	0.725	0.195				
<b>July</b>	0.452	0.718	0.863	0.200	0.799			
<b>August</b>	0.532	0.646	0.751	0.225	0.981	0.831		
<b>September</b>	0.073	0.248	0.094	0.225	<b>0.040*</b>	<b>0.040*</b>	<b>0.047*</b>	

**Table 3.** Numbers of individuals of nose-horned vipers with undeveloped follicles and developed eggs/embryos / numbers of all females of the respective class caught in the given month.

Month	Individuals with undeveloped follicles		Individuals with developed eggs/embryos
	Subadults	Adults	Adults
<b>March</b>		2 / 2	1 / 2
<b>April</b>		2 / 2	1 / 2
<b>May</b>	4 / 4	14 / 14	7 / 14
<b>June</b>	3 / 3	7 / 8	5 / 8
<b>July</b>	10 / 10	23 / 23	4 / 23
<b>August</b>		9 / 9	4 / 9
<b>September</b>	2 / 2		
<b>Unknown</b>	4 / 4	18 / 18	7 / 18
<b>Sum</b>	<b>23 / 23</b>	<b>75 / 76</b>	<b>29 / 76</b>

In 29 of 76 (38.1%) adult females which were collected between March and August, we found developed eggs/embryos (Table 3). The distribution of frequencies did not differ from a theoretical triennial reproduction frequency ( $\chi^2 = 0.38$ ,  $df = 1$ ,  $p = 0.536$ ). In two individuals, a single developed egg, and in one individual two developed eggs were found. Since they represent outliers we did not use them in analyses. The average litter size was  $9.1 \pm 2.5$  ( $n = 26$ , range 5–17). There were significantly less ( $Z = 2.69$ ,  $p = 0.007$ ) developed eggs/embryos in left oviducts ( $3.8 \pm 1.5$ ) compared to the right ( $5.3 \pm 1.87$ ). Also, there was a statistically significant difference in sum volumes of developed eggs/embryos between left and right ovaries+oviducts ( $Z = 2.48$ ,  $p = 0.013$ ). Volumes increased significantly from March to August: Kruskal-Wallis test:  $H_{(5, N=198)} = 44.81$ ,  $p < 0.001$  (Fig. 3). We found a statistically significant positive correlation between maternal body sizes (SVL) and the numbers of developed eggs/embryos they were carrying ( $r = 0.395$ ,  $p = 0.046$ ).

### Diet

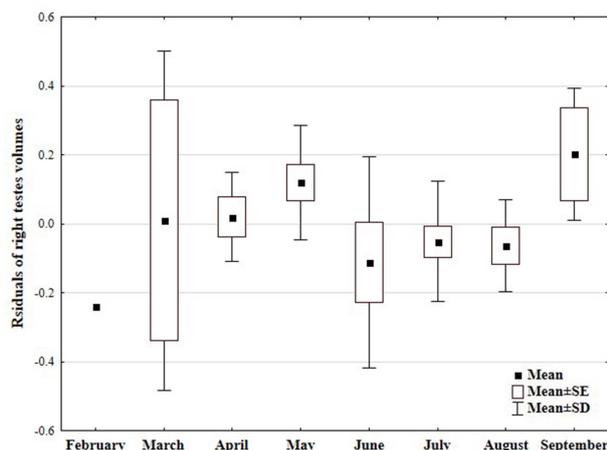
The majority of individuals with prey (97.5% of the sample) had only one type of food, and only three specimens had two different types (lizard and mammal). Of individuals with food, in 41.8% of males remains were found in the stomach, and in 89.6% in the intestine, whereas food was found in the stomach in 40.4% of females, and 76.9% had food remains in the intestine

(Table S1). The dominant prey types of *V. ammodytes* were lizards (64.7%, e.g. *Podarcis muralis*, *Lacerta viridis*, *Anguis fragilis*), followed by mammals (31.9%). Other types of prey (birds, snakes (*Natrix tessellata*) and centipedes (*Scolopendra cingulata*)) were negligible in diet composition (3.4%) (Table S1).

### Comparison of the sexes and age categories

Approximately half of the dissected specimens (53.6% and 46.4% of males and females, respectively) had food remains in their digestive systems. The relative numbers of males and females with and without food remains were similar ( $\chi^2 = 1.22$ ,  $df = 1$ ,  $p = 0.270$ , all age classes pooled). There were no differences between gravid and non-gravid females in presence of food items ( $\chi^2 = 1.10$ ,  $df = 1$ ,  $p = 0.298$ ).

In the general analysis of diet composition, we found no differences between the sexes ( $\chi^2 = 5.11$ ,  $df = 4$ ,  $p = 0.276$ ). However, when we considered only the two most dominant types of prey (lizards vs. mammals), analyses of inter-sexual differences showed that males more frequently consumed lizards than mammals ( $\chi^2 = 6.28$ ,  $df = 1$ ,  $p = 0.012$ ), while in females both types of prey were present in a frequency not different from the null hypothesis ( $\chi^2 = 1.46$ ,  $df = 1$ ,  $p = 0.227$ ). Thus, we separated sexes in further analyses of two age categories. We found differences in the frequency of lizards and mammals as prey between immatures and adult males



**Figure 2.** Volumes of right testes of the nose-horned vipers (residual scores for regression of the right testes volumes ( $\log_{10}$ ) against snout-vent length ( $\log_{10}$  SVL)) vs. month of the activity period (only adult males).

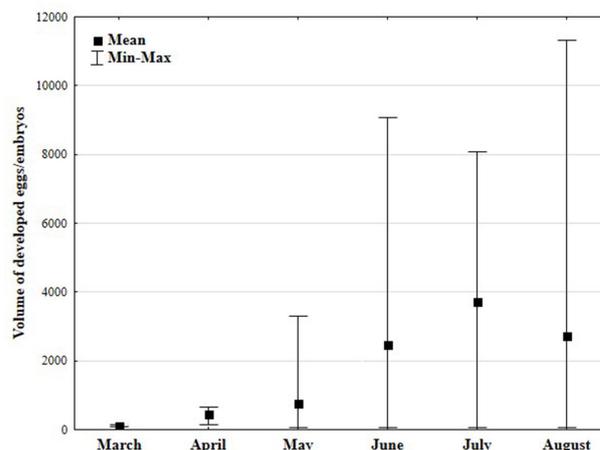
(Yates corrected  $\chi^2 = 3.95$ ,  $df = 1$ ,  $p = 0.047$ ), but not in females of two age classes (Yates corrected  $\chi^2 = 0.97$ ,  $df = 1$ ,  $p = 0.324$ ).

#### Seasonal differences in diet composition

Seasonal change in diet composition was statistically significant in males ( $\chi^2 = 14.10$ ,  $df = 6$ ,  $p = 0.029$ ), but not in females ( $\chi^2 = 4.48$ ,  $df = 6$ ,  $p = 0.611$ ). In the spring season, immature males fed almost exclusively on lizards and adults had a more diverse diet, while in summer both age categories fed on lizards and mammals. Opposite to males, immature and adult females had a diverse diet both in spring and in summer (Fig. 4). In February (males), as well as in August and September (females), *V. ammodytes* fed exclusively on lizards, while other prey items (birds, snakes and centipedes) were found in May and June, exclusively.

#### Fat reserves

Results of Mann-Whitney U tests showed that there were no inter-sexual differences in the amount of fat reserves in both subadults ( $Z = -1.48$ ,  $p = 0.140$ ) and adults ( $Z = -0.96$ ,  $p = 0.336$ ). Pregnant females had similar amounts of fat reserves as the non-pregnant ones ( $Z = -1.57$ ,  $p = 0.117$ ). Adult individuals had more fat reserves than subadults both in males ( $Z = 4.12$ ,  $p < 0.001$ ) and in females ( $Z = -2.51$ ,  $p = 0.012$ ). Kruskal-Wallis test showed that the amount of fat reserves was not different among months in adult males ( $H_{(7, N = 52)} = 5.07$ ,  $p = 0.651$ ), non-gravid females ( $H_{(5, N = 34)} = 2.81$ ,  $p = 0.730$ ) and gravid females ( $H_{(5, N = 21)} = 2.14$ ,  $p = 0.830$ ). The highest amount of fat bodies in adults was in early spring (February – March in males, March – April in females). The lowest amount in adult males was in May and remained relatively constant until the end of the season (Fig. 5a). Amount of body reserves varied more in non-gravid than in gravid females but was high in both groups until the end of August (Fig. 5b).



**Figure 3.** Volumes of developed eggs/embryos through months in adult females of nose-horned vipers.

## DISCUSSION

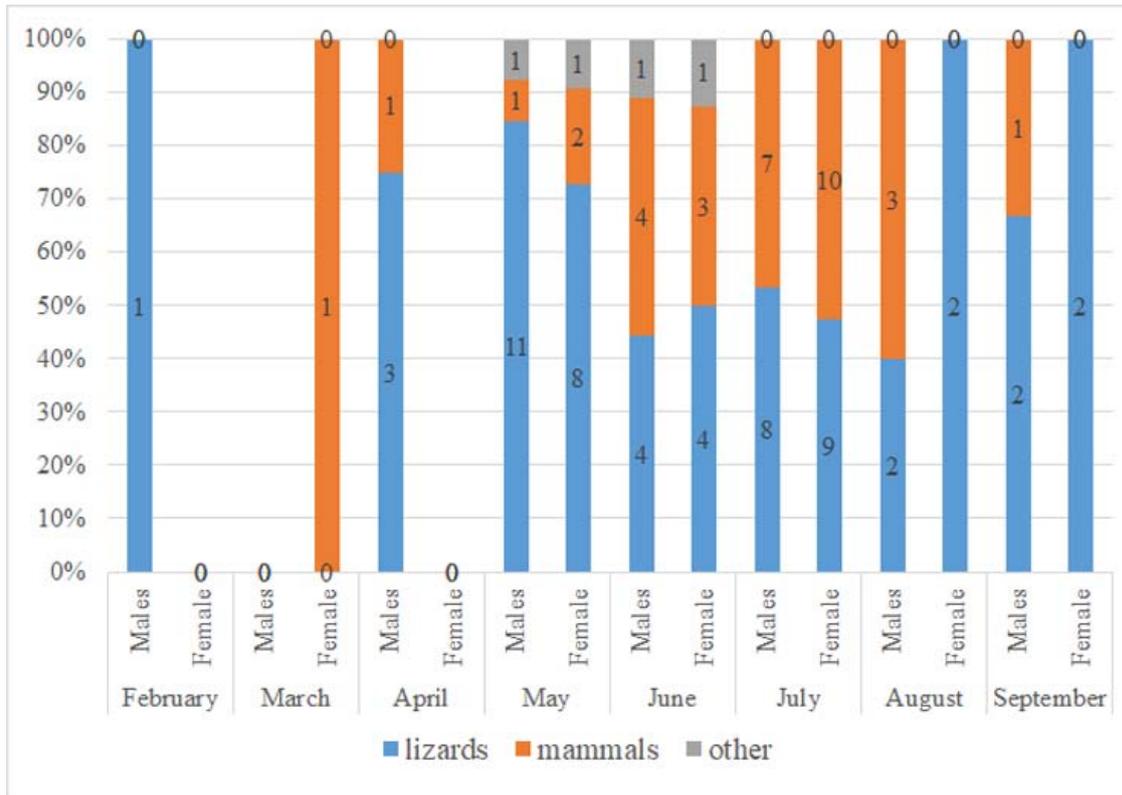
Our study shows that anatomical dissections from museum specimens can provide fundamental information about size at maturity, reproductive output, diet composition and amount of body reserves for snakes. Information about life-history traits are essential for species conservation (Filippi & Luiselli, 2000; Pleguezuelos et al., 2007; Shine et al., 2014). Analyses of museum specimens have both advantages (e.g. inexpensive method, large samples from an important part of the species range) and limitations (e.g. preservation status of specimens precludes accurate measurements of some body parts, unequal representation of specimens from different parts of the species range, sex-related biases due to different activity patterns) (Crnobrnja-Isailović et al., 2007; Luiselli & Amori, 2016).

#### Reproductive characteristics

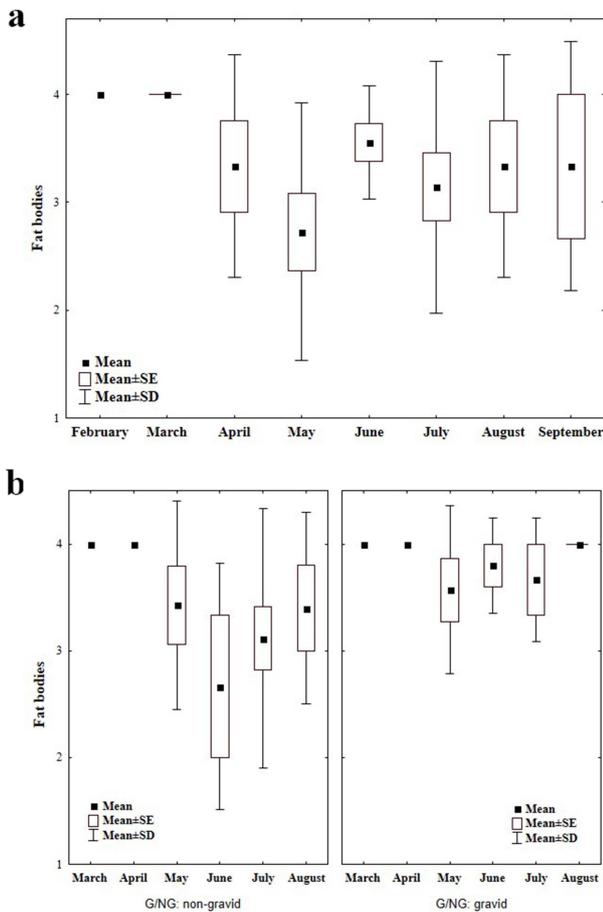
Although sexual dimorphism in nose-horned vipers was previously confirmed (Tomović et al., 2002) and adult males are generally larger than females (e.g. Beškov, 1977), results of our study showed that both sexes attain sexual maturity at approximately the same body size.

#### Males

Testes (relative size) were significantly larger in adult males than in subadults, but only the right side organs. In male snakes, there is a significant increase in testes size with the onset of reproductive maturation (White & Kolb, 1974). To the best of our knowledge, the only precise information regarding the size of testes at the onset of sexual maturation in vipers is available for *Vipera latastei* (Pleguezuelos et al., 2007, Fig 1A). However, this species attains a much smaller adult body size than *V. ammodytes*. Although we did find enlarged testes in individuals larger than 43 cm (SVL), our data did not show a clear difference in testes sizes between subadult and adult males. Therefore, we could not precisely set the threshold body size values for sexual maturity in males of the nose-horned viper.



**Figure 4.** Numbers of different prey types in males (M) and females (F) through months of all age groups of nose-horned vipers.



**Figure 5.** Amount of fat reserves through months in **a)** adult males; **b)** adult gravid and non-gravid females of nose-horned vipers.

Our finding that right testes of *V. ammodytes* were significantly longer (and relatively more voluminous) than the left (like in *Thamnophis* sp. – White & Kolb, 1974; Shine et al., 2000), was most probably the consequence of the general asymmetry of internal organs in snakes (Shine, 1977; Shine et al., 2000; Gribbins & Rheubert, 2011). Also, our study found seasonal variation in testes volume in adult males: testicular growth began in March–April, peaked in May, dropped in summer (June–August), and then increased again in September (Fig. 2). It is generally known that testes size is variable during the reproductive cycle in vipers (e.g. Nilson, 1980; Saint Girons, 1992; Pleguezuelos et al., 2007), as well as in other snake species in temperate regions (Duvall et al., 1992; Pleguezuelos & Feriche, 1999; Sivan et al., 2016). In *V. ammodytes*, mating was recorded only in spring (Crnobrnja-Isailović et al., 2007; Tomović, 2015), which differs from *V. latastei* where copulation was observed both in spring and autumn, depending on the geographic region (Pleguezuelos et al., 2007). Our results indicate that in nose-horned vipers, perhaps both vernal and autumnal spermatogenesis occur (Fig. 2), but the small sample size precluded us from a definite conclusion. Further research is needed to understand if there is dissociated timing of male and female reproductive cycles in *V. ammodytes*, and whether short-term (in females) or long-term (in males) sperm storage (Saint Girons, 1992; Aldridge & Duvall, 2002; Pleguezuelos et al., 2007) is characteristic for this species across its entire distributional range.

## Females

Results of this study enabled us to set the threshold body size value for sexual maturity in females of nose-horned vipers: the smallest female with developed eggs/embryos had 43.8 cm SVL (49.0 cm TotL). The total body length of the smallest gravid female in our sample was lower than the values reported by some other authors (52.0 cm (Luiselli & Zuffi, 2002) and 59.5 cm (Beshkov, 1977)), probably due to large geographic variability in growth rates and adult body sizes, often resulting from environmental factors (Tomović et al., 2002; Zuffi et al., 2009), as well as because of larger sample sizes in our study.

Undeveloped follicles were found in most subadult and adult females in our sample, but only 38.1 % of adult females were gravid (had developed eggs/embryos). The latter stands for both the complete sample and for the subsample of adult females caught during the reproductive season (May, June, July and August). This result implies that *V. ammodytes* in the central and western parts of the Balkans reproduce on a biennial or even triennial basis (see Table 3), as was reported for some other parts of the species' range (Luiselli & Zuffi, 2002). Accordingly, *V. ammodytes* displays a capital breeding strategy (Bonnet et al., 1998), typical for other European vipers (e.g. Zuffi et al., 1999; Bonnet et al., 2002; Luiselli & Zuffi, 2002). Less-than-annual reproduction was shown to contribute to the species' vulnerability to extinction (Pleguezuelos et al., 2007; Tomović et al., 2015).

In our study, the average number of developed eggs/embryos was 9.1, while the range was between 5 and 17. This average was much higher compared to literature data (5.6 – Luiselli & Zuffi, 2002; 7.3 – Beškov, 1977; 7.0 – Dushkov, 1978). Also, the observed range of numbers of developed eggs/embryos was wider than the published for the same species (4–8 – Luiselli & Zuffi, 2002; 4–12 – Beškov, 1977; 5–10 – Dushkov, 1978). This could be the consequence of the quantitative and qualitative differences in the sample structure. Previously published data were based on relatively good population samples from rather narrow locations/regions: 17 females (Luiselli & Zuffi, 2000) and six females (Beškov, 1977) from the same population, as well as 11 females from three close regions (Dushkov, 1978). Unfortunately, we do not have dense population samples, and the second drawback was that our sample covered a very wide distribution range (territory of Ex-Yugoslavia), with (implicitly) huge variation of abiotic and biotic conditions and factors. Possible explanations for the numbers of developed eggs or embryos smaller than expected (observed in three individuals not included in analyses) may be a reproductive failure (egg and embryo resorption as a compensatory mechanism for minimising nutrient losses during failed attempts at reproduction: Bonnet et al., 2008) or result from other constraints such as dehydration, protein deficiency or the presence of parasites (Lourdais et al., 2015; Dyugmedzhiev et al., 2018 and references therein).

As expected, we found a significant correlation between maternal body size (SVL) and numbers of developed eggs/embryos (Luiselli & Zuffi, 2002; Zuffi et

al., 2009). It is known that the reproductive success of females is dependent on body size because larger females have more abdominal space (Shine, 1978, Madsen & Shine, 1994). Our result that more undeveloped follicles and developed eggs/embryos were found in right ovaries + oviducts, could also be the consequence of the general asymmetry of internal organs in snakes (Shine, 1977; Shine et al., 2000; Gribbins & Rheubert, 2011).

## Diet

Approximately half of the nose-horned vipers we dissected had remains of food in their digestive systems. This suggests that *V. ammodytes* has low feeding frequency, which is also observed in other European vipers (Santos et al., 2007 and references therein). Nose-horned vipers almost exclusively consumed lizards (64.7 %) and mammals (31.9 %). Only a few individuals (3.4 %) ate birds, snakes and centipedes, which was already known from the literature (Clark, 1967; Beškov, 1977; Luiselli, 1996; Arsovski et al., 2014). A very high percentage of lizards in the diet of nose-horned vipers in our study is quite different from the only detailed analysis of the diet composition of this species in the literature (Beškov, 1977). In south-western Bulgaria, *V. ammodytes* individuals of all age categories fed predominantly on mammals (60.9 %), then on lizards (22.8 %), far less on centipedes (12.0 %) and the least on birds (3.3 %) (Beškov, 1977). Within European vipers, *V. latastei* and *V. ammodytes* are the most similar species in regards to general diet composition (Bea et al., 1992), with the smallest proportion of small mammals in the diet (Saint Girons, 1980; Bea et al., 1992). The reason could be the fact that both species inhabit predominantly Mediterranean and Submediterranean habitats with similar prey type availability. However, *V. latastei* shows significant variation in prey preferences along a latitudinal cline (Santos et al., 2008) with more mammals eaten in humid areas and a more diverse diet in drier and Mediterranean regions.

## Comparison according to sex and age category

Our results showed no difference in the percentage of female and male *V. ammodytes* with and without food, although it has been reported that, generally, their activity patterns differ (Crnobrnja-Isailović et al., 2007). Our data were contradictory to the literature in that we found a higher proportion of individuals with food than those without food (Nilson, 1980; Madsen & Shine, 1993), suggesting that adult males still feed during the mating season. Also, there were no differences between gravid and non-gravid females regarding the presence of food items, contrary to published data which showed that reduced feeding in gravid females (at least in viviparous snakes) is a common phenomenon, probably due to limitation of abdominal space to accommodate food and embryos (Gregory et al., 1999; Lourdais et al., 2002). However, many studies show that female snakes occasionally feed during pregnancy (e.g. Barron et al., 2013; Claus & Bauwens, 2019).

In our analysis of complete diet composition, we found no difference between the sexes. However, when

we considered only the two most dominant types of prey (lizards vs. mammals), analyses of inter-sexual differences showed that in the diet of males, lizards were significantly more prevalent than mammals, while in females both types of prey were equally present. These results are different from those obtained for *V. latastei* where no intersexual differences in diet composition were found (Brito, 2004; Santos et al., 2007). Sexual dimorphism could also be the cause of diet differences, as reported for other snake species (Camilleri & Shine, 1990; Vincent et al., 2004; Meik et al., 2012). In nose-horned vipers, sexual dimorphism was recorded for head size, with females having higher values of head width (Tomović et al., 2002) and thus, they may be able to feed on larger prey (i.e. mammals). Another possible reason for a greater percentage of mammals than lizards in the female diet may be due to the higher energetic demands of females since they must allocate more resources to reproduction, and mammals supply more energy per biomass than reptiles (Shine, 1989).

Results of our study may imply a subtle ontogenetic shift in diet (significant only in males), where towards adulthood the share of mammals increases at the expense of lizards. Similar results were obtained for other viper species (Saint Girons, 1980; Luiselli, 1996; Beškov, 1977; Bea et al., 1992; Santos et al., 2007, 2008). The ontogenetic shift in diet composition may be the consequence of the gape limitations of small snake individuals, which have a narrower range of prey types suitable for ingestion (e.g. Shine, 1991; Shine & Madsen, 1997).

#### Seasonal differences in diet composition

We found seasonal variation in the diet composition of nose-horned vipers. The consumption of lizards decreased from spring to summer in both sexes, while for mammals the trend was the opposite. However, this result should be taken as tentative given the limited sample of subadult individuals. Most probably, the prey spectrum depends on the availability of prey at a given moment, as previously shown for *V. latastei* (Santos et al., 2007, 2008). Our data needs to be taken cautiously given that result may include possible seasonal differences in the diet related to climatic heterogeneity across the wide distribution range of nose-horned vipers, as already recorded for some other viper species (e.g. Santos et al., 2008).

#### Fat reserves

The largest amount of fat reserves in adult individuals of both sexes was recorded in early spring (February – April) and it remained relatively constant until the end of the season (Fig. 5a,b). Gravid and non-gravid females did not differ in the amount of fat reserves. A possible explanation for similar amounts of fat reserves between gravid and non-gravid females may be feeding during gestation (Lourdais et al., 2002), that was confirmed in this study, i.e. there was no difference in the percentage of diet in the gut between gravid and non-gravid females. Results of our study showed that nose-horned vipers (like all other vipers) are capital breeders which are capable

of storing large fat reserves for reproduction (Bonnet et al., 1998). However, we also found pregnant females with prey, which means that this species may also be an income breeder and that capital and income attainment of energy may not be alternative, but additive strategies (Pleguezuelos et al., 2007). Feeding activity during and after gestation increases fitness and reproductive output; if food intake during gravidity is reduced or absent, females may not reproduce (have bi-annual or less frequent reproduction) (Zuffi et al., 1999; Luiselli & Zuffi, 2002; this study).

We did not find differences in the amount of fat bodies between the sexes, and this character is probably species-specific (Bonnet et al., 1998). Adults had a higher amount of fat reserves compared to subadults, probably because they have a bigger gape and greater range of prey types that can be ingested, or a higher feeding success (Shine, 1991; Shine & Madsen, 1997).

## CONCLUSION

According to our knowledge, this is the most comprehensive analysis of reproductive characteristics, diet composition and amount of fat reserves in *V. ammodytes*. It represents the basis for future field-oriented population (Capture-Mark-Recapture) studies that could reveal the influences of geographic/ecological variability on life-history characteristics of nose-horned vipers. The obtained data about reproductive traits (i.e. biennial or even triennial reproduction) and dietary specialisation demonstrate the vulnerability of this species (Tomović et al., 2015), generally regarded as “very common” and non-threatened (Tomović, 2015).

## ACKNOWLEDGEMENTS

This study was supported by the Ministry of Education, Science and Technology of the Republic of Serbia (grant nos. 451-03-9/2021-14/ 200007 and 451-03-9/2021-14/ 200178).

#### Author Contributions:

MA and SN performed laboratory work. SN and MA analysed data and wrote the initial draft. All authors analysed and discussed the data. MA and LT wrote the final manuscript. All authors read and approved the final manuscript.

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Accepted: 15 March 2021

**Please note that the Supplementary Materials are available via the Herpetological Journal website:  
<https://thebhs.org/publications/the-herpetological-journal/volume-31-number3-july-2021>**