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Research Article

Seasonal Changes in Ovarian Follicle Growth in Iran Viper (*Vipera albicornuta*)

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The *Vipera albicornuta* is an economically important snake of Iran, its venom is used for antivenin production, and we need to breed the snake in the captivity. In order to know about the viper's reproductive biology, seasonal alterations in ovarian weight, morphology, and the follicles developmental stages in *Vipera albicornuta* were studied using macroscopic parameters and histological examination of reproductive tissues during a year. Twenty-four female vipers were collected from mountainous zone of Bostanabad, East Azerbaijan, and Tarom in Zanjan through years 2011-2012. Evaluation of the reproductive parameters of this viper is performed for the first time in Iran. Our observation revealed that vitellogenesis cycle begins in autumn and continues till early summer; ovaries and follicles are in previtellogenesis stage in autumn and vitellogenesis in winter and during spring. Weight of ovary is heaviest in spring, ovulation occurs in late spring till early summer, and copulation is prior to ovulation.

1. Introduction

Vipera albicornuta, locally named Zanjani viper, belongs to Viperidae family, *Vipera* genus, and *Montivipera* subgenus. The geographical distribution of this species is in Northern Iran, covering several areas of the Northern Zagros and provinces of Gilan, Qazvin, Zanjan, and East Azerbaijan [1]. The aim of this study is to provide data on the reproductive biology of female *V. albicornuta* using histomorphologic and histomorphometric investigation on the female reproductive organs through a year.

Female reproductive system consists of one pair of the long ovaries that sometimes contain follicles, which are located at back by abdominal mesoderm and are in anterior oviducts that open to cloacae [2]. The right ovary is located slightly higher than the left ovary [3]. The ovaries in the reptiles are composed of stromal and parenchymal tissues. Structural and morphological changes in ovaries size and weight occur during reproductive cycle. Reptilian reproductive cycles are normally affected by environmental factors, among which temperature and light are clearly environmental cues controlling the reproductive cycles of lizards and snakes [4]. The reproductive cycle of female snakes is in relation to reproductive cycle of male and climate. In this regard there are prenuptial and postnuptial cycles. In the prenuptial cycle, female ovulation follows the peak of spermatogenesis but in postnuptial cycle there is a latency period over winter, from completion of spermatogenesis to fertilization, but there is no such latency in the prenuptial cycle [5].

Ovarian follicles are 3 types: previtellogenic, vitellogenic, and atretic. The development of the egg yolk in the follicles starts when estrogen stimulates the liver to start converting lipids from the body's fat stores, creating vitellogenin. During atresia the theca interna is greatly hypertrophied and is loaded with lipids consisting of first phospholipids and then phospholipids and finally triglycerides, cholesterol and its ester, and little phospholipids. Eventually the oocyte, zona pellucida, granulosa cells, and most of the cells of theca interna regress and disappear, leaving behind some residual cells of theca interna. Histological examination of ovaries in

monocled cobra *Naja kaouthia* in May shows high number of pyriform cell in the granulose layer. Pyriform cell is classified into the 3 sizes, including small, medium, and large [6]. The squamate granulose contains a pyriform cell that is in direct contact with a developing oocyte apparently involved with early steps in oocyte's development as granulose cells either degenerate or transform into typical granulose cells soon after the onset of vitellogenesis as the time of ovulation approaches. The granulose cells and some theca cells accumulate cholesterol-positive lipids and, following ovulation, proliferate and luteinize to form the corpora lutea [7]. Development of the primary oocyte consists of 3 phases: previtellogenic, vitellogenic, and maturation step [8]. During vitellogenesis the yolk is added to the oocyte arrested in meiosis diplotene stage and maturation leads to ovulation; after that the ovulation female gamete is called ovule. At this time oocytes are large and yolk is in large quantities also and chorion is surrounding them. Vitellogenesis in the *Bothrops insularis* occurs in spring and ovulates in late spring. In most of snakes vitellogenesis begins after waking up from hibernation in spring and is peak at ovulation time [9]. In a study on development of follicles in brown snake *Storeria dekayi* it is reported that the follicles at the different developmental stages have different length, the length of primary follicles was 1.0–1.5 mm, that of vitellogenic follicles 1.6–3.0 mm, developing follicles during late winter or early spring in mature female, and that of developed follicle 6.1–12.0 mm [10]. Many of the follicles that grow in the ovary and lead to ovulation have diameters not more than 10 mm. In some species one or two follicles are ovulated and others are resolved and absorbed in ovary [11].

Ovulation in spring is compatibility to finalize pregnancy in female; for the reason that environment temperature is high during this season embryonic growth is rapid and abnormal growth is decreased [12]. Lizard that has delayed in reproduction has enough stored abdominal fat that has been able to make large size clutch [13]. There is the relationship between body size and clutch size [14].

The lizards that have large body cavities are largest and have greatest potential to foster more pups or produce more ovum and species that have diversity in body size also are expected to be various in clutch size [15]. Variation of clutch size is ancestral state in reptiles [16]. Food and available resources in reptile have the positive effect on clutch size [17].

2. Materials and Methods

In this research, ovaries morphology and stages of ovarian follicle growth were examined in *Vipera albicornuta* during 4 seasons for this purpose, so twenty-four *V. albicornuta* snakes were collected from Bostanabad in Azerbaijan and Tarom districts in years 2011 to 2012. Samples were collected and transferred to the Laboratory of the Venomous Animals Department in RAZI Institute and the snakes were parasiticide with antimite ivermectin 1% and kept in temperature between 20 and 30°C and to be sure of their health they were caged for quarantine. Snakes were anesthetized with lidocaine 1% injection around cloacae, and then they were killed with alcohol 96%. Body weight and length (SVL, Snout-vent length) and tail length (TL) were measured with the meter. The snake was dissected and abdominal region was incised in the sterile condition and abdominal fat was removed and weighed. Ovaries were removed, their weight was measured by digital scale (OSK, FX 3000, 0.0001 g) and length, width, and thickness were measured by a digital caliper [18]. Numbers of ovarian follicles were counted and their diameters were measured by micrometer under stereomicroscope (SZX-1LLB2-200) as described by Betz [19]. For histological examination, samples were fixed in formalin 10% MERK and histological processing of tissues was performed on paraffin section stained with hematoxylin and eosin method and the tissue was cut in cross section to 5 μm; then slides were examined under light microscope same as Luna [20].

2.1. Samples Collection Areas. Bostanabad is located in the Eastern Azerbaijan Province of Iran, a mountainous zone with cold winter and temperate summer and altitude from sea level is 1740 meters. The coldest temperature for two years in February 2012 was −25/6 and in January 2013 −18/6°C (Figure 1). Tarom is located in Zanjan Province of Iran and is located in high mountains and has cold and snowy winter, mild and dry summer (Figure 2).

2.2. Statistical Analysis. Student's t-test was performed for comparison of two values by Quick Graph software in windows. Descriptive values were presented as mean and standard deviation.

3. Results

The morphometrical parameters of viper are shown in Tables 1 and 2. The results show that body weight is increasing in fall and it is decreasing in winter during rest and during vitellogenesis, and in spring it rises up and descends in summer again (Tables 1 and 2). Maximum body weights of Bostanabad's samples were 213.33 ± 30 and 204.06 ± 1.32 g and for Tarom's samples were 142.82 ± 31.41 and 136.66 ± 30.5 g in spring and fall, respectively. Maximum average of body length in Bostanabad's samples was 68.43 ± 2.98 cm and for Tarom's was 63.96 ± 3.77 cm (Tables 1 and 2). Our result indicated that the right ovarian weight and numbers of follicle were more than the left ovary in Bostanabad viper as well as in Tarom snake. Maximum ovarian weight of Bostanabad's snakes was observed in spring and minimum weight in winter and for Tarom's samples is in spring and summer (Tables 3 and 4). Long length of ovarian follicles in Bostanabad's vipers was 6.20 ± 1.60 mm in spring and short length is 4.43 ± 1.85 mm in right ovary whereas in the Tarom's snakes the long follicles with the length of 7.05 ± 2.34 mm were observed in the left ovary in spring and the short follicles with the length of 3.75 ± 1.43 mm were seen in the right ovary in summer. The size of ovarian follicles in the Bostanabad snakes was largest and may have ovulated between late May and June (Figure 3) and in the Tarom's snakes largest follicles and ovulation time were seen in May (Figure 4). Histological study revealed that vitellogenic follicles were observed in the ovaries from

(a) (b)

Figure 1: Geographical map of East Azerbaijan Province (a), Bostanabad (b).

(a) (b)

Figure 2: Geographical map of Zanjan Province (a); Zanjan, Tarom (b).

Table 1: Mean values of morphometric parameters from Bostanabad Zanjani viper in four seasons (M ± SD).

Parameters	Season			
	Spring	Summer	Autumn	Winter
Body length (cm)				
Total	73.87 ± 3.58	71.25 ± 2.88	71.13 ± 1.58	65.61 ± 5.73
SVL	68.43 ± 2.98	67.01 ± 3.07	64.63 ± 2.99	60.66 ± 5.34
TL	5.43 ± 0.67	5.1 ± 0.37	5.38 ± 0.16	4.95 ± 0.39
Weight (g)				
Body	213.33 ± 30.00	162.72 ± 36.76	204.06 ± 1.32	126.83 ± 45.27
Fat	24.45 ± 4.33	15.05 ± 10.03	24.93 ± 5.62	13.11 ± 11.59
Body diameter (cm)				
Neck	4.91 ± 0.21	4.78 ± 0.19	4.86 ± 0.05	4.28 ± 0.29
Vent	8.50 ± 0.59	7.75 ± 0.71	8.39 ± 0.74	6.78 ± 0.70
Upper caudal	4.85 ± 0.36	4.73 ± 0.33	5.01 ± 0.63	4.08 ± 0.30
Orifices diameter (mm)				
Cloacae	4.25 ± 1.42	3.40 ± 0.83	2.63 ± 0.45	2.52 ± 0.09
Vagina (right)	2.03 ± 0.76	1.47 ± 0.33	1.76 ± 0.37	1.84 ± 0.31
Vagina (left)	2.05 ± 0.52	1.49 ± 0.48	1.55 ± 0.37	1.89 ± 0.32

Table 2: Mean values of morphometric parameters from Tarom Zanjani viper in four seasons (M ± SD).

Parameters	Season			
	Spring	Summer	Autumn	Winter
Body length (cm)				
Total	64.02 ± 4.83	67.66 ± 2.76	64.55 ± 6.89	61.16 ± 1.23
SVL	59.28 ± 4.49	63.96 ± 3.77	59.31 ± 6.61	56.08 ± 1.11
TL	4.91 ± 0.53	4.88 ± 0.31	5.24 ± 0.50	5.08 ± 0.35
Weight (g)				
Body	141.82 ± 31.41	136.66 ± 6.83	131.66 ± 30.51	107.49 ± 1.18
Fat	13.93 ± 3.98	12.93 ± 3.61	12.41 ± 3.55	9.09 ± 2.46
Body diameter (cm)				
Neck	4.20 ± 0.31	4.66 ± 0.20	4.51 ± 0.27	4.23 ± 0.14
Vent	7.12 ± 0.71	7.40 ± 0.41	7.55 ± 0.68	7.23 ± 0.00
Upper caudal	4.24 ± 0.32	4.35 ± 0.20	4.37 ± 0.26	4.13 ± 0.18
Orifices diameter (mm)				
Cloacae	3.48 ± 1.42	3.18 ± 0.85	2.40 ± 0.47	2.42 ± 0.20
Vagina (right)	2.07 ± 0.80	1.30 ± 0.60	1.89 ± 1.04	1.81 ± 0.50
Vagina (left)	1.89 ± 0.64	1.24 ± 0.54	1.71 ± 1.09	1.96 ± 0.14

Table 3: Mean values of ovaries dimensions of Bostanabad Zanjani viper in four seasons (M ± SD).

Ovary parameters	Season			
	Spring	Summer	Autumn	Winter
Right ovary				
Length (mm)	71.29 ± 10.70	54.12 ± 13.25	58.44 ± 3.80	46.91 ± 6.10
Width (mm)	9.19 ± 2.59	6.23 ± 3.32	6.81 ± 0.62	6.78 ± 1.91
Diameter (mm)	3.77 ± 1.06	2.68 ± 1.42	3.25 ± 0.19	2.98 ± 1.06
Volume (mm^3)	3493.2 ± 1515.11	1819.13 ± 206.39	1440.83 ± 203.66	1069.43 ± 485.78
Weight (g)	1.79 ± 0.74	1.03 ± 0.46	0.98 ± 0.26	0.76 ± 0.28
Left ovary				
Length (mm)	49.86 ± 11.63	35.70 ± 10.33	37.83 ± 0.43	32.01 ± 8.92
Width (mm)	8.99 ± 2.24	5.60 ± 2.37	6.99 ± 0.04	7.59 ± 1.99
Diameter (mm)	3.92 ± 1.02	2.72 ± 1.14	2.63 ± 0.41	3.49 ± 1.17
Volume (mm^3)	3182.32 ± 1408.54	951.56 ± 351.56	1097.68 ± 951.15	1005.28 ± 450.5
Weight (g)	1.30 ± 0.55	0.59 ± 0.15	0.73 ± 0.24	0.64 ± 0.24

Table 4: Mean values of ovaries dimensions of Tarom Zanjani viper in four seasons (M ± SD).

Ovary parameters	Season			
	Spring	Summer	Autumn	Winter
Right ovary				
Length (mm)	54.61 ± 13.01	53.44 ± 8.65	45.39 ± 8.64	42.71 ± 14.68
Width (mm)	6.80 ± 2.22	4.11 ± 0.48	5.66 ± 1.88	5.73 ± 1.03
Diameter (mm)	3.00 ± 0.94	1.48 ± 0.24	3.22 ± 1.09	2.81 ± 0.61
Volume (mm^3)	1376.26 ± 508.91	478.54 ± 139.96	894.78 ± 158.09	771.53 ± 107.4
Weight (g)	1.01 ± 0.61	0.41 ± 0.08	0.70 ± 0.19	0.6 ± 0.20
Left ovary				
Length (mm)	41.81 ± 11	31.80 ± 4.49	32.70 ± 6.56	33.56 ± 0.23
Width (mm)	7.66 ± 2.56	5.19 ± 1.14	6.27 ± 1.62	5.04 ± 1.46
Diameter (mm)	3.45 ± 1.71	1.66 ± 0.52	2.82 ± 0.53	2.69 ± 0.03
Volume (mm^3)	1588.5 ± 446.76	471.90 ± 208.90	709.15 ± 115.19	471.61 ± 49.98
Weight (g)	0.95 ± 0.73	0.34 ± 0.10	0.54 ± 0.15	0.40 ± 0.16

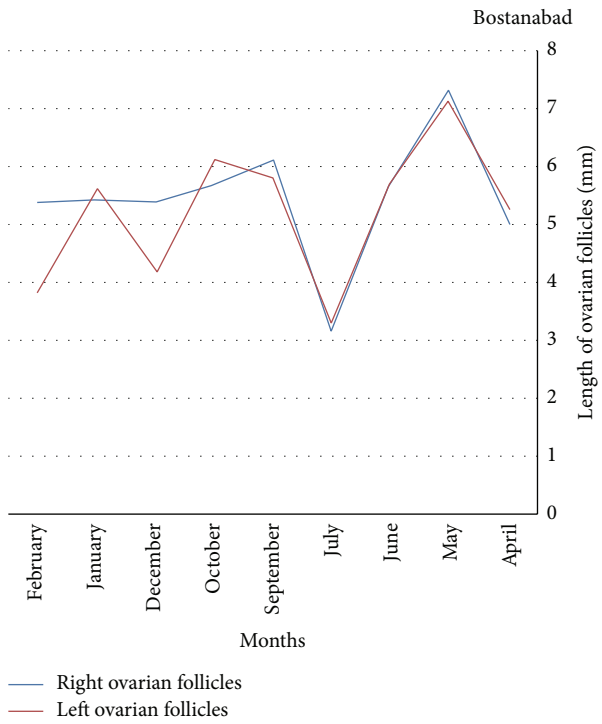

Figure 3: Size of ovarian follicles in Bostanabad *V. albicornuta*.

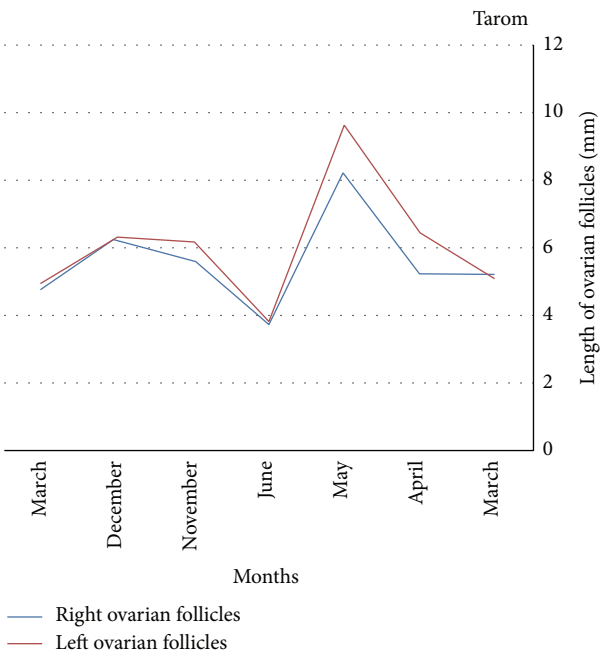

Figure 4: Size of ovarian follicles in Tarom *V. albicornuta*.

early May to June (Figure 5(a)) and postovulatory follicles in July (Figure 5(b)) which revealed that ovulation occurred in spring (early May till June). Previtellogenic follicles had many type pyriform cells in the granulose layer: small, medium, and large but vitellogenic follicles were only small pyriform cell (Figure 6(a)). Weights of right ovaries were more than left at $p < 0.001$ and dramatically increased in spring. In

Table 5: Mean size and number of the ovaries follicles of Bostanabad Zanjani viper in four seasons (M ± SD).

Follicles	Season			
	Spring	Summer	Autumn	Winter
Right ovary				
Number	23.86 ± 7.08	21.82 ± 3.66	21.58 ± 1.53	21.96 ± 9.60
Size (mm)	6.20 ± 1.60	4.43 ± 1.85	5.81 ± 0.30	5.40 ± 0.03
Left ovary				
Number	18.14 ± 4.85	16.82 ± 3.97	13.66 ± 2.35	16.06 ± 5.10
Size (mm)	6.18 ± 1.35	4.50 ± 1.72	6.05 ± 0.20	4.95 ± 0.99

Table 6: Mean size and number of the ovaries follicles of Tarom Zanjani viper in four seasons (M ± SD).

Follicles	Season			
	Spring	Summer	Autumn	Winter
Right ovary				
Number	19.25 ± 3.77	21.33 ± 5.75	16.11 ± 10.15	19.16 ± 6.83
Size (mm)	6.26 ± 1.75	3.75 ± 1.43	5.59 ± 2.97	5.62 ± 0.86
Left ovary				
Number	15.13 ± 1.96	17.50 ± 1.87	9.67 ± 3.24	14.25 ± 6.01
Size (mm)	7.05 ± 2.34	3.86 ± 1.65	6.21 ± 2.96	5.55 ± 1.11

Table 7: Mean percentages of different types of follicles in the ovaries of Bostanabad Zanjani viper in four seasons (M ± SD).

Follicles type (%)	Season			
	Spring	Summer	Autumn	Winter
Right ovary				
Type I	51.09%	80.93%	51.47%	59.78%
Type II	43.95%	15.81%	48.53%	40.22%
Type III	4.96%	3.25%	0	0
Type IV	0	0	0	0
Left ovary				
Type I	51.74%	82.23%	44.73%	64.56%
Type II	42.65%	16.44%	55.27%	35.44%
Type III	6.29%	1.31%	0	0
Type IV	0	0	0	0

Bostanabad samples length of ovarian follicle was large in summer (June and May) (Table 5), and the follicular length for Tarom's was at peak in early summer (June) and it was decreased in midsummer (July) (Table 6). The percentages of different types of ovarian follicles of the viper are presented in Tables 7 and 8.

4. Discussion

Snout-vent length (SVL) is commonly reported for body size in snakes [21]. SVL is correlated with litters or clutch's size in many species [13, 16]. Maximum mean SVL of Bostanabad *Vipera albicornuta* was 68.43 ± 2.98 cm and for Tarom samples 63.96 ± 3.77 cm. The previous studies have reported the SVL of 55.86 ± 1.32 cm for female *V. albicornuta* [22] and 73.56 ± 3.77 cm for male *V. albicornuta* [23], for pit viper,

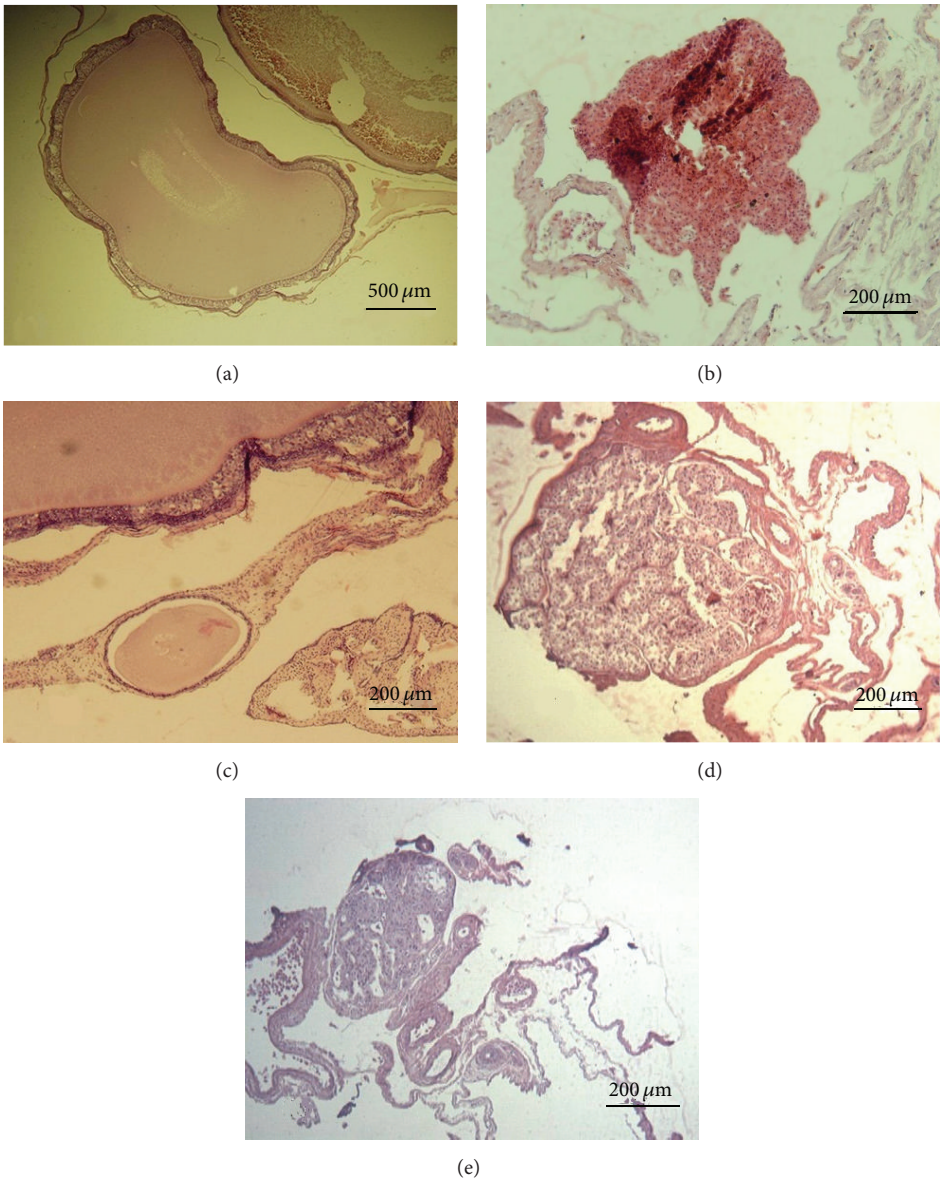

Figure 5: Micrographs of the *Vipera albicornuta* ovary. (a) Developing follicle in spring (July); (b) postovulatory follicle in summer; (c) previtellogenic follicle in fall; (d) the corpora lutea that regressed (Feb.); (e) corpora atretica in winter (Mar.). Scale bars: (a) 500 μm; (b), (c), (d), and (e) 200 μm.

Gloydius halys caucasicus, from Northwestern Iran 45.07 ± 2.83 cm [24], and body length in *Crotalus durissus terrificus* was reported as 89.00 ± 1.26 cm [25]. In Bostanabad *Vipera albicornuta* mean of tail length was 5.43 ± 0.67 cm and for Tarom samples 5.24 ± 0.50 cm similar to other studies, 5 cm [1], 4.61 ± 0.9 cm in female [22] and 5.0 ± 0.49 cm for male [24] Zanjani vipers and 6.07 ± 0.85 cm for *Gloydius halys caucasicus*, but compared to the female *Elaphe rufodorsata*, 10.7 cm and in male 11.2 cm were smaller [26]. Body size is the important biological trait for snakes, reproductive behavior such as fecundity in female or greater rates of mating in male [27]. Maximum average body weight of the Bostanabad *V. albicornuta* was 213.33 ± 30 g in spring (May), fat weight 24.93 ± 5.62 g in fall (November), and in the Tarom viper it was 142.82 ± 31.41 g in spring and fat weight 17.36 ± 3.55 g in the fall; therefore the *V. albicornuta* compared to the *Gloydius halys caucasicus*, 51.5 ± 10.42 g [24], is larger snake, although the results revealed that *V. albicornuta* is medium size snake [28] and Bostanabad *V. albicornuta* is larger than Tarom's one. Body fat reserves have shown the phase of hibernation and reveal a significant increase [29]. Body weight of female snakes shows an increase in fall could be due to fat storage for hibernation and in spring as a result of their feeding after emergence to prepare for motherhood. Snakes with weak bodies cannot produce the good clutch during reproductive seasons like *Elaphe longissima* [30], *Vipera aspis* [31]. Body fat of viviparous cortalid is reduced during the vitellogenesis, the hibernation, and also pregnancy period and lack of nutrition [11, 15, 32, 33]. Weight of body fat is reduced during the pregnancy and vitellogenesis in the *V. albicornuta* that is

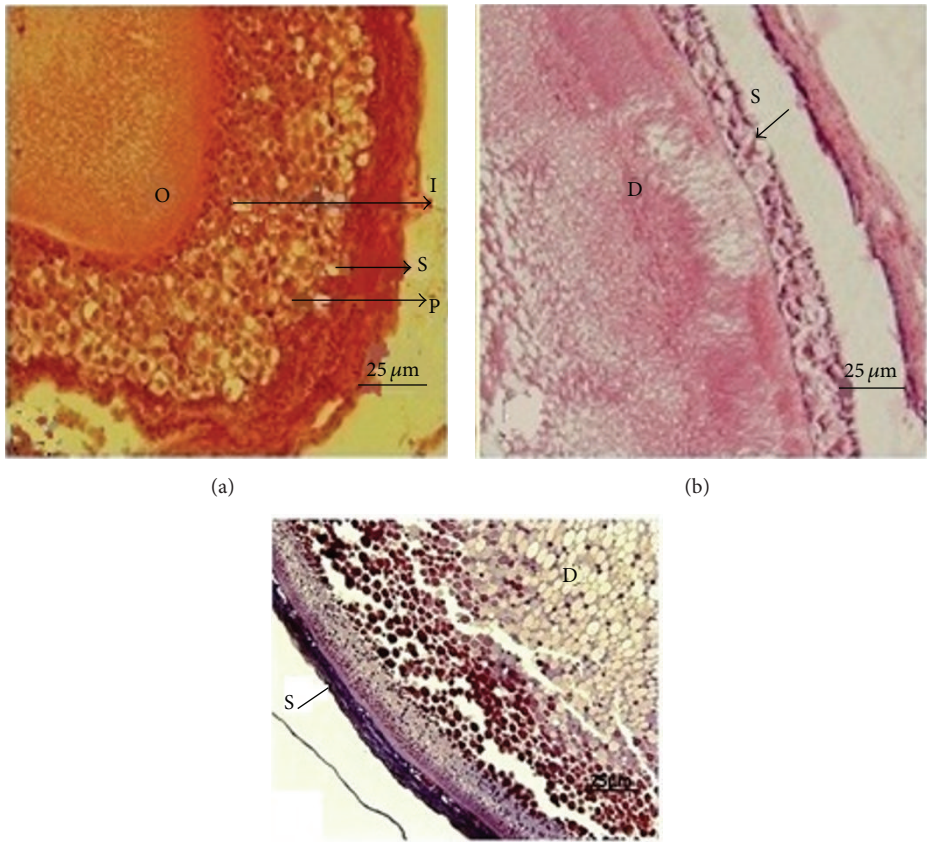

Figure 6: Sections of *Vipera albicornuta* ovary (H × E). (a) Stage I follicle (primary follicle): the former is <5 mm in length. No deutoplasmic granules were found in the ooplasm. The granulose contained pyriform, intermediate and small cells. (b) Stage II follicle: 5–15 mm in length. Deutoplasmic granules started to form and 2-3 layers of small cells existed in the granulose. (c) Stage III follicle: 15–25 mm in the length. The number and size of deutoplasmic granules increased, but there was only one layer of follicular cells. D: deutoplasmic granules; I: intermediate cell; O: ooplasm; P: pyriform cell; S: small cell. Scale bars = 25 μm.

Table 8: Mean percentages of different types of follicles in the ovaries of Tarom Zanjani viper in four seasons (M ± SD).

Follicles type (%)	Season			
	Spring	Summer	Autumn	Winter
Right ovary				
Type I	62.79%	88.77%	50.34%	50.34%
Type II	33.72%	11.23%	49.66%	49.66%
Type III	20.09%	0	0	0
Type IV	0	0	0	0
Left ovary				
Type I	52.23%	93.90%	40.22%	40.22%
Type II	40.29%	6.10%	59.77%	59.77%
Type III	11.94%	0	0	0
Type IV	0	0	0	0

similar to *Trimeresurus stejnegeri stejnegeri* [4]. In spring ovarian volume of Bostanabad's samples was at peak in July and decreased in August. The maximum ovarian weight and volume were observed in Tarom's snakes in late May to early June. As previously reported by Goldberg and Beaman [34] in *Crotalus enyo* and *Crotalus oreganus* snakes ovulation occurs in June [35]. Our study revealed that ovarian follicular growth in Bostanabad's Zanjani viper starts in fall and is at peak in spring, and mate is in late autumn, and the development of ovarian follicles continues till winter, because mating appears to have an obligatory role in the onset of vitellogenesis [36]. In aspic viper early vitellogenesis is reported during March-April [5]. Ovarian follicles continue to evolve in late spring when ovulation occurs. Development of ovarian follicles in Tarom's *Vipera albicornuta* is similar to *Agkistrodon piscivorus* of temperate regions [37, 38]. In female viper *Bothrops insularis* the vitellogenesis occurs in spring and ovulation occurs in late spring [9] as *Tantilla coronata* from the temperate region of South America [33]. Our result revealed that in Bostanabad's *V. albicornuta* ovulation probably occurred between June and July and in Tarom's vipers between May and June. The ovulation and mating in vipers usually occur at time that infants grow in the appropriate conditions. In some species, ovulation occurs in early summer so babies born in late summer [37, 38]. Our results show that there is difference at ovulation time between Tarom's and Bostanabad's vipers.

This difference could be due to differences in the geographical conditions. Typically, ovarian cycles and follicular growth are correlated with periods of light, rain, moisture, and food sources [39, 40]. Overall follicular growth in *V. albicornuta* is similar to nontoxic *Storeria dekayi* snake of Southeast Canada [10]. However, in the Zanjani viper, ovaries and follicles are at previtellogenesis stage in autumn, vitellogenesis in winter and during spring, ovulation occurs in late spring till early summer, and mating is prior to ovulation.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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