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Annual Study on Ovarian Structure of *Astacus leptodactylus* (Eschscholtz, 1823) in Arass Dam Lake, Iran

[1]*Seyed-Mehdi Mirheydari,* [2]*Marina Paolucci,* [3]*Abbas Matinfar,* [4]*Mehdi Soltani,*
[1]*Abolghasem Kamali and* [5]*Asadpour-Ousalou*

[1]Department of Fisheries, Science and Research Branch, Islamic Azad University, Tehran, Iran
[2]Department of Biological, Geological and Environmental Sciences,
University of Sannio, Via Port'Arsa, 11, 82100 Benevento, Italy
[3]Iranian Fisheries Research Organization, Tehran, Iran
[4]Department of Aquatic Animal Health,
Faculty of Veterinary Medicine, University of Tehran, Tehran, Iran
[5]Iranian Artemia Research Center, Golmankhaneh Harbor, Urmia, Iran

Abstract: The annual changes in the reproductive organ of female *Astacus leptodactylus* were investigated by the morphometric characteristics of the ovaries and seasonal fluctuations in the gonadosomatic index (GSI) which were confirmed by the histological analysis of the ovaries. A total of 10-12 mature size females of *Astacus leptodactylus* were collected seasonally from the Aras Dam Lake located at northwest Iran from June 2011 until January 2012. GSI fluctuated within an extended range (between 0.6 and 13.5% from June to January). The morphologic characteristic of the ovaries during the reproductive cycle were compared with the histological sections of ovaries. Both of synchronous and asynchronous ovaries were seen in August sampled ovaries; however asynchronous form was higher than another.

Key words: *Astacus leptodactylus* · Ovaries · Histology · Morphology · Reproductive Organ · Gonadosomatic Index

INTRODUCTION

Decapoda is the largest order of crustaceans and when compared to most other crustaceans are relatively large size animals. Decapods include four super-families, including of Parastacoidea, Enoplometopoidea, Nephropoidea and Astacoidea, respectively based on their importance [1].

Astacoidea consists of two main families; Combaridae and Astacidae. Astacidae is an important crayfish family whose main mentioned genera are *Astacus*, *Austropotamobius* and *Pacifastacus* [1]. The narrow-clawed crayfish *Astacus leptodactylus* is the only crayfish species in Iran, an important commercial species that has been exported to Europe since 1985 due to very low domestic consumption of crayfish in Iran [2]. It lives in cold clear Anzali lagoon and Aras Dam Lake in northern Iran, although it has been introduced to other reservoir in entire Iran in recent decade; Voshmgir Dam Lake, Sheikh Ali Kelaye Lagoon and Ghouri Gol Lake [3]. Haft Barm Lake, Izad khast water reservoir, Khamiran water reservoir, Malek Kian water reservoir, Shahid Yaghoobi water reservoir, Yam water reservoir, Shoorabil Lake and Khandaghloo Lake [2].

Abdolmalaki [4] reported a dramatic reduction in crayfish number in Anzali Lagoon due to following factors; Outburst of Caspian seawater with 12 ppt salinity into the lagoon, entering of urban, industrial and agricultural pollutants into Anzali lagoon, low depth and vegetarian growth, drought, eutrophic condition. As well

Corresponding Author: Seyed-Mehdi Mirheydari, Department of Fisheries Sciences, Faculty of Agriculture and Natural Resources, Science and Research Branch, Islamic Azad University, Tehran, Iran.
Tel: +98 912 8097438.

because of drought between 1999 and 2001, there was a reduction in crayfish harvest in Aras Dam reservoir in these years. Therefore survey on complicated reproductive system of this species is required to its propagation [2].

The reproduction cycle of this animal in Iranian inland waters can be explained as follows; mating in Anzali lagoon begins in early November and release Juveniles in early May [5]. Mating in the Aras Dam Lake conducted in early December and release Juveniles in early June [6]. In the Iranian coast of the Caspian Sea, the first gravid females were observed in early march and by the second decade of August the Juveniles were seen who had stuck to their mother's pleopods [7]. Spawning of each of the mentioned populations of *A. leptodactylus*, takes place only once a year. However, if there were fully-developed eggs within the spent ovaries, in winter, it could be due to the potential of multiple spawning during a year. For this purpose, ovarian examining should do in four seasons a year, while it is unprecedented about the species *A. leptodactylus* up to now. Despite of the previous studies on various populations of *A. leptodactylus* broodstock in Iranian inland waters, have mainly focused on the mating season, relationship between female size and egg size, the minimum size of gravid crayfish [5-6], length-weight relationship [5-8], mean of pleopodal fecundity [5-7], the differences between pleopodal and ovarian fecundity, sex ratio, [5-6] and molting sequences of males and females [6]. However morphologic and histological studies on ovarian tissue in the current study could play an important role in identifying of reproductive biology of *A. leptodactylus*.

The mature female reproductive system is a Y-shaped ovary which is placed in the cephalothorax over the heart and towards a cephal and along with the intestine. The ovary of crayfish is composed of a posterior lobe and two anterior lobes, covered by thin muscle and connective tissue layers [9]. The anterior lobes extend toward rostrum in the cephalothorax with the approaching of spawning season and form a Y-shaped organ. The ovaries are associated with short, paired oviducts that lead to paired gonopores, which open at the base of the third pereiopods [10].

Ovarian development divided into for separated previtellogenesis, vitellogenesis, oocyte development and ripeness [11]. The secondary oogonium moves from the germinal zone and develops up to previtellogenic oocyte throughout the previtellogenesis [12]. Thereafter during vitellogenesis, accumulation of organic and non-organic ingredients of yolk occurs in a progressing oocyte [13]. Throughout the oocyte progressing and vitellogenesis processes, follicle cells leave the germinal epithelium towards the growth region to stuck to each oocyte, result in oogenetic pouche formtion. Based on the results of Ando and Makioka[14] an oogenetic pouch consists of a sole ripe egg or a sole previtellogenic/or vitellogenic oocyte. An oogenetic pouch contains of a single mature egg or single previtellogenic or vitellogenic oocyte. According to oogenesis division stated by Adiyodi and Subramoniam [15] oogenesis process separated into two sequent stages: the proliferative phase and the differentiative phase. Throughout the first stage of oogenesis (proliferation), occurrence of mitotic divisions results in increasing of secondary oogonia in the germinal region. During the second stage of oogenesis (Differentiation), the prophase of meiosis starts and the secondary oogonium transforms to primary oocyte and moves toward the growth region.

MATERIALS AND METHODS

Seasonal sampling carried out from June, 2011 (sub-adult) to January, 2012 (spawned-adult). 10-12 specimens of mature size female *A. leptodactylus* (mean weight; 61.40, 92.42, 42.95, 54.72g and mean carapace length; 57.19, 62.34, 34.98, 50.75 mm, respectively from June 2011 to January 2012) were collected from Aras Dam Lake, Western-Azerbaijan province, Iran.

Female crayfish killed in the laboratory by cold treatment. Ovarian morphologic characteristic recognizing carried out by visual examination and individual ovarian histology analyzing carried out by microscopic examination.

Gonads were initially assigned, on the basis of their macroscopic appearance using the verified staging of [16] for the *C. quinquecarinatus*, to one of nine stages. The nine stages were: I- virgin (immature), II- maturing virgin/recovering, III- developing, IV- developed, V- mature; VI- ripe (gravid)-1; VII- ripe (gravid)-2; VIII- ripe (gravid)-3 and IX- spent. Subsequent histological verification of the macroscopical changes in gonads was done (June, 2011 to January, 2012).

Dissected ovaries fixed in Bouin's fixative for 24-h. The tissues were serially dehydrated with graded alcohol ethanol series, cleared in xylene and embedded in paraffin wax in a 65°C oven. The 5-6 µm thick sections made by rotary microtome, placed onto microscope slides and

stained with hematoxyline-eosin [17], impregnated with glue (Entellan, Merck) and finally mounted for morphological analyses of seasonal ovarian changes.

RESULTS AND DISCUTION

Exterior Appearance of Ovary: What was seen in the early stages of ovarian development consist of very small massive/tumor-formed ovaries; approximately lentils size and dark and bright orange. There was a middle slot at the end part of the gland which opened gradually to form Y-shaped ovaries in the following stages (in August).

November sampled ovaries with black-green colour thickened and progressed toward tip of rostrum. These ovarian colour changes are towards green, pale yellow and orange, respectively. Y-shaped ovaries developed towards the rostrum, finally containing yellowish-orange oocytes.

The full accumulation of yolk stocks in the ripe eggs was seen in the cytoplasm of ripe oocytes in November. Thus due to dehydration process of the ovary samples by serially grades of ethanol alcohol for tissue passage, oocytes contents were fragile and dried; therefore just oocyte walls were remained after exfoliation by Rotary Microtome. Therefore preparing of histological slides from November samples was not possible (Figure 1).

Four final stages of the ovarian oocytes are classified according to the oocyte diameter and colour and different embryonic shapes. For example, the developed embryo is well-recognized in the stage VIII oocyte (Figure, 2)

The oocyte stages were identified on the viewing screen of a microscope equipped by a profile projector. The oocyte diameter was measured by using the profile projector (Nikon- Japan) as well. All oocyte stages were identified on a viewing screen of microscope equipped by a profile projector. The oocyte diameter frequencies were measured by using a profile projector (Nikon- Japan). Both of synchronous and non-synchronous ovaries were seen in August samples ovaries. Synchronous type ovaries were mainly composed of stages 3 and 4 (Figures 3A, C), while non-synchronous type ovaries were containing of all 4 initial stages along with together (Figures 3B, D). However the first type (Synchronous type) was dominated in the seasonal samples.

Histological Comparisons of *A. leptodactylus* Ovaries During the Annual Reproductive Cycle: Spent ovaries (In January), were thicker than immature and maturing ovaries (In June and August), it could refers to oocyte diameter in ovaries; microscopic oocytes of mixed sizes were discernable in immature ovaries (Figures 4A, B, C, D) compare to mature size eggs remained after spawning in spent ovaries (Figure 4G). In fact the histological analyses of ovaries reflect a direct relationship between the microscopic development of oocytes during of oogenesis stages and macroscopic characteristics of ovaries (Table 1).

Based on the findings of the current study, the crayfish ovary is composed of four structural parts:

- Lumens are covered by the ovarian epithelium, were including of germaria. However Vogt [18] reported just a central lumen for the marbled crayfish, which it could be due to differences between species.
- Oogenetic pouches that are located within the follicular epithelium were containing of a sole (provitellogenic or vitellogenic) oocyte or a sole ripe egg, as demonstrated by [14-18].
- The ovarian wall-tissue consisting of an extended network of the muscular cells and connective tissue which are sporadic between hemal sinuses.
- The ovarian envelope that is made of the muscle cells, connective tissue and hemal sinuses, was thickened considerably in January rather than immature ovaries.

During of the annual maturation process oogonia migrate through the ovarian compartments, as follows, respectively: germarium, lumen, oogenetic pouch and again lumen.

Due to the lack of direct contact between main of oocytes and hemal sinuses, all oocytes required to synthesize or transport yolk stocks through the follicular cells as the pathways towards the extensive network of hemal sinuses. This result confirms previous findings of Vogt [18] with the marbled crayfish.

The ovarian envelope plays an important role both as an external protective that forming the ovarian shape and as an important protective against entering the pathogens [18].

Ackefors [19] classified egg maturation via colour and diameter changes during the developing eggs. However, changing of egg colours in the various oocyte stages of *A. astacus* up to maturation differed with findings of the current study (from black to orange vs. from white to brown). This could be due to the differences between species (narrow-clawed crayfish *A. leptodactylus vs*. noble crayfish *A. astacus*).

Table 1: Macroscopic and histological descriptions of different oogenesis stages during ovarian development of female *A. leptodactylus*

Ovarian stage	Period of occurrence	Macroscopic description	Maximum oocyte Diameter (µm)	Histological description
I. Immature (Virgin)	June.	Very small ovaries; Dark orange colour. A middle slot at the end part of the gland was seen	295	Oogonia and previtellogenic occytes dominate. post-spent ovaries also contain follicular epithelium
II. Maturing virgin	August.	Ovaries slightly thickened; with bright orange colour is easily discernable	570	Previtellogenic oocytes undergone primary vitellogenesis. Oogonia oocytes still present
III. Developing	August.	Growing thickened Ovaries; with bright orange colour	750	Yolk vesicle materials were present from near the epithelium to near the nucleus of theses oocytes, indicating secondary vitellogenesis. Primary vitellogenic and previtellogenic oocytes still present
IV. Developed	August	Ovaries slightlywere swollen Y-shaped; With clear oocytes becoming pale opalescent /milky colour.	950	Yolk vesicles dominate throughout the cytoplasm indicating full vitellogenesis. Folicullar epithelium surrounds oocytes
V. Ripe (Gravid) -1	November	Y-shaped Ovaries developed towards the front of rostrum, containing of mainly black oocytes.	1000	Cytoplasm of oocytes dominated by yolk vesicles.
VI. Ripe (Gravid) -2	November	Y-shaped Ovaries developed towards the front of rostrum, containing of mainly green oocytes.	1150	Cytoplasm of oocytes dominated by yolk vesicles.
VII. Ripe (Gravid) -3	November	Y-shaped Ovaries developed towards the front of rostrum, containing of mainly yellow oocytes.	1180	Cytoplasm of oocytes dominated by yolk vesicles
VIII. Ripe (Gravid) -4	November	Y-shaped Ovaries developed towards the front of rostrum, containing yellowish orange oocytes	1250	Cytoplasm of oocytes dominated by yolk vesicles.
IX. Spent	January	These (approximately lentils size) ovaries were thinner compared to virgin ovaries.	225	Post-ovulatory follicles present along with large unextruded oocyte.

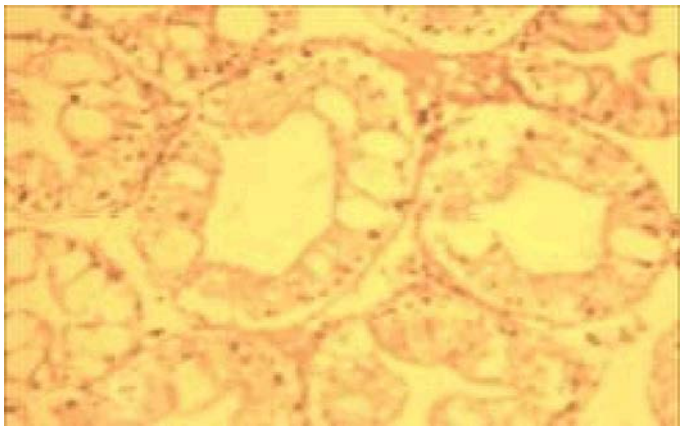

Fig. 1: The remained cell walls of mature eggs after tissue passage process (stages V, VI, VII and VIII)

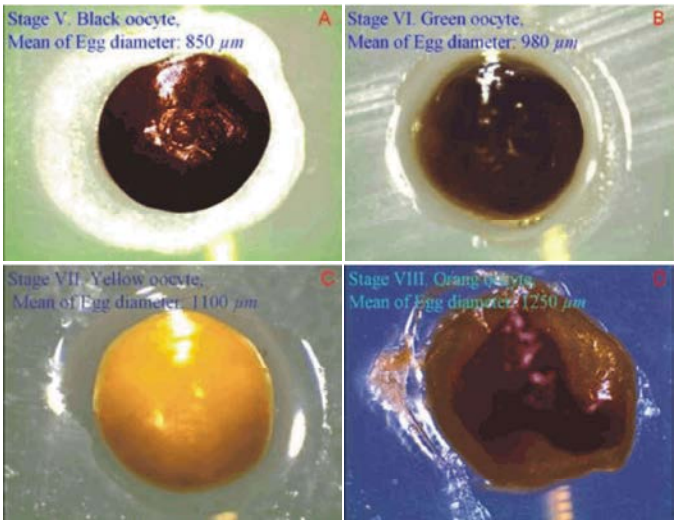

Fig. 2: Morphological changes of the last ovarian eggs detected with light microscopy

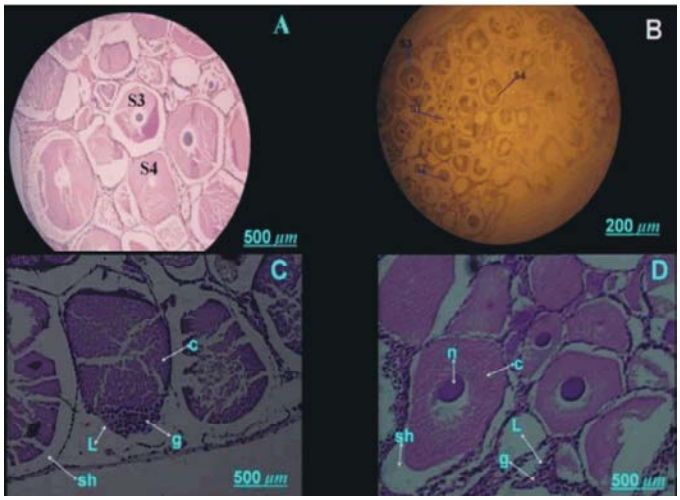

Fig. 3: Comparison of ovarian development between a synchronous ovary (TL; 90.67 mm) (A) and a non-synchronous ovary (TL; 88.91 mm) (B). Close up view of a synchronous ovary (C) and a (D) close up view of a non-synchronous ovary (S1, stage1; S2, stage2; S3, stage3; S4, stage4; sh, Outer muscular sheath; g, germanium; n, nucleus; L, lumen; c, cytoplasm).

Table 2: Morphological changes in the testes and *Vas deferens* of *A. leptodactylus* caught from June 2011 until January 2012 (see Figs. 4A-G). Due to histological problems, preparing slide from autumn (November) specimens was not possible

Organ		June	August	January
Ovaries	Atresia			+
	Oogonia	+		+
	Previtellogenic oocytes	+	+	+
	Primary vitellogenic oocytes	+	+	+
	Secondary vitellogenic oocytes			+

In the present study, mature orange-colored eggs were found for the first time in late November, which could be simultaneous with the presence of the second vitellogenic oocytes. But, it was seen in the histological sections of ovaries in the January (and a few of August sampled ovaries). Although as it is mentioned above, due to the histological problem occurred in November, it wasn't possible to prepare histological slides from November sampled ovaries, but based on the observation of secondary vitellogenic oocytes in both August and January, presence of these oocytes in autumn ovaries was proved.

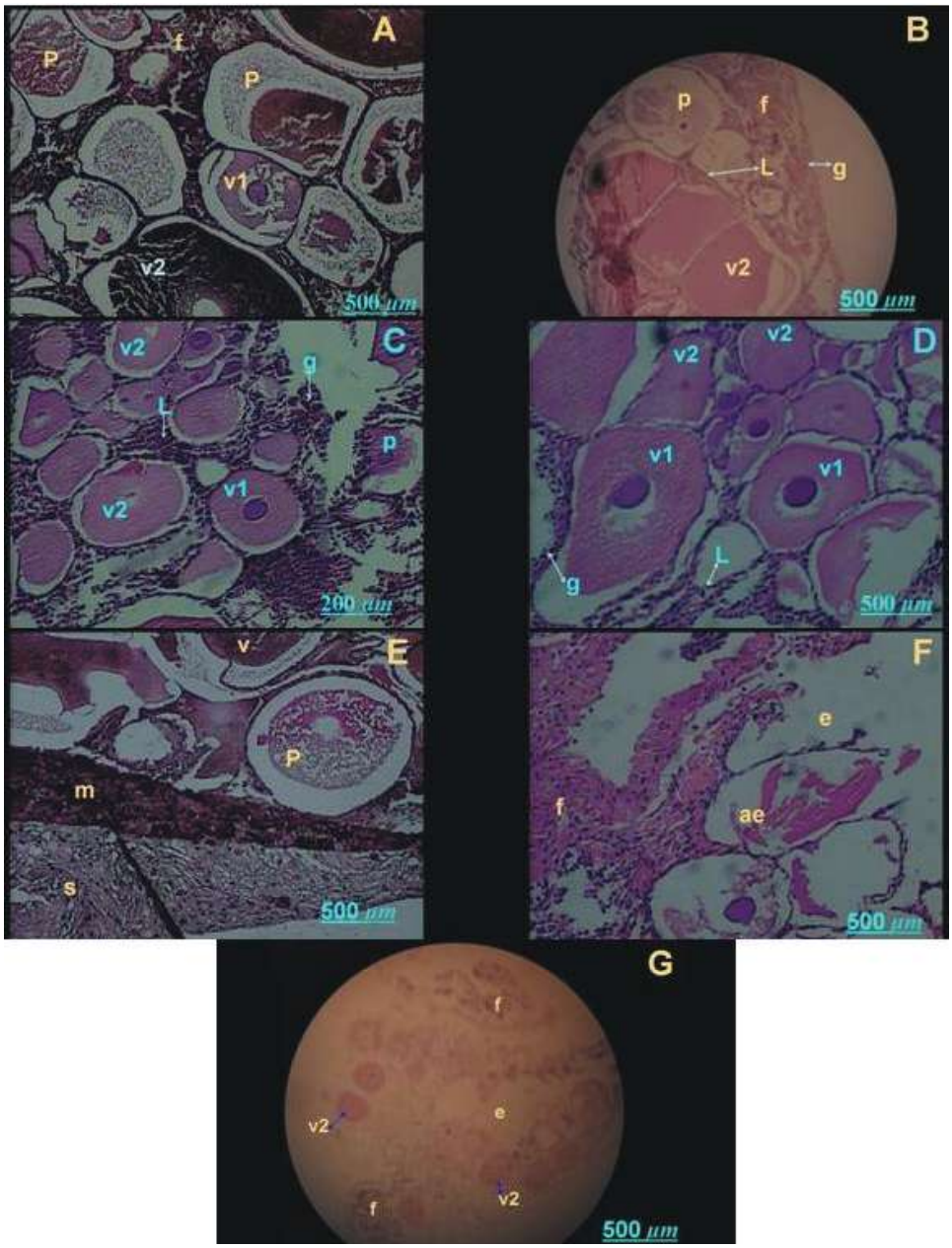

Fig. 4: Ovaries of the *A. leptodactylus* caught in June, 2011 (A), August, 2011 (B, C, D) and January, 2012 (E, F, G) from Aras dam reservoir. Due to histological problems, preparing slide from autumn (November) specimens was not possible. ae–absorption of unspawned eggs; e–empty oogenetic pouch; f-follicular epithelium; oe-ovarian envelope; v1–primary vitellogenic oocytes; v2–secondary vitellogenic oocytes; g–germarium. m-muscle fiber, s-surface layer, L-lumen. G; General view of a spent ovary of *A. leptodactylus* caught in January. These ovaries consist of un-spawned eggs.

The lack of immature white eggs throughout the entire ovaries of gravid crayfish in November was confirmed by the visual examination in this study. However it was in contrast with the findings of Ackefors [19] and Lucic [20] with *A. astacus*. This difference could be due to the diversity between species (narrow-clawed crayfish *A. leptodactylus vs*. noble crayfish, *A. astacus*) or environmental differences (Asia, Iran, Aras dam Lake *vs.* Sweden, Sweden small Lake and Croatia, Vukovina gravel pit, respectively).

Mcrea and Mitchell [21] and Lucic [20] reported that remained immature eggs in spent ovaries could get mature during the next reproductive season or be reabsorbed. Also in this study most of the un-spawned mature and/or immature oocytes were reabsorbed by ovarian tissue in January.

The process of egg maturation was begun by the presence of oogonia in June, while histological analysis demonstrated that previtellogenic oocytes were growing, simultaneously (Table 2)

The mean gonad weight increased approximately 22 times (in November) compared to the gonad weight at the beginning of reproductive cycle (in June) and gonadosomatic index was near to 12.6%. It was near to findings of Lucic [20] with *A. astacus* who reported increasing of ovary weigh and GSI during reproductive cycle, 25 times and 12.3% respectively.

A thin muscle layer covered the ovaries in June; this structure became much thicker after spawning in January. This is in contrast with the hypothesis of Adiyodi and Subramoniam [15] and the result of Lucic [20] who reported the absence of the mentioned layer. Although, this could be due to the differences between species (*A. leptodactylus vs. A. astacus*).

The presence of the ovarian lumen is in contrast with the findings with many other decapod species [10]. However Vogt [18], stated a similar result with the marbled crayfish (an unknown species), as well.

Yolk dispersed first around the nuclei in the cytoplasm, in contrast with other crayfish species *A. astacus* [20] and prawn *Penaeus japonicus* [22] whose reported yolk accumulation, first in the peripheral cytoplasm.

Some different stages of ogenesis cells was synchronized within ovaries which indicated by dominance of previtellogenic, vitellogenic oocytes and oogonia in ovaries of different months (Table, 2). It was in agreement with the result of [20]. However different stages of ripe eggs were seen in gravid crayfish in November which were recognized according to their colors (black, green, yellow and orange, respectively) and diameters. This means that all of oogenesis cells became mature in November via increasing of steroids and environmental parameters in this season which were affecting on egg maturation. As well, all ripe eggs were released in January, completely.

CONCLUSION

This research represents the first study on the annual reproductive cycle of *A. leptodactylus*. Both of the asynchronous and synchronous ovaries were seen in this crayfish population; however asynchronous ovaries were dominated in Aras dam Lake, based on our histological analyses. Development of *A. leptodactylus* ovaries is synchronized by the growth of the ovaries, (0.6.5% and 13.5%, respectively from June to November, respectively).

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