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Survey of Seasonal Histology of Male Reproductive Organ in Narrow-Clawed Crayfish *A. leptodactylus* in Aras Dam Lake, Iran

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Abstract: The annual reproductive cycle of male *Astacus leptodactylus* was surveyed by study on the seasonal changes of the external appearance of the testes and vasa deferentia, fluctuations in the gonadosomatic index (GSI) and the histological analysis of the male reproductive system. A total of 10-12 mature size males of *A. leptodactylus* were collected seasonally from the Aras Dam Lake, located at northwest Iran, from June 2011 to January 2012. The testis of *A. leptodactylus* is composed of a paired of anterior lobules and a posterior lobule which is located dorsoventrally in the cephalothorax. Vasa deferentia were separated to three different structural compartment; proximal vas deferens (PVD), middle vas deferens (MVD) and distal vas deferens (DVD). The seasonal total body lengths of Crayfish were measured respectively 139.64, 140.07, 100.29, 118.04 mm and weighted respectively 88.28, 99.58, 58.72, 64.58 g. GSI changed seasonally within a limit range (from 0.50, 0.52, 1.21 to 1.12). The morphological characteristics of the testes and vasa deferentia throughout the annual study were emphasized with the findings of histological analysis. Histological differentiation of male crayfish was separated into 5 stages, from stage I to stage V. The immature testis of crayfish with stage I was composed of immature cell mass. Increasing in the number and differentiating towards acini of testis gland. Acini are containing of spermatogonia at stage II. Simultaneous spermatogenesis at stage III resulted in three different spermatogenesis cells, spermatocyte transformed to spermatid at stage IV and to spermatozoa that release from the testis at stage V, were resulted. The findings suggested asynchronous testis in the species *A. leptodactylus*. The cell forms of vasa deferentia layers transformed seasonally toward mating period which might be due to the ejaculatory duct, to producing spermatozoa and maintaining it alive or to transferring down the spermatozoa. The presence of primary spermatophore layer may help keeping spermatozoa alive while the secondary spermatophore layer may produces spermatophore or synthesize of a cellular material which forms spermatophore.

Key words: *Astacus leptodactylus* % Gonadosomatic index % Testis % Vasa deferentia % Morphology % Histology

INTRODUCTION

The narrow-clawed crayfish (*Astacus leptodactylus*), an important species from the Astacidae family, is widely distributed in European countries [1].

Early studies carried out about spermatogenesis and spermatophore formation of this species [2-3]. However, with exception for a few studies on male reproductive organ with *Astacus* genus [4-5], there is not enough detailed study on the exterior appearance and histology of the testis in Astacidae family.

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Some preliminary studies on the morphology of the testis, spermatogenesis and the annual cycle of sperm production have been done in Cambaridae family [6-7-8], *Pacifastacus* genus [3], *Orconectes* genus [9-10] and *Combarus* genus [11-12].

The testis includes of a paired of anterior lobes and a posterior lobule, which are covered by a thin layer [13]. Testicular lobule is including of some seminiferous tubules which are covered by subsidiary cells. Some hemal sinuses are located around of these tubules. Formation and structure of the seminiferous tubules vary with the stage of spermatogenesis in terms of. Spermatogenesis is occurred within seminiferous tubules and some special sections of the testes [14]. The flagellated spermatozoa have low-density nucleus and various number of pin-shape structures [13]. Tubules act as gathering sperms, from the seminiferous tubes and then transfer them to the *vas deferens* organ. The *vas deferens*, a very long organ, is incorporated to the testis. By means of a paired tubules spermatozoa transfers from the testicular collecting tubes to the gonopores, microgenital pores whose opening is located at the base of the last pair of periopods. They consist of a secretive cortex and connective tissues are located around them [15]. When spermatozoa enter the *vas deferens organ* they strengthen the sperm mass and secrete non-cellular spermatophore wall layers [16].

The spermatozoa which were collected in the collecting tubules of the testes transmitted to the ejaculatory duct by means of the vas deferens and then ejaculated to the gonopods to transfer to ventral-thoracic cavity of female during mating period [5].

Based on the results of this study; The explanation of the annual changes in the testis and the doubled spermatophore layer within the *vas deferens*, help understanding the temporal trend of the reproductive cycle of the species *A. leptodactylus*.

MATERIALS AND METHODS

This study carried out in order to survey the morphologic and histological changes in the seasonal reproductive cycle of male *A. leptodactylus*. Sampling was done by opera house traps. In the sampling Opera house traps, silken ropes 200mm diameters. Nylon ropes 8 mm diameter; GPS, folderable plastic baskets, grab sampler and electro shockers were used. The specimens were transported by air in a styrofoam box with meshed ice to the laboratory complex of Islamic Azad University (Tehran, Iran).

From ten to twelve mature size males were collected each season. Individuals weighed to the nearest 0.01 g and mean carapace length were measured to the nearest 0.01 mm. The carapace length (CL, from the rostrum's tip to the posterior middle edge of cephalothoraxe) and wet weight (W) of crayfish were measured to the nearest 0.01 mm and 0.01 g, respectively, as described by [17]. After moving away the carapace, the testes and *vasa deferentia* were then dissected out and also weighed to approximately 0.01 g. The seasonal gonadosomatic indices (GSI) of mature and immature male *A. leptodactylus* were determined using the following equations; (wet weight of gonad /total body weight) × 100 [18]. Gonads were initially assigned, on the basis of their macroscopic appearance using the verified staging of Taketomy [19] for the *Procambarus clarkii*, to one of five stages. Animal sex was recognized by visual evaluation.

To identify spermatogenesis phases, subsequent histological operation was done prior to and during the mating period (from June 2011 to January 2012). After 48 hours of catching males, all dissected testes and *vasa deferentia* were fixed in Formalin 10% solution for 24-h. The tissues were serially dehydrated with graded alcohol ethanol series, cleared in xylene or toluene and finally were embedded in paraffin wax in a 60°C oven, twice. The 5-6 µm thick sections made by rotary microtome, placed onto microscope slides and stained with hematoxylin-eosin, impregnated with entellan glue and finally mounted for morphological analysis of seasonal testes and *vasa deferentia* changes.

RESULTS AND DISCUTION

Macroscopic Changes in Testis Appearance: The crayfish *A. leptodactylus* has a tri-lobed testis which is located inside the cephalothorax. Two anterior lobules located in both sides of the cephalothorax and connected to the hepatopancreas, finally. Under the cephalothoraxes, both of the anterior lobules jointed to the posterior lobule altogether and then extended along with the intestine. The anterior lobules of the testis were not as long as the posterior lobule. The mean total length of the testis increased from 20 mm (on June), 34 mm (on August), 51 mm (on November) and 41 mm (on January) and its color changed from bright white (on June and August) to dark white (on November and January).

Histological Studies on Male Reproductive Organ

Testes: Mature swollen testes were observed for the first time in November. The mean weight of the gonads

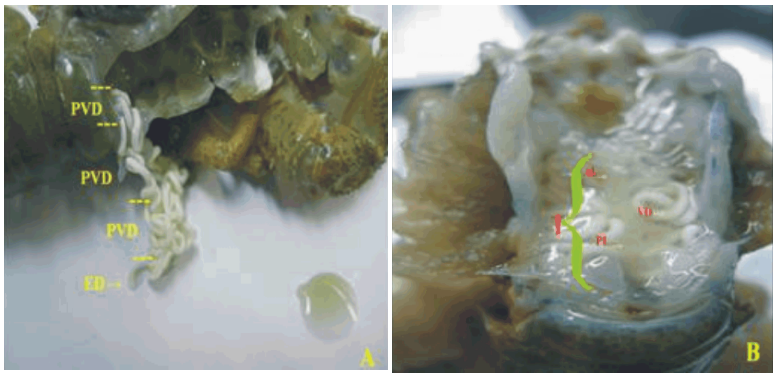

Fig. 1: A, shows three distinctive zones; proximal (near) vas deferens (PVD), medial vas deferens (MVD) and distal (away) vas deferens (DVD). B, General view of a tri-lobed testis (T) and a paired of vas deferens attached to the testis from the sides,, the tri-lobed testis consists of two anterior lobules (AL) and a posterior lobule (PL), vas deferens (VD) and ejaculatory duct (ED).

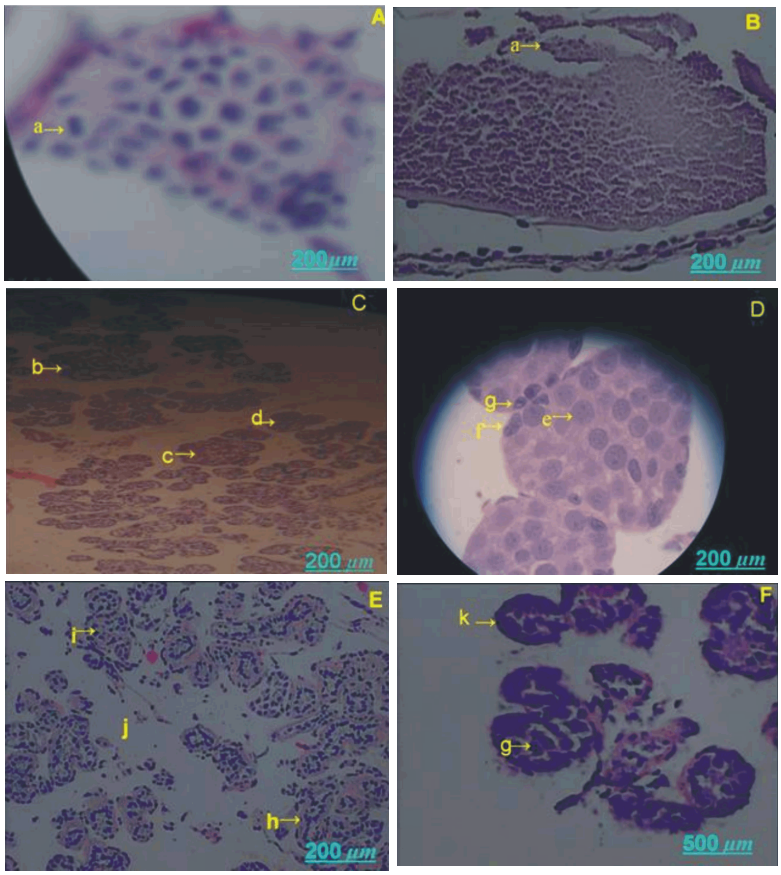

Fig. 2: A cross section of the testis in *A. leptodactylus* caught on June, 2011 (A, B), August, 2011 (C), November, 2011 (D) and January, 2012 (E, F) from Aras dam reservoir. A, general view of immature testis a, gonadal cell mass; B, proliferation and differentiation in cells of immature testis toward becoming mature testis; C, general view of the testes showing the acini (b); containing of spermatogonia (c) alongside of collecting duct (d). D, the acini containing of primary spermatocytes (e) and secondary spermatocytes (f) in meiotic synchrony and sub-cells with flattened nuclei (arrowhead) spermatid and/or spermatozoa (g). E, The heart form acini (h) showing spermatids (i) alongside of hemal sinus (j). F, spermatozoa (g) accumulated in the cluster form acinus (k)

Table 1: Mean of gonadosomatic index (GSI) and mean of gonad weight (GW) in males of *A. leptodactylus* caught from June 2011 until January 2012 from Aras Dam Lake

	Gonadosomatic Index (GSI)	Mean Gonad Weight (GW)
June	88.28	0.5026
August	99.58	0.5227
November	58.72	1.210
January	64.58	1.124

increased after the mating season resulted in GSI enhancement, gradually (Table 1). The gonadosomatic index of males calculated from June to January within a very limit range, between 0.50 (on June) and 1.21% (on November) (Table 1).

The testes of *A. leptodactylus* were classified by a similar pattern described by Taketomi [20]. Early in stage I, the immature testis was undeveloped (Fig. 2A). Late in stage I, the proliferation (increasing) and differentiation (transform) of testis cells to the acini was began (Fig. 2B).

During the stage II, the acinus was contained of a few spermatogonia cells in acini. In this stage the testis was bigger than previous stage (Fig. 2C).

During the stage III, the testes were ripped, approximately. Throughout the stage III, plenty of meiotic divisions occurred in the acini. Simultaneous primary and secondary meiotic divisions' occurred in per acinus of this stage (Fig. 2D). Late in stage III, spermatids and mature spermatozoa were observed accompanied by primary and secondary spermatocytes (Fig. 2D).

In stage IV, the number of acinus cells decreased but each cell enlarged. Late in stage IV secondary spermatocytes (Fig. 2D), transformed into spermatid (Fig. 2E), thus the volume of the acinus increased, considerably.

The appearance of the testes in stage V is the same as in stage IV (Figs. 2E, F). Spermatid were transformed into spermatozoa in the last stage (V. Spermatozoa were accumulated in the collecting tubules with cluster form acini (Fig. 2F) while spermatid were accumulated in heart form acini.

Vasa Deferentia: Classification of different segments of *vas deferens* could be as follows:

The first compartment of the *vas deferens* is the proximal *vas deferens* (PVD), dark white in color, whose diameter was ranging from 0.3 to 0.7 mm from spring to winter, respectively.

The second segment of the vas deferens was the medial *vas deferens* (MVD), bright white in color whose diameter ranging from 0.5 to 0.9 mm throughout the annual study.

The DVD, the brightest white segment, was the last and widest segment (approximately 1.0-2.1 mm diameter. from spring to winter, respectively) of the vas deferens.

Seasonal changes were observed from various segments of vas deferens are presented as follows.

Spring Observations: There were some red spots, called as phallic papilla in all segments of VD. Primary and secondary layer of spermatophore were not seen in this season Spermatozoa and other a cellular material were discharged in all compartments of VD in spring. PVD, MVD and DVD were consisting of horizontal cells with sporadic nuclei, advanced horizontal cells and transformed columnar cells, respectively (Fig. 3A&B and C).

Summer Observations: Spermatozoa and other a cellular material were not seen in VD compartments in summer. PVD, MVD *and* DVD were consisting of columnar, advanced elliptic columnar cells and, respectively cells (Fig. 3E&F). Primary and secondary layers were as thin lines in this segment (Fig. 3G).

Autumn Observations: Spermatozoa and other a cellular material were seen with low concentration in VD compartments in autumn. Primary layer of spermatophore was becoming obvious in MVD (Fig. 3J). PVD, MVD and DVD were consisting of string like cells, fibrous cells and unknown cells, respectively (Figs. 3I, J and K). Primary and secondary layers of spermatophore were not obvious in this season (Fig. 3L).

Winter Observations: Spermatozoa and other a cellular material were seen with high concentration in VD in winter. PVD, MVD and DVD were consisting of fibrous cells, fibrous columnar cells and string like cells (Figs. 3M, N and O). Primary and secondary layers of spermatophore were becoming obvious in this compartment (Fig. 3N).

Ejaculatory duct is consisting of a narrow epithelial layer and thick muscular layer surrounding the first layer. The muscular layer consists of a longitudinal layer and a circular layer (Figs. 3D, H, L and P).

Identification of testis different phases carried out based on a modified version of the method described by Taketomi [20] with crayfish *Procambarus clarkii*. The immature testis was obvious in stage I. The small size of immature testis could be due to low activity of cells in stage I. In this stage the cells of immature testis proliferated and differentiated to transform immature gonad into well-developed testes. It seems that male

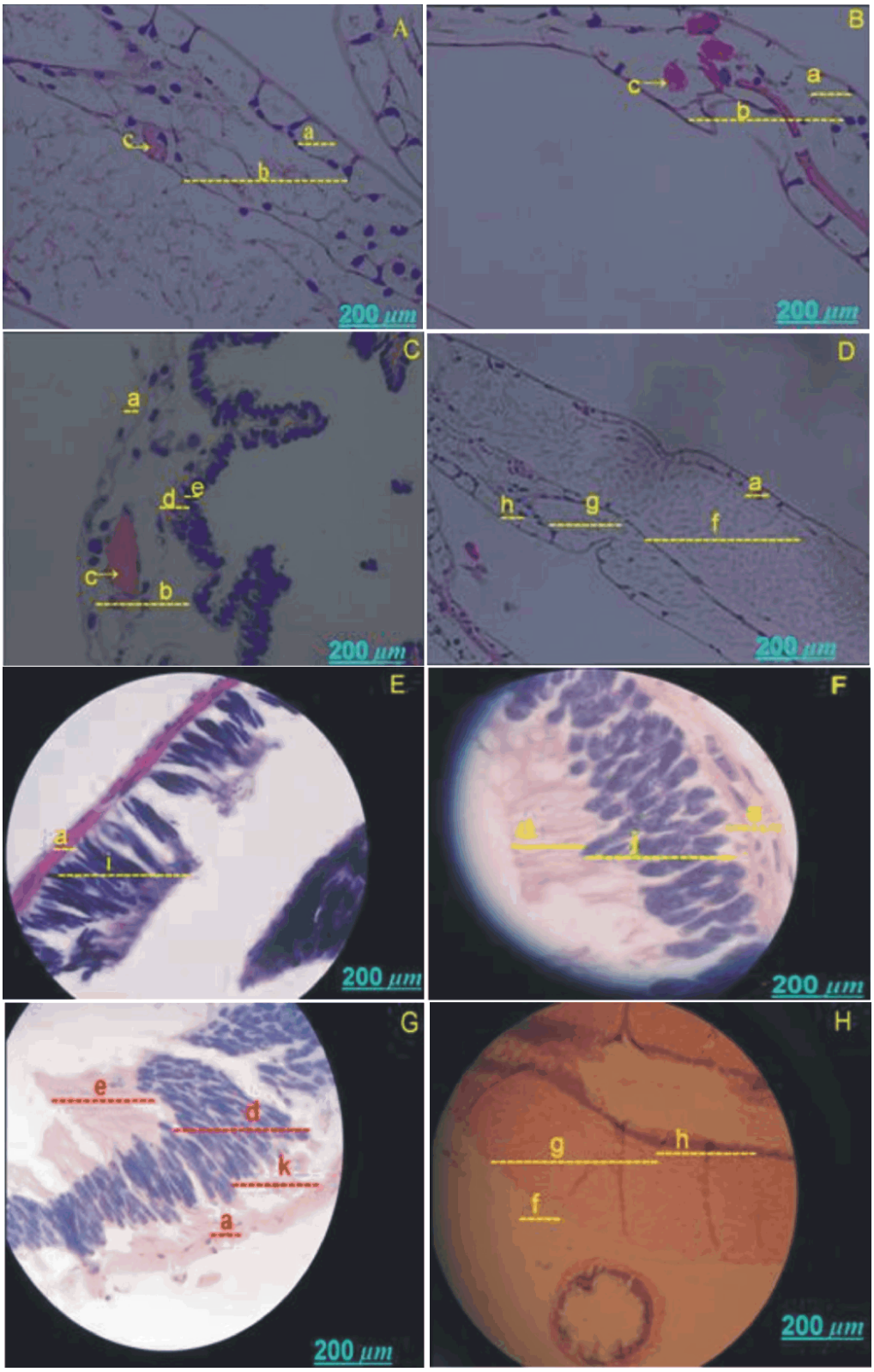

Fig. 3: Continued

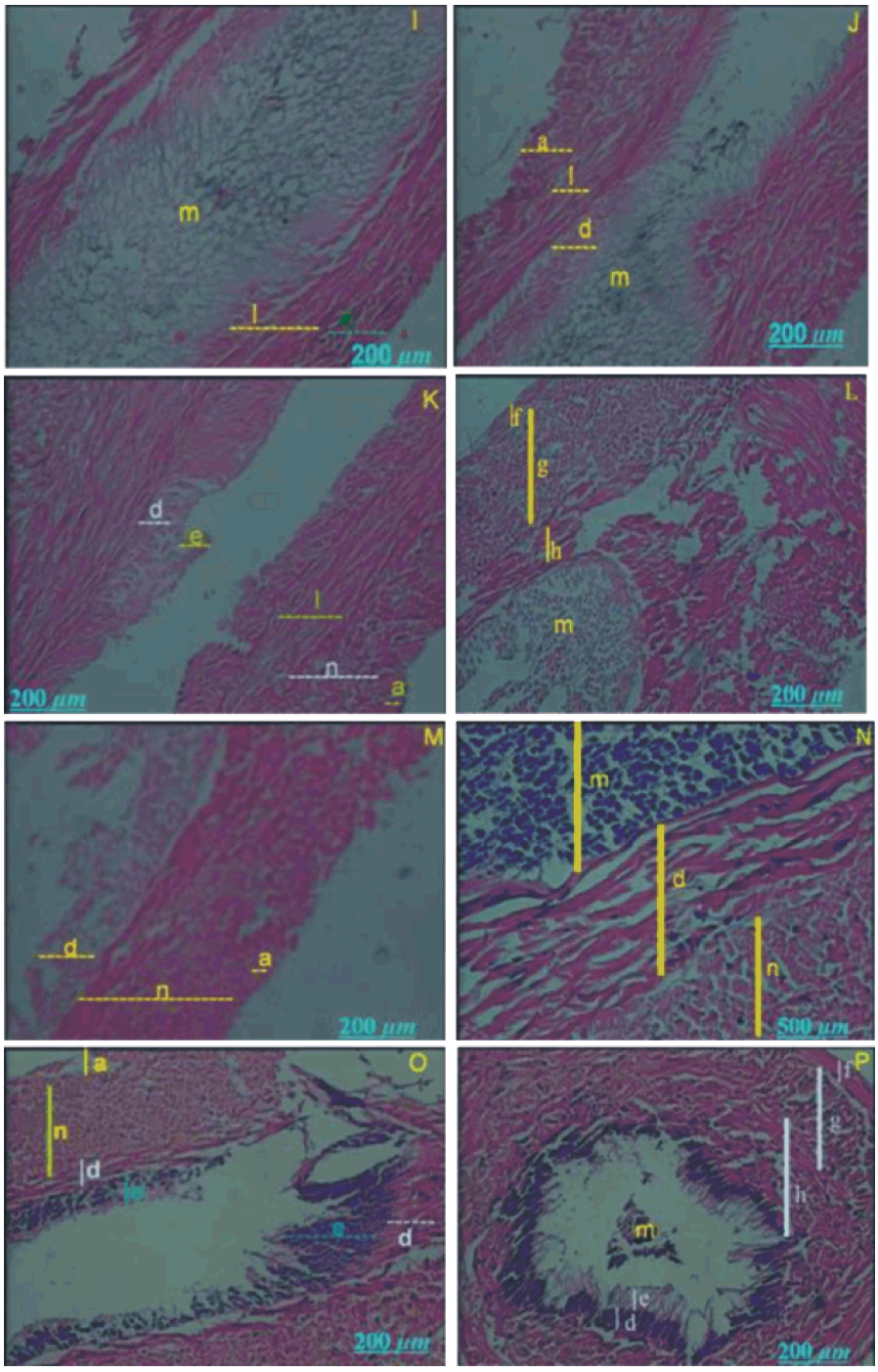

Fig. 3: *Vas deferens* of *A. leptodactylus* caught in June2011 (A, B, C, D), August 2011 (E, F, G, H), November 2011 (I, J, K, L) and January 2012 (M, N, O, P) from Aras Dam reservoir. Staining; was done by Hematoxylin + Eosin, in all seasons. Samples of Proximal *vas deferens* were containing of (A, E, I, M), Samples of Medial *vas deferens* were containing of (B, F, J, N,), Samples of Distal *vas deferens* were containing of (C, G, K, O) and Samples Ejaculatory duct were containing of (D, H, L, P). a; fibrous connective tissue, b; horizontal cells with sporadic nuclei, c; phallic papilla, d; primary spermatophore layer, e; secondary spermatophore layer, f; circular layer, g, longitudinal layer, h; epithelial layer, i; columnar cells, j; elliptic columnar cells, k; unknown cells, l; string-like cells, m; spermatozoa, n; fibrous cells

Table 2: Morphological changes in the testes and *vas deferens* of *A. leptodactylus* recorded from June 2011 until January 2012 (see Fig. 2A-P)

Organ			June	August	November	January
Testis		Spermatogonia	+			
		Spermatocyte (1)		+		
		Spermatocyte (2)		+		
		Spermatid			+	
		Spermatozoa		+		+
Vas deferens	PVD	Spermatophore	+		+	+
		Primary spermatophore layer				+
		Secondary spermatophore layer				
		Type of cells	Horizontal cells	Columnar cells	String-like cells	Fibrous cells
	MVD	Spermatophore			+	+
		Primary spermatophore layer		+	+	+
		Secondary spermatophore layer				
		Type of cells	Horizontal cells	Columnar cells	String-like cells	Fibrous cells
	DVD	Spermatophore			+	+
		Primary spermatophore layer	+	+	+	+
		Secondary spermatophore layer	+	+	+	+
		Type of cells	Horizontal cells	Unknown cells	Fibrous cells	Fibrous cells
	Ejaculatory duct	Spermatophore			+	+
		Epithelial layer	+	+	+	+
		Longitudinal layer	+	+	+	+
		Circular layer	+	+	+	+

sexual hormones had an inducing role in the appearance of the primary sexual characteristics, viz gonadal morphology. Stage II was continued along with the stage I with the proliferation and differentiation of spermatogonia in the acini. The presence of high-active cells in stage III resulted in both of primary and secondary cell division simultaneously.

In Stages for IV and V cells didn't differed significantly. In stage IV spermatid cells were collected within heart form acini. During stage V spermatozoa activity became higher in the cluster form acini. Spermatogenesis process was still observed in the well-developed testes of stage V when spermatid transforming to spermatozoa.

The number of these high-activity cells increased significantly during the winter, in contrast with a few number in spring. This was in a reversed form with the finding of [19-20] with the crayfish *Procambarus clarkii.* This diversity could be due to the differences between the species (*A. leptodactylus vs. P. clarkii*) and/or environmental parameters (e.g. Aras dam Lake *vs.* Lake Edzu).

The testis morphology of *A. leptodactylus* was different from that was reported for *Pacifastacus* [3]; *Cambarus* [22]; *Orconectus* [23]; *Procambarus* [20-24] and *Cambarus* [25].

During spermatogenesis two forms of the acini's maturation have been observed in decapod crustaceans; however either synchronous or asynchronous maturation of the testis are based on the crayfish species. Despite of the finding of Erkan [5] which introduced *A. leptodactylus* as a synchronous species; the result of the current study proved asynchronous type of testis of this species due to the occurrence of three developmental degrees of spermatogenesis in acinus of November sampled testes. However it seems that Erkan [5] couldn't find the asynchronous statue in acini probably due to their sole-season study. Although synchronous form of acini is prevalent in crayfish [13-14-26], however, asynchronous form of testis has reported previously for some crayfish genus like *Samastacus* [27], *Parastacoides* [4], *Cherax* [28] and *Parastacus* [29].

Mature testes were observed first on November, which is respectively three and two month later than reports of Taugbol [30] in Norway and Lucic [26] in Croatia with *A. astacus*. According to the fact that preparation for the mating period in winter depends on temperature decreasing, it occurs earlier in the Europe (Norway and Croatia) than in Asia (Iran, Western-Azerbaijan province). However the difference between species (*A. astacus* vs. *A. leptodactylus*) could play an important role in this delay of maturation, as well.

Vas Deferens: Vas deferens is separated into three segments (proximal, medial and distal vas deferens) according to its structural differences in *A. leptodactylus*.

The responsibilities of these segments are previously described by Erkan [5] as following; spermatophore formation; spermatophore transportation and sperm conservation up to fertilization.

In spite of the finding of Erkan *et al*.[5] who reported that the proximal vas deferens (PVD) of *A. leptodactylus* is made by a simple columnar epithelium. However in the current study, the structural cells which made this layer, differed seasonally from horizontal cells with sporadic nuclei (June, 2011), columnar cells (August, 2011), string-like cells (November, 2011) to fibrous cells (January, 2012). This difference could be due to stage by stage structural preparation of *vas deferens* to synthesize the sperm-packs within spermatophore layers.

In *A. leptodactylus*, epithelial cells of the medial *vas deferens* (MVD) were taller than those in the PVD in August, November and January samples. The epithelial cells of the MVD differed seasonally from a single layer of horizontal cells with sporadic nuclei (June, 2011), elliptic columnar cells (August, 2011), string-like cells (November, 2011) and fibrous cells (January, 2012), but in *C. quadricarinatus* the epithelial cells of MVD range from cubic to flat [28].

In *A. leptodactylus*, the primary layer of spermatophore is first observed at the beginning of the MVD as reported previously by Erkan *et al*. [5], however with exception for June samples which both primary and secondary layer of spermatophore were seen in DVD. The secondary layer of spermatophore first appears near t he end of the MVD and/or the beginning of DVD of *A. leptodactylus* as reported previously in *A. leptodactylus* [5] and in *Cherax albidus* [31].

Although the structure of the distal *vas deferens* (DVD) in *A. leptodactylus* did not vary considerably from medial *vas deferens* (MVD), thickening of both the primary and secondary spermatophore layers and the expansion of the epithelium show that the activation of epithelium increased in DVD in order to complete the spermatophore maturation.

Spermatophore transfers to the ejaculatory duct as stated by Krol *et al*. [13]. It seems that DVD showed a folded-shape in the mating season as stated previously by Erkan *et al.* [5]. The curvature of DVD could be created during of sperm-packs depletion on January which continues until June to discharge of remained spermatophore from *vas deferens*, although it (DVD) became smooth gradually on November.

In June sampled vasa deferentia, the red spots known as phallic papilla were obvious in all segments of vas deferens. Hobbs [4] reported that these spots were designate only to discharge remained sperm-packets after mating season.

The sperm-pack was surrounded by three spermatophore layers in *Pacifastacus leniusculus* [3], while Lopez-Greco [28] reported only two spermatophore layers, covering the sperm-pack in crayfish *Cherax quadricarinatus*. However, according to our results, *A. leptodactylus* belong to the second group.

The primary layer of spermatophore may help keeping spermatozoa alive [32], while the secondary layer of the spermatophore was present at the same time (on November and January); thus the second layer of spermatophore may has an important role for formation of spermatophore (as a capsule that is surrounded spermatozoa) and/or its another role could be de novo synthesize of a cellular material which forms spermatophre accompanied by Sperm-pockets.

Some histological differences, particularly about cellular types of various segments of vas deferens, was seen between the current study and previous study with *A. leptodactylus* [5], it could be due to genotype changes which may be occurred in different populations of the same species.

The fluctuations of gonadosomatic index (GSI) of immature males *A. leptodactylus* up to mating season was observed in decapod species [26-32-33]. In the present study, the gonadosomatic indices (GSI) of males fluctuated within a very small range during the annual reproductive cycle (0.50, 0.52, 1.21 and 1.12% from spring to winter, respectively), which confirmed the previous findings Yamaguchi [34] with *Uca lactea* and Lucic [26] with *A. astacus*.

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