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Histological Comparison of the Edible Water Frog (*Pelophylax ridibundus* Pallas, 1771) Gonads Before and After Reproduction Period

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ABSTRACT

In this study, testicular and ovarian structures of *Pelophylax ridibundus* (Pallas, 1771) were histologically examined before and after reproduction period in male and female individuals. Forty-eight (24 ♂♂, 24 ♀♀) adult frogs were collected from Gölbaşı Lake in Hatay. The average weight and length values of female frogs were found to be 56.61±19.59 g and 79.54±7.07 mm; while, the average weight and length values of male frogs were 36.63±12.84 g and 69.29±9.15 mm, respectively. Frogs were brought to the frog farm established in Aydıncık and placed in breeding ponds with a width of $1m^2$. Frogs in the ponds were brought to the laboratory of Iskenderun Technical University in different periods, before breeding (March) and after breeding (June). Then, histological samples were taken from the ovary and testis. The female frogs were determined ready for reproduction. Moreover, a large number of mature oocytes in the before breeding ovaries in vitellogenic stage, while after reproduction oocytes in primary structure and oocytes which have atresia status observed. Also, an increase in the thickness of the theca layer was determined. In the male frog seminiferous tubules containing a large number of spermatogonia, spermatocyte, spermatid, and a small number of spermatozoons including sperm bundles and leydig cells were found before reproduction. After the reproduction, the density of spermatogonia, spermatocyte, and spermatids were decreased; while, the density of spermatozoon and sperm bundle were increased in the seminiferous tubules. This study will contribute to the determination of mating and spawning in frog breeding by revealing the histological status of the gonad structure of *P. ridibundus* in the breeding process.

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Introduction

Examination of the histological status of the gonads in the reproduction period of economically important species provides an advantage in breeding studies. The changes occurring in the liver or gonads before and after the reproduction period and the differences caused by environmental effects can be followed by histological research (Gernhofer et al., 2001). Histological changes seen in the gonads have been considered as an important factor for understanding the life cycle of frogs and the developmental basis of reproductive cells (Erazo et al., 2016). In particular, it is of great importance to explain the levels of gonadal development and gonadal differentiation in economically important breeding frog species. Also, knowing the histophysiological structure of the species belonging to the *Ranidae* family, which is economically cultivated, makes an important contribution to the production rate (Arauco et al., 2007; Bambozzi et al., 2004).

There are few reports on the histology of testis and ovary of *Rana cyanophlyctis*, *Rana hexadactyla*, *Bufo melanostictus* (Saidapur, 1989), *Rana curtipes* (Gramapurohit, 2004), and *Rana cyanophlyctis* (Saidapur and Nadkarni, 1975; Pancharatna and Saidapur, 2009). Oocytes are classified into six stages based on their appearance, colors and size (Dumont, 1972). Following the formation of spermatogenesis in the *Ichthyophis tricolor* and *Uraeotyphlus cf.*, firstly primary and secondary spermatogonia, primary and secondary spermatocytes, round and long spermatids, and lastly, sperm were detected in the testicles (Smita et al., 2004).

Intense histological changes in the gonads are observed during the reproduction period between spring and autumn. Reproduction in frogs continues from the first week of April until the end of May depending on the temperature change (Samantha and Stephen, 2013). While protein, fat, and glycogen reserves during the hibernation of the frogs

are relatively enough for sustaining life, reproductive action must be carried out before the summer months to restore these essential nutrients for continuing levels in the reproduction process (Rugh, 1951). The seasonal gonad cycle of most amphibian species is evident and their breeding activities are highly sensitive to environmental changes. It is known that the hormones lead to, in amphibians, the production and growth of spermatic cells and ovarian follicles, environmental effects, and nutrition (Lofts 1974; Saidapur, 1989). *P. ridibundus* is one of the most common frog species in our country and it is exported to many countries in Europe in great amounts due to its economic value (Şereflişan and Alkaya, 2016). Moreover, any information regarding the reproduction of this species is important for our country.

The aim of study, indicate the histological changes in the gonads before and after frog reproduction period. Also, knowing the changes in the gonads during the reproduction period are thought to contribute significantly to the production in the frog breeding studies.

Material and Method

In this study, edible male and female frogs (*P. ridibundus*) which have economic importance was used. For this study forty-eight (24 ♂♂, 24 ♀♀) adult frogs were collected from Gölbaşı Lake in Hatay (36°30'16.4"N 36°29'39.0"E) in March 2015. During the frog collected process temperature 19°C and humidity was 40% measured. Frogs were brought to the frog farm established in Aydıncık (36°08'39.2"N 33°19'19.4"E) and placed in breeding ponds with a width of 1m^2. Frogs in the ponds were brought to the laboratory of Iskenderun Technical University in different periods, before breeding (March) and after breeding (June). The average weight and length of female frogs collected from nature were found to be 56.61±19.59 g and 79.54±7.07 mm; while, the average weight and length of male frogs were 36.63±12.84 g and 69.29±9.15 mm, respectively. Gonadosomatic index (GSI) was calculated for each frog March and June. GSI was based on the weight ratio of each gonad to the body weight. The average GSI value of female March and June 7.35±1.53; 1.27±1.02 that the average GSI value of male frogs March and June 0.29±0.04; 1.47±0.99 respectively.

The female and male frogs were kept in ice-containing containers for 30 minutes and then treated with 25 ml chloroform spilled cotton for the competition of anesthesia procedure in the laboratory. Then, the dissection procedure was carried out and removed gonads. The specimens were fixed in 10% formaldehyde. Samples were then dehydrated through a series of graded alcohols, cleared in xylene, infiltrated, and embedded into the paraffin. Gonad's paraffin wax blocks were cut into 4 µm thick sections, stained with hematoxylin and eosin, and they were examined under Olympus CX 41 microscope (Akiyoshi and Inoue, 2012; Seixas Filho et al., 2013). Images were captured Olympus DP 20 digital camera. Pictures of gonads were taken from the most homogeneous fields possible.

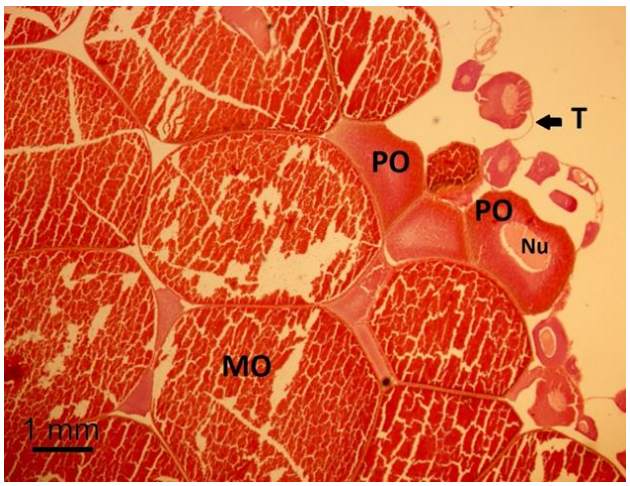

Figure 1. Female frog ovarium before breeding (MO: Mature Oocyte, PO: Primary Oocyte, Nu: Nucleus, T: Theca. H&E)

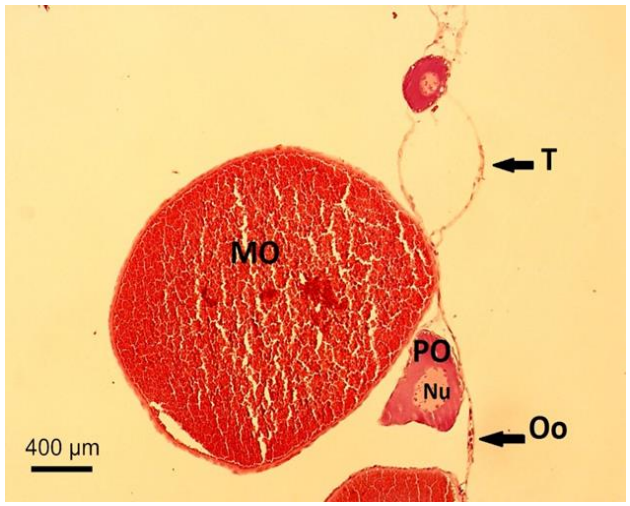

Figure 2. Female frog ovary, previtellogenic and vitellogenic oocytes in before breeding (MO: Mature Oocyte, Oo: Oogonia, PO: Primary Oocyte, Nu: Nucleus, T: Theca. H&E)

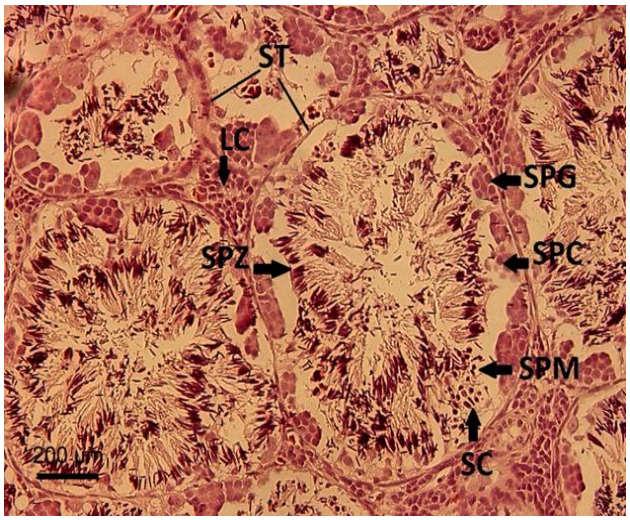

Figure 3. Male frog testis before breeding (LC: Leydig Cell, SC: Sertoli Cell, ST: Seminifer tubule, SPG: Spermatogonia, SPM: Spermatid, SPC: Spermatocyte, SPZ: Spermatozoon. H&E)

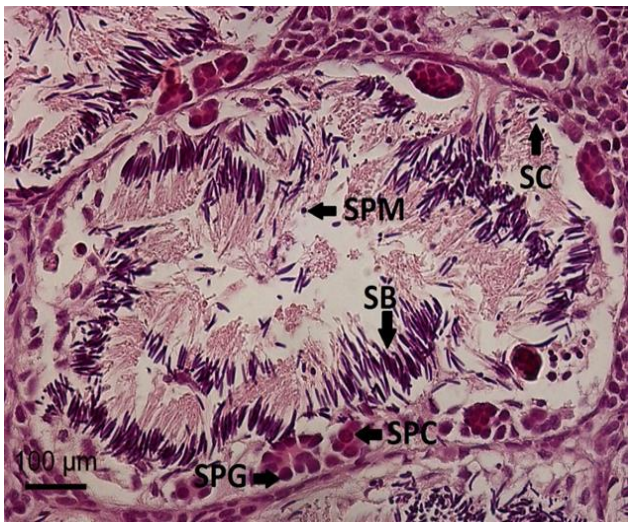

Figure 4. Male frog testis before breeding (SB: Sperm Bundle, SC: Sertoli Cell, SPG: Spermatogonia, SPM: Spermatid, SPC: Spermatocyte. H&E)

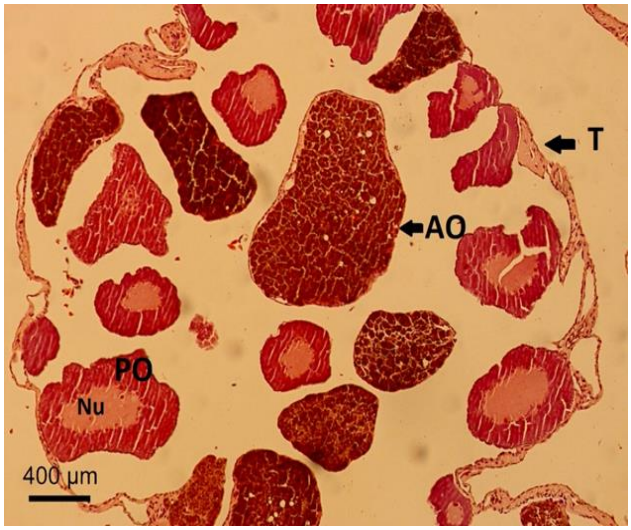

Figure 5. Female ovaries after breeding (AO: Atresia Oocyte, PO: Primary Oocyte, Nu: Nucleus, T: Theca. H&E)

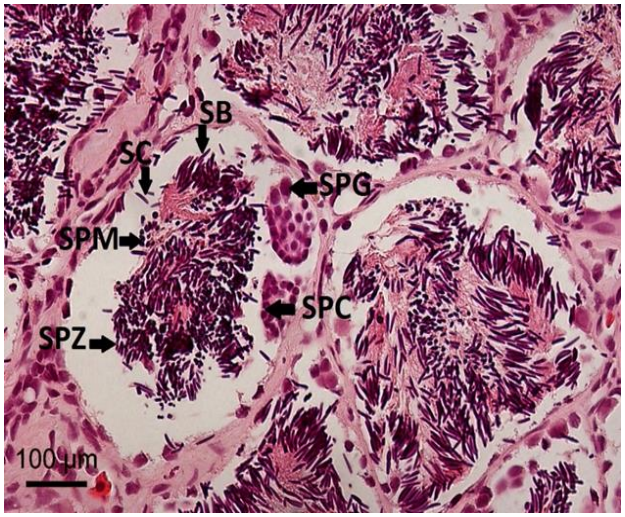

Figure 6. Male frog testis after reproduction (SB: Sperm Bundle, SC: Sertoli cell, SPG: Spermatogonia, SPM: Spermatid, SPC: Spermatocyte, SPZ: Spermatozoon. H&E)

Results

Histological Status of Frog Ovaries Before the Reproduction Period

When the samples of ovaries were taken before the reproduction period in female frogs, a large number of mature oocytes were detected in the ovaries in the vitellogenic stage. Therefore, it was determined that female frogs were ready for reproduction. In this period, it was observed that oocytes coexist in different stages of vitellogenic stage and primary structure belonging to the previtellogenic stage in ovaries (Figure 1). In addition, it was determined that oocyte production continues and the theca was found as a thin structure (Figure 2).

Histological Status of Frog Testis Before the Reproduction Period

When the testicles of male frogs collected from nature were examined at the beginning of the reproduction period (March); a large number of spermatogonia, spermatocyte, spermatid, and a small number of spermatozoons and sperm bundles were detected in the seminiferous tubules (Figure 3). In March, a large number of leydig cells were seen in the intertubular areas between the seminiferous tubules (Figure 4). Leydig cells are known to contribute to the continuation of reproduction by secreting testosterone hormone.

Histological Status of Frog Ovaries After the Reproduction Period

After spawning from the frogs in breeding ponds in April and May, the ovaries of female frogs were examined histologically. In June, the most common in the ovaries of female individuals were primary oocytes (Previtellogenic) and atresia oocytes (browning and degradation of oocytes) (Postvitellogenic). Also, the thickness of the theca was increased (Figure 5).

Histological Status of Frog Testis After the Reproduction Period

From the male frogs placed in breeding ponds, the histological status of the testicles was examined after reproduction (June). After the reproduction, it was found that the density of spermatogonia, spermatocyte, and spermatids were decreased in the seminiferous tubules of male frogs, but the density of spermatozoon and sperm bundle were increased (Figure 6).

Discussion

The development stages of the ovaries throughout the year and the eggs matured in six stages and that the mature oocytes were not observed in the November to February, but it was determined in the ovary samples taken from the March-October period (Sretarugsa et al., 2001). Annual reproductive cycle investigated with *Fejervarya limnocharis* that identified a large number of mature oocytes in female individuals in March, April, and May (reproduction period) (Othman et al., 2011). In this study, similarly with other studies, a large number of mature oocytes were detected before reproduction took place in the ovaries of female frogs grown in nature (March). Oogenesis was divided in two fundamental stages:

previtellogenic and vitellogenic. Oogonias are the elements that form the nests germinative cells and are located in the periphery of the ovary (de Oliveira and de Souza Santos, 2004). Germinative cells in female frogs are classified into five types: oogonias, previtellogenic oocyte, vitellogenesis step oocyte, vitellogenic and postvitellogenic oocytes (Iturriaga et al., 2012). In this study, oocytes belonging to different stages of previtellogenic (primary structure oocytes) and vitellogenic stage (mature oocytes) were detected in the ovaries of female frogs that were collected from nature and brought to the frog farm before reproduction took place.

One of the studies with *Rana leptoglossa* detected oogonia, primary oocytes, and mature oocytes before the breeding period in the female ovaries. In addition, primary and mature oocytes were detected in the reproduction period in ovaries; on the other hand, only oogonial cells were detected after the reproductive period (Saha and Gupta, 2011). In this study, before reproduction takes place in the ovaries of female frogs grown in nature; oogonia, primary oocyte, and mature oocytes are detected; while, oogonia, primary oocytes, and atresia oocytes are detected after reproduction. The appearance of stage I and II or previtellogenic oocytes throughout the year as in other anurans (Sklavounou and Loumbourdis,1990). Oocytes in the developmental stage I. and III. (Primer) are detected every term during the year in *Rana tigrina* ovaries (Hoque and Saidapur, 1994). Similarly, primary oocytes were observed in the ovaries of female frogs, which were collected from nature and brought to the frog farm, both before and after reproduction. On the other hand, these findings that there is always a reserved pool of oocytes.

It has been reported that after spawning in *R. tigrina*, oocytes were detected in the postvitellogenic atresia status (Hoque and Saidapur, 1994; Pancharatna and Saidapur, 1985). In another study, reported that the structure of some oocytes in the ovaries was disrupted and transformed into atresia oocytes during the reproduction period in amphibians (Masood-Parveez, 1987). Moreover, the melanocyte pigments in gonadal structures participated in gonadal regression observed after the maturation of germinative cells (Besseau and Faliex 1994; Grier and Taylor, 1998). That atresia oocytes may be seen in *R. tigrina* at any time of the year, but their number was increased with the decrease of mature oocytes in the VI stages after the most reproduction period (Sretarugsa et al., 2001). A study of female *Pelophylax bedriagae*, decreased the number of mature oocytes in the ovary after spawning (Akef, 2012). However, in this study after the reproduction period (June), mature oocytes were not observed in the ovaries of female frogs. Also, previtellogenic oocytes containing atresia status were increased, and the thickness of the theca was increased.

During a year, it has been reported that *R. cyanophlyctis* is observed in the atresia structure of follicles in its ovaries and their number is affected especially hormones (gonadotropins), by the stress of growing undernutrition and culture (Pancharatna and Saidapur 1992; Saidapur 1989). During the present study, it is thought that oocytes in the atresia structure seen in ovaries are had reproductive and hormonal origins (Hoque and Saidapur 1994; Sretarugsa et al., 2001). Reported that female frogs completed vitellogenesis in a summer study with *Rana palustris* (Resetarits and Aldridge, 1987). The number of mature oocytes in *P. bedriagae's* ovaries decreased after spawning period and that vitellogenesis was active throughout the year and was seen many times outside the reproductive and reproductive period (Akef, 2012). In this study, it can be said that developing oocytes can continue to vitellogenesis since it is known that female frogs with primary oocytes that have not completed their development in the ovaries after reproduction can produce eggs several times during the same reproduction period.

In the seminiferous tubules of male frogs both before reproduction (March) and after reproduction (June); spermatogonia, spermatocyte, spermatid, spermatozoon, and sperm bundle were detected. Similar spermatic structures were also identified as a result of the study conducted in *Rana catesbeiana* testicles (Sretarugsa et al., 2000). Moreover, seminiferous tubules of *P. ridibundus* has the structural pattern found in most anurans (Duellman and Trueb, 1994; Kardong, 2002). In comparison to *P. ridibudus* observed in the present study, in a study carried out by Kalt (1976) testes of male *X. laevis* and found 11 stages of germ cells. In a study carried out by Smita et al., (2004) spermatogenesis was completed after seven stages. Also, primary and secondary spermatogonia, primary and secondary spermatocytes, round and long spermatids, and lastly spermatozoon were observed in the testes following the formation of spermatogenesis in *I. tricolor* and *U. narayani* species. Examined the structural features of *E. planirostris* gonads histologically during spermatogenesis of germinal cells and divided spermatogenesis of germinal cells by six types: spermatogonia, spermatocyte I, spermatocyte II, early and late spermatids, and spermatozoon (Iturriaga et al., 2012).

Although in the seminiferous tubule's spermatozoon, spermatocyte and spermatids are observed, it contains mostly spermatogonia in January-February (before the reproduction period). Further, the development of gonad between March and June (reproduction period), secondary sexual characters and that most of the spermatogonia are transformed into spermatocytes. Which are detected secondary spermatogonia have been reported in the lumen of the testicular epithelium (Raucci and Di Fiore, 2007). In this study, it was found that the seminiferous tubules of male frogs collected from nature contain a large number of spermatogonia, spermatocyte, spermatid, and a small number of spermatozoon and sperm bundle before reproduction. It was determined that the number of spermatogonia, spermatocytes, and spermatids were decreased in the seminiferous tubules of male frogs, but the number of spermatozoon and sperm bundle were increased after the reproduction. Moreover, a study with *Rana ridibunda* that this situation was effective in the frog's continuous reproductive potential (Kaptan and Murathanoğlu, 2008).

Studies with *R. ridibunda* and *R. leptoglossa*, it has been reported that the number of spermatids increased in the testicles of male frogs during the reproduction period (April-June), and spermatids decreased after reproduction (Saha and Gupta, 2011; Sklavounou and Loumbourdis, 1990). However, in this study, it was found that the density of spermatids before reproduction was higher in the seminiferous tubules of male frogs collected compared to post-reproduction. Reported that male individuals

increased their sperm count in March, April, and May (reproductive period) in their study with *F. Limnocharis* (Othman et al., 2011). In this study, it was determined that the density of spermatozoon and sperm bundle increased in June in the frogs. The reproductive cycle will be related to prevailing climatic conditions in habitats, and even slight annual changes in environmental conditions can disrupt reproductive cycles (Sretarugsa et al., 2000). Even in the same species, it has been stated that histological differences may occur in testicles and ovaries in the reproductive period (Rastogi et al., 1978). However, in this study, the present findings suggest that any histological differences between frogs result from climatic conditions were not detected.

Conclusion

As a result of histological observations in testicles and ovaries before and after reproduction; It has been determined that female and male individuals of this species (*P. ridibundus*) have gonadial cells necessary for reproduction and ready for reproduction from March. Moreover, after the reproduction atresia oocytes were detected with the start of gonadial regression in females, while sperm germ cells decreased in males and sperm remained in bundles. Therefore, this study will contribute to the breeding process by revealing the histological status of the species gonads in the reproduction process of frogs.

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