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Histological analysis of ovary development in narrow-clawed crayfish *Astacus leptodactylus* Esch. 1823 (Crustaceae, Decapoda, Astacidae), reared in ponds in South Bulgaria

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Abstract. The study was carried out for determination the age and body weight in which the females of freshwater narrow-clawed crayfish *Astacus leptodactylus* Esch. reached maturity, as well as of the duration of the different stages of the annual reproduction cycles of the matured individuals in the conditions of carp ponds. The study was carried out on juvenile and matured female crayfish. As a result of the study it was found that female narrow-clawed crayfish reaches sexual maturity during the first year of its life (6-7 months old), at body weight above 9.5 g and body length above 7 cm. The annual ovarian cycle of sexually mature females in the region of South Bulgaria, is following: January-May - eggs incubation, resorption of unovulated oocytes in the ovary, presence of previtellogenic and early vitellogenic oocytes, endogenic vitellogenesis; June-November - exogenic vitellogenesis, active accumulation of vitellogenin into the oocytes from August till November; November-December - copulation, ovulation, fertilization of the eggs.
Key Words: crayfish, *Astacus leptodactylus,* ovarian development**.**

Zusammenfassung. Das Ziel der Untersuchung war es, das Alter und die Koerpergroesse zu bestimmen, bei dennen der Sumpfkrebs *Astacus leptodactylus*, gezuechtet in Karpfenteichen geschlechtsreif wird. Die Dauer der einzelnen Perioden der Ovariumentwicklung waehrend des Jahresreproduktioncyclus bei geschlechtsreifen Krebsen wurde auch bestiummt. Es wurde nachgewiesen, dass der Sumpfkrebs waehrend des ersten Jahres seines Lebens (6-7 Monate alt) geschlechtsreif wird, und das bei einer Koerpermasse von ueber 9.5 g und Koerperlaenge von ueber 7 cm. Das jaehrliche Reproduktionscyclus bei den Weibchen unter den Bedingungen der Karpfenteichzuechtung in Suedbulgarien koennte folgenderweise verteilt werden: Januar-May – Eierinkubation, Resorption von nichtovulierten Eiern, Anwesenheit von previtellogenen Oozyten, endogene Vitellognese; Juny-November – exogene Vitellogenese, aktive Akummulation von Vitellogenin in den Oozyten vom August bis November; November – Dezember – Kopulation, Ovulation, Eierbefruchtung.
Schluesselwoerter: Sumpfkrebs, *Astacus leptodactylus,* Ovariumentwicklung.

Rezumat. Prezentul studiu s-a demarat în vederea determinării vârstei și greutății corporale la care femelele de rac din specia *Astacus leptodactylus* Esch. ating maturitatea, precum și pentru aflarea duratei diferitelor stadii ale ciclului anual de reproducere la indivizii maturi în condiții de creștere de natura bazinelor de crap. Cercetările s-au efectuat pe indivizi juvenili și femele mature de rac. Ca rezultate, s-a constatat că femelele acestei specii ating maturitatea sexuală în primul lor an de viață (la vârsta de 6-7 luni), la o geutate corporală de sub 9,5 g și la o lungime de sub 7 cm. Ciclul anual ovarian al femelelor mature din sudul Bulgariei este: ianuarie-mai – incubarea ouălor, resorbția oocitelor neovulate la nivelul ovarelor, prezența oocitelor previtelogenetice și vitelogenetice timpurii, vitelogeneza endogenă; iunie-noembrie – vitelogeneza exogenă, acumularea activa de vitelogenină în oocite (din august până în noembrie); noembrie-decembrie – copulația, ovulația și fertilizarea ouălor.
Cuvinte cheie: raci, *Astacus leptodactylus*, dezvoltare ovariană.

Introduction. The narrow-clawed crayfish *Astacus leptodactylus* is one of the three fresh water species of the genus *Decapoda*, which inhibit the inland water bodies in Bulgaria.

Data concerning the annual changes in the gonad development have been established for almost all species – the local European crayfish species from cultivated

and natural populations (*Astacus astacus:* Lahti & Lindqwist 1981; Ackefors 1999; Lucic et al 2006; *Astacus leptodactylus*: Stucki 1999), as well as for the introduced species (*Procambarus clarkii:* Castanon-Cervantes et al 1995; Gutierrez-Yurrita & Montes del Olmo 1999).

The annual cycle of ovary development in the various freshwater crayfish can be determined from the characteristic features of the species, as well as from the concrete conditions of inhabitance.

As far as our latitude is concerned, the sexual maturity in narrow-clawed crayfish in natural water bodies can be observed after the third year (Nechaev 1935). Zaikov (1999, 2006) indicates that depending on their inhabitance this might happen earlier – during the second or third year. The different authors show a various minimum size for reaching sexual maturity in female crayfish from Central and Eastern Europe: 5-6 to 8.5 cm (Holdich & Lowery 1988; Alekhnovich & Kulesh 1996; Alekhnovich et al 1999; Zaikov 1999; Souty-Grosset 2006).

To be familiar with the reproduction characteristics of given species is the main and obligatory prerequisite for its controlled reproduction, as well, with the purpose of its further cultivation.

The rearing of the narrow-clawed crayfish in polyculture in carp ponds determine a somewhat different temperature regime and trophyc conditions than in other natural water bodies like rivers and lakes, and these two factors are the main ones for reaching sexual maturity in juvenile crayfish and set the limits of the different stages in the annual reproduction cycle of sexually mature specimen.

The great economical significance of the narrow-clawed crayfish as an object in the aquaculture, as well as the lack of data about the oogenesis of this species when reared at the specific conditions of carp ponds has imposed to carry out this investigation.

The aim of this investigation is to determine the age and body weight, at which the female crayfish reach sexual maturity, as well as the duration of the separate stages of the annual reproduction cycle of sexually mature specimen, at conditions of polyculture in carp ponds.

Material and Method. The investigation has been done upon female juvenile and sexually mature narrow-clawed crayfish *Astacus leptodactylus* Esch. reared all the year round in carp ponds.

In order to determine the minimum size, at which female reach sexual maturity, 52 one-year old specimen have been investigated during the period December-April. The crayfish have copulated at natural conditions in a carp pond during the autumn-winter period and have been caught at the end of April. As a marker for reaching sexual maturity, the presence of pleopodal eggs along the internal part of the abdomen has been used.

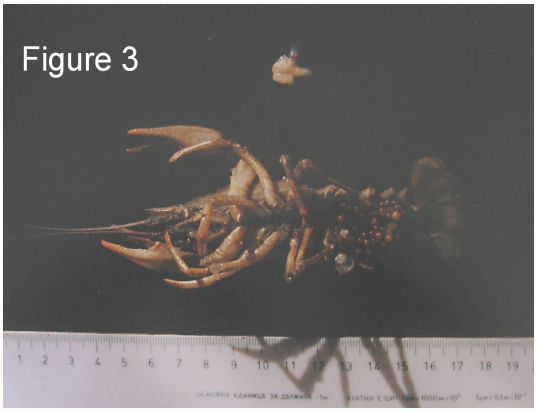
In order to trace ovary development in an age and annual aspect, GSI,% (gonad somatic index) has been calculated and histological sections have been prepared. After body dissection, ovaries at a various age have been isolated: 212 from one-summer old crayfish, 20 from one-year old crayfish, 20 from two-summer old crayfish and 32 from sexually mature specimen from the brood stock.

Ovarian samples from the juveniles have been taken during the spring and autumn, when the crayfish come of the respective age, and from the sexually mature specimen – every month.

The fixed material (4% formalin) has been imbedded in paraffin and processed by using the methods of the classical histological technique. After removing the fixing agent by running water, the tissues have been dehydrated in an increasing alcohol series: 50%, 70%, 80%, 96% and 100% of ethanol. The clarification of the preparations has been done in cedar oil, after which they have been imbedded in paraffin (with melting point of 52-54^{o}C). The sections of 5-6 μm thickness have been deparaffinated in xylol and hydrated in a decreasing alcohol series of 100^{o}, 96^{o}, 80^{o}, 70^{o} and 50^{o} of ethanol and running water. Samples were stained with hematoxylin-eosine (HE).

The histological sections have been evaluated by using a light microscope Nikon MICROPHOT-SA, equipped with a microphotocamera Nikon FX-35DX.

Results and Discussion. *Morphology of the Ovary.* The ovary of the narrow-clawed crayfish is Y-shaped and consists of paired anterior ovarian sacks and of an unpaired medial posterior sack (Figure 1). It is located in the cephallothorax dorsally to the stomach (see Figure 2).



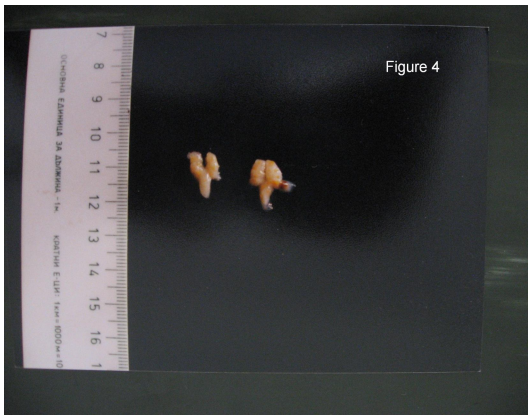


Figure 1. Ovary of narrow-clawed crayfish before ovulation, November. Figure 2. Location of the ovary in the body of female crayfish, November. Figure 3. Narrow-clawed crayfish with ovulated eggs and ovary after ovulation, February. Figure 4. Ovary after ovulation. Oviduct with non-ovulated oocyte, February.

The ovary of the various species of crayfish during the different stages of vitellogenesis consist mature oocytes with different size and color, which is their species-specific feature (Adiyodi 1985; Ando & Makioka 1998; Ackefors 1999; Gutierrez-Yurrita & Montes del Olmo 1999).

Concerning the narrow clawed crayfish females with white eggs in the ovary can be observed all the year round. Since June, the eggs start maturing and initially they have a yellow color, and in August – September they change their color to orange or orange-brownish and enlarge their size, reaching definitive sizes (see Table 1).

Immediately before ovulation the ovary contains a few hundreds of eggs, which are orange-brownish in color and have reached their maximum sizes. Its maximum length, at this moment, is 5.2 cm and its weight 3.97 g (average value of 1.96 ± 0.56 g, n = 88).

Table 1

Some reproductive characteristics in mature female crayfish, *Astacus leptodactylus* Esch., reared in carp ponds

Month	*I*	*II*	*III*	*IV*	*V*	*VI*	*VII*	*VIII*	*IX*	*X*	*XI*	*XII*
Eggs colors in the ovary												
White	√	√	√	√	√	√	√	√	√	√	√	√
Yellow									√			
Orange-Brownish										√	√	
Pleopodal eggs												√
Copulation											√	
Hatching of juveniles					√							
Average water temperature, °C	4	2.7	7	12	18	23	23.5	23	20	18	5	1.5

The copulation in the narrow clawed crayfish can be observed at the end of November and the beginning of December. The ovulation of the mature eggs occurred immediately after it or within the interval of 1 month after spermatophore disposition.

After the ovulation, the ovary contains only small previtalogenic oocytes and single non-ovulated oocytes (Figures 3 and 4).

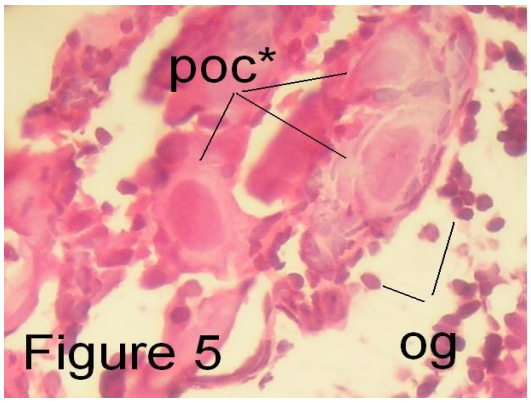
Minimum sizes of the individuals at first maturation. The body weight of the one-summer old (6-7 months) females investigated varies from 9.47 to 19.44 g, the average value being 13.66 g, and its body length is from 7 to 8.9 cm, or the average value being 7.94 cm. The carapax length varies from 3.4 to 4.3 cm and its width from 1.7 to 2.2 cm.

The presence of pleopodal eggs during November and December has been established in all specimen investigated. The average working fecundity is 38.77 pleopodal eggs, varying from 2 to 86 in the different individuals.

As a result of the investigation, it has been established that female narrow-clawed crayfish, at conditions of carp pond rearing can reach sexual maturity during the first year of their life, at body weight above 9.47 g and body length above 7.0 cm.

Annual cycle of the ovary development in immature crayfish in age aspect up to reaching sexual maturity. Concerning the one-summer old crayfish (August-September), having a body weight of 5-11 g in August, GSI,% is 0.47, oogonia have been established in the ovary on a mass scale, as well as single previtellogenic oocytes (Figure 5).

The primary oocytes have approximately one and the same size, their nucleus is oval, centrally positioned and fills up almost the whole ooplasm (Figure 5). The oogonia are positioned in nests (Figure 6) among the primary oocytes.



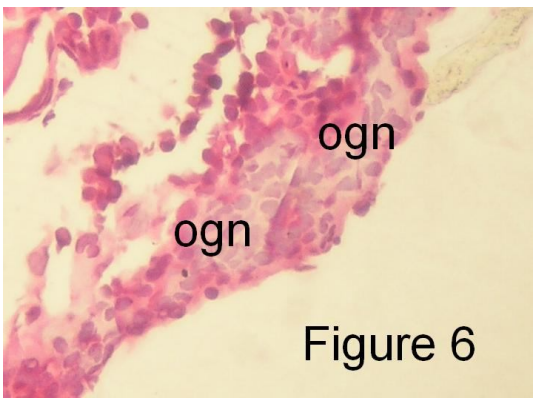
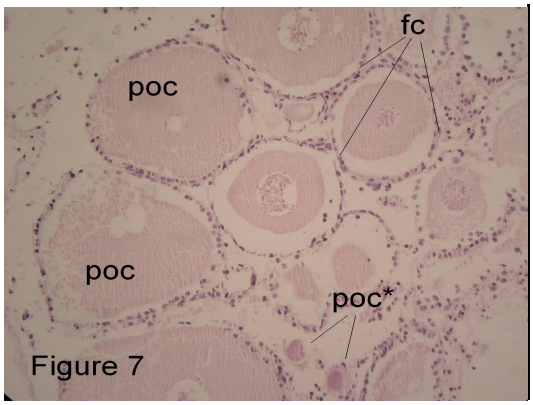


Figure 5. Part of ovary of one-summer old crayfish, August, early previtellogenic oocytes (poc*) and oogonia (og), HE, Magnification x 400. Figure 6. Part of ovary of one-summer old crayfish, September, nests of oogonia (ogn), HE, Magnification x 400.

Differentiation of oocytes as regards their size has been established on the histological sections in September (BW (body weight) =7-12 g). Oocytes with smaller and bigger sizes have been observed (Figure 7). Their ooplasm is vacuolisated by one or more layers of vesicles (vacuoles) located at the periphery of the oocyte. Similar structures have been described in ovary of juveniles by Ando & Makioka (1988). In January (BW = 8.34 g) GSI,% has values, similar to those of one-summer old crayfish in September-0.46 %. For this period, differentiated distribution of previtellogenic oocytes in the ovary has been established: the bigger ones positioned in its periphery, and the smaller ones in the internal part of the ovary (see Figure 8).



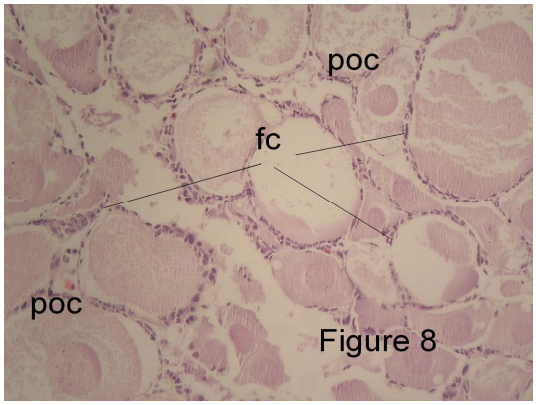


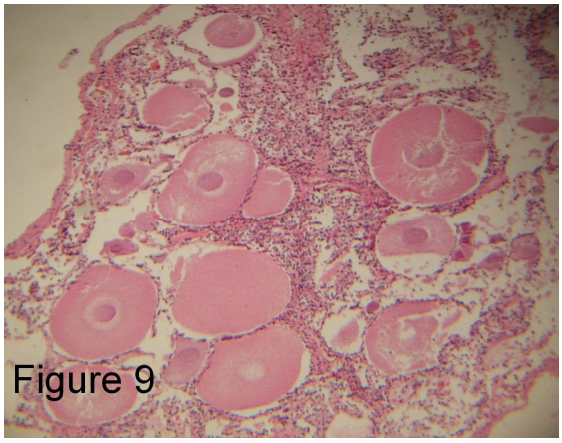
Figure 7. Part of ovary of one-summer old crayfish, September, previtellogenic oocytes (poc), poc* - early previtellogenic oocytes, HE, Magnification x 200. Figure 8. Part of ovary of one-summer old crayfish, January, different location of previtellogenic oocytes (poc), fc - primary follicle cells layer, Magnification x 200., HE – Hematoxylin - Eosine.

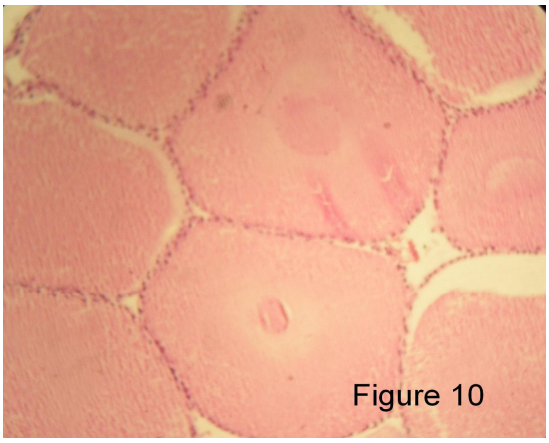
The oocytes have a well-shaped primary follicle cells layer. The ovary wall in the one-summer old crayfish is narrower, built of one layer of cells (Figure 9), and no muscle layer is formed, in contrast to the ovary in *Procambarus clarkii*, where besides a multilayer epithelium wall the ovary possesses a muscular one, as well (Ando & Makioka 1998).

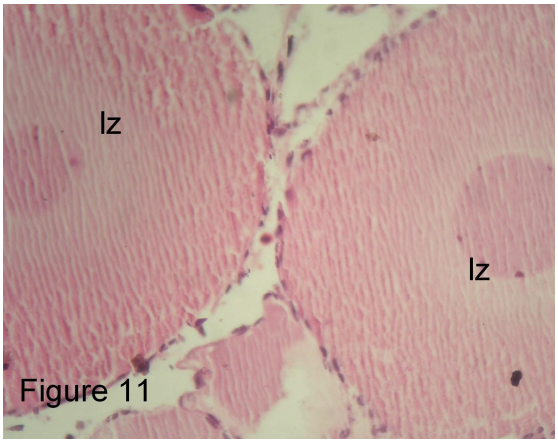
In one-year old crayfish (BW=12-16 g), from March till August, the changes in the ovary concern mainly oocytes preparation for vitellogenin accumulation in the ooplasm, as a result of which they start enlarging their size (Figure 10). A characteristic feature for the oocytes at this stage of development is the differentiation of a lighter zone around the nucleus located centrally in the ooplasm, which has been also observed in *Orconectes limosus* (Juchno & Chybowski 2003) (Figure 11).

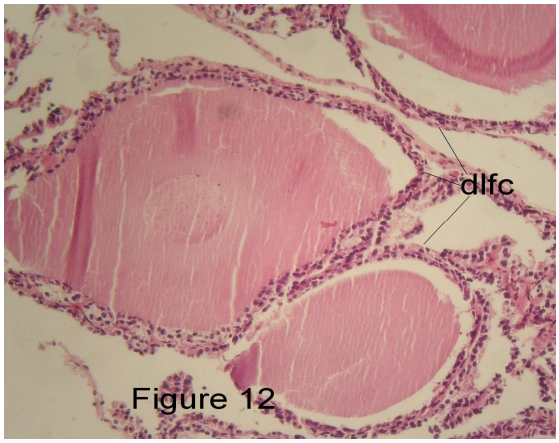
In two-summer old crayfish (BW=24.86 g), in September, vitellogenic oocytes with already formed secondary follicle cell layer around them are the mass fraction in the ovary (Figure 12). GSI,% has reached values of 3.37 %.

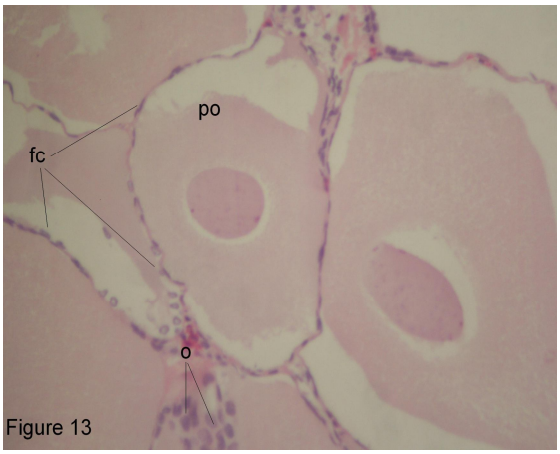
A characteristic feature for all one-summer old females is that some individuals, like the male crayfish (Hubenova et al 2004), after reaching a body weight of approximately and above 10 g in September, have reached maturity during the first year of their life yet, by forming vitellogenic oocytes, which after copulation with the male crayfish during the autumn-winter period have ovulated. This is in accordance with the already established regularity in one-summer old male crayfish, as well, which after reaching a body weight above 10 g (in some cases above 6 g, too) are mature and can participate in the reproduction process (Hubenova et al 2004). The females with body weight of 10 g were about 32% from all investigated individuals and the number of the isolated pleopodal eggs in these crayfish was 4-7 times lower than that in older mature individuals.











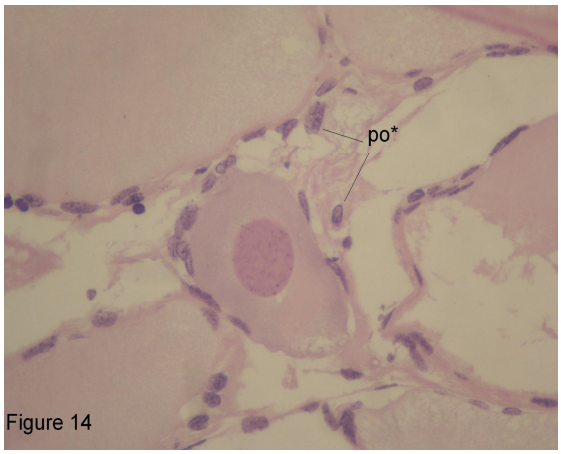


Figure 9. Part of ovary of one-summer old crayfish, January, ovary wall, HE, Magnification x 200. Figure 10. Part of ovary of one-summer old crayfish, May, endogenic vitellogenesis, HE, Magnification x 200. Figure 11. Part of ovary of two-summer old crayfish, August, endogenic vitellogenesis, light zone around the nucleus (lz), HE, Magnification x 200. Figure 12. Part of ovary of two-summer old crayfish, September, vitellogenic oocytes (vo) with secondary follicle cells layer (dlfc), HE, Magnification x 200. Figure 13. Part of ovary of mature crayfish, February, previtellogenic oocytes (po) with primary follicle cells layer (fc), HE, Magnification x 200. Figure 14. Part of ovary of mature crayfish, February, early previtellogenic oocytes (po*), HE, Magnification x 200.

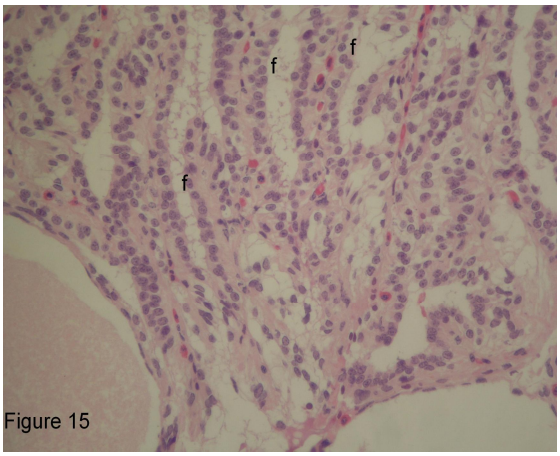


Figure 15. Part of ovary of mature crayfish, February, empty follicles (f), HE, Magnification x 200.
Figure 16. Part of ovary of mature crayfish, March, different location of previtellogenic oocytes (po), HE, Magnification x 200.

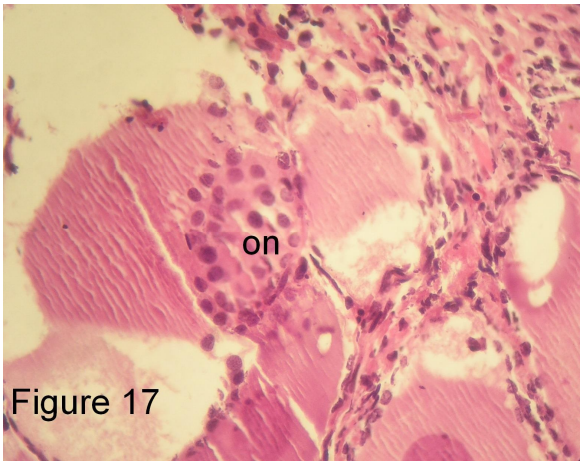
The earlier maturity of the narrow clawed crayfish, reared in ponds, according to our opinion, has been the result of the rearing conditions effect - mainly, the abundance of food in the ponds and the higher water temperature in them, which has a considerable influence upon the development of their reproductive system.

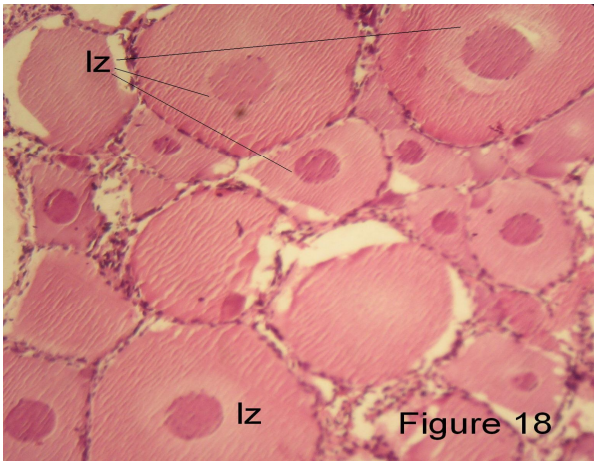
Annual cycle of ovary development in sexually mature crayfish. Concerning South Bulgaria, the copulation in *A. leptodactylus* is carried out from the middle of November till the beginning of December, depending on water temperature (Hubenova et al 2002) and is in accordance with the investigations of (Cukersis 1989; Daniels et al 1994) etc for the narrow-clawed crayfish.

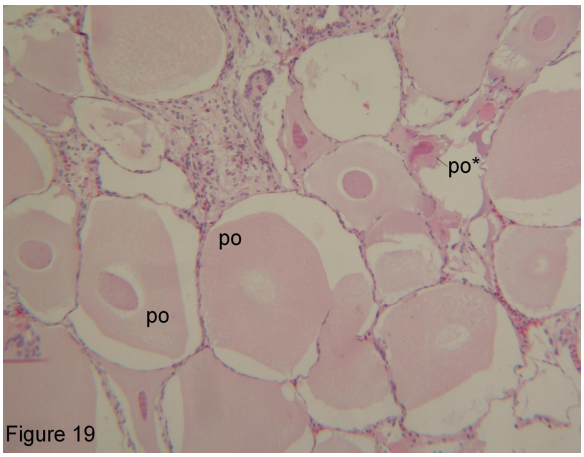
Immediately after copulation, within some days up to two-three weeks, the ovulation and the fertilization of the ovulated eggs has begun.

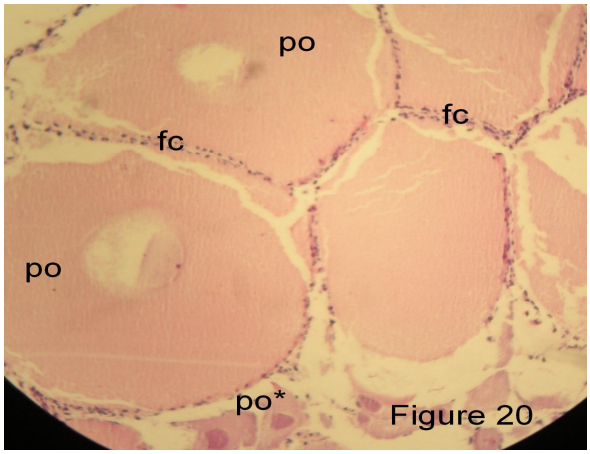
After the ovulation (November-December) till February, the relative weight of the ovary expressed by means of the GSI,% is the lowest one (0.73%).

The following histological picture can be observed in the ovary: previtellogenic oocytes represent the main fraction, surrounded by one layer follicle cells with elongated shape (Figure 13). The nucleus of the oocytes is big, oval, with many nucleoli located in the periphery. The oocytes have approximately one and the same size. Oogonia nests located centrally in the ovary, close to the early previtellogenic oocytes, (Figure 14) are presented at that time in the ovary too. Some non-ovulated oocytes and empty follicles, both in process of atresia and desorption are a characteristic structure in the ovary during February (Figure 15). In March, similar structures to those of the previous month have been found in the ovary (Figure 16) and the GSI,% for that period is 0.94. The most characteristic feature in the ovary is the enlarging of the volume of prevetellogenic oocytes. Oocytes with various sizes can be observed: the bigger ones occupy the periphery of the ovary, and the smaller ones – its internal parts. Oogonia proliferation continues (Figure 17). No atretic follicules have been found and the number of the empty follicles has decreased. In April, the histological picture has not undergone great changes. The differentiation in previtellogenic oocytes location, depending on their sizes has been preserved. The nucleus of the oocytes has been positioned centrally and the number of the nucleoli has not changed considerably (Figure 18). A characteristic feature for this period is the formation of a lighter zone in the ooplasm, around the nucleus, where according to many authors (Adyodi & Subramonian 1983; Papatanasiou & King 1984; Juchno & Chybowski 2003) cellular organells have been concentrated. This process is considered as the beginning of the endogenic vitellogenesis, characterized by structures synthesis, which will prepare the oocytes for the following exogenic yolk accumulation into the oocytes. No atretic follicules and empty follicles have been found. The GSI,% for this period is 0.75.









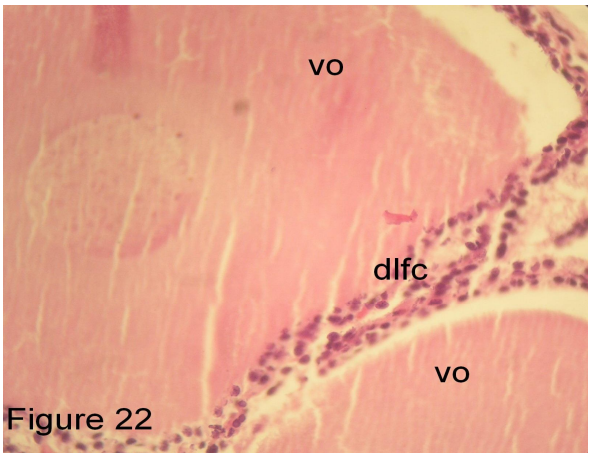


Figure 17. Part of ovary of mature crayfish, March, oogonia proliferation (on), HE, Magnification x 200. Figure 18. Part of ovary of mature crayfish, April, endogenic vitellogenesis, light zone around the nucleus (lz), HE, Magnification x 200. Figure 19 and 20. Part of ovary of mature crayfish, May, previtellogenic oocytes (po) with primary follicle cells layer (fc), early previtellogenic oocytes (po*), HE, Magnification x 200. Figure 21. Part of ovary of mature crayfish, August, Exogenous vitellogenesis, vitellogenic oocytes (vo) with primary layer of sphericle follicle cells, HE, Magnification x 200. Figure 22. Part of ovary of mature crayfish, September, vitellogenic oocytes (vo) with secondary follicle cells layer, HE, Magnification x 200.

In May, the maturing oocytes have occupied the ovary periphery, and the previtellogenic oocytes and oogonia, which represent the new wave of cells, which will be differentiated during the next reproduction period have been located its interior (Figure 19)**.** The GSI,% is 0.88. The endogenic vitellogenesis still goes on (a light zone around the nucleus of the oocytes has been established). The follicle cells around the oocytes have been located more closely to each other and they have gradually changed their shape from elongated (in February-April) to a spherical one (Figure 20)**.** The elongated shape is characteristic

for the stage, in which the follicle cells "overgrow" around the oocyte. Farther in time, when they begin to fulfill their typical function, namely, to contact with the blood vessels and to transfer the vitellogenin into the ovocyte, their shape will be changed into a spherical one. That is how, we can draw the conclusion that the exogenic vitelogenesis starts in May and the primary vitellogenic oocytes are formed. Together with this process, the process of endogenic vitellogenesis still goes on, because the light zone around the nucleus of the oocyte can be observed during this period, as well. In June-July, follicle cells change of shape to spherical and the endogenic synthesis of vitellogenin still go on. The GSI,% is 0.85. The follicle cells layer around the oociytes becomes thicker and in August the follicle cells have turned completely into spherical ones, and the light zone around the nucleus has begun to decrease (Figure 21). An active accumulation of vitellogenin has been carried out in the maturing oocytes. The GSI,% is 0.92.

The presence of a two-layer follicle cells has determined the differentiation of the primary vitellogenic oocytes into secondary ones (Figure 22). The oocytes gradually enlarge their size, their color changes towards yellow-orange and they are positioned very closely to each other.

Ovary maturation starts in August and the beginning of September. The active vitellogenin accumulation in narrow-clawed crayfish, at the climatic conditions of Bulgaria continues up to the end of October and the beginning of November. Evidence for this is the increase of GSI,% values from 2.88 in September to 5.29 in November.

During the ovulation the oocytes leave the ovary through the oviducts positioned at the base of the anterior ovarian sacks, symmetrically on its both sides. The oviducts are covered with a thin muscle membrane, which during muscular filaments shortening helps for oocytes transportation.

The changes in the cellular structure of the ovary of the narrow-clawed crayfish can be divided in two clearly expressed stages: the first one can be seen during the greater part of the year (from January till August), when previtellogenic or early vitellogenic oocytes can be found in the ovary, and the second one is characteristic for the period August – November, when vitellogenin accumulation in the oocyte can be observed.

Conclusions. From the results obtained, the following conclusions can be made: The female narrow-clawed crayfish reaches sexual maturity during the first year of its life (6-7 months old), at body weight above 9.5 g and body length above 7 cm. The annual ovarian cycle of sexually mature females in the region of South Bulgaria, is following: January-May - eggs incubation, resorption of unovulated oocytes in the ovary, presence of previtellogenic and early vitellogenic oocytes, endogenic vitellogenesis; June-November - exogenic vitellogenesis, active accumulation of vitellogenin into the oocytes from August till November; November-December - copulation, ovulation, fertilization of the eggs.

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