

Gonadogenesis in Chub *Squalius (Leuciscus) cephalus* (L. 1758)

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The chub is an opportunistic, rheophilic cyprinid species and popular gamefish distributed throughout almost all of Europe, South Caucasus and part of Anatolia. It is most abundant in small rivers and large streams. The process of anatomical and cytological differentiation was analyzed in chub gonads. Material used in the study was derived from artificial spawning of adult individuals caught in the Lyna River in April 2002. The results showed that gonadal sex differentiation develops faster in females than males. Morphological differentiation of gonads was noted about 98 days after hatching when fish weight varied between 0.483 g and 1.014 g and body length varied between 3.7 to 4.8 cm. Developing ovaries were spindle-shaped and male gonads were pear-shaped. Final cytological differentiation took place 154 days after hatching when single previtellogenic oocytes were observed in ovaries, contrary to males in which testes filled with spermatogonia not before the 190th day post hatching.

Key words: *Squalius cephalus*, gonadogenesis, PGCs, oogonia, spermatogonia.

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The chub, *Squalius cephalus* (L.), is a rheophilic cyprinid fish, preferring mostly lotic habitats (ARLINGHAUS & WOLTER 2003). This ubiquitous species inhabits various ecological/environmental niches throughout almost all of Europe, South Caucasus and parts of Asia Minor. It is naturally absent from Italy and the Adriatic basin. It is the most abundant eurythermic fish among all cyprinids, resistant to thermal pollution and occurring both in cold mountain streams and warm, slow-flowing, lowland rivers (DURAND *et al.* 1999; KOTTELAT & FREYHOF 2007). In Turkish reservoirs, chub is mainly caught for consumption, and thus has an economic value. It is also popular as a gamefish (KOÇ *et al.* 2007). Males reproduce for the first time at two – four years, females at four – six years (KOTTELAT & FREYHOF 2007). Sexual maturation is influenced by environmental conditions and geographical location and some individuals may mature much later (MANN 1976) or much earlier (SASI 2004).

Gonads are composed of somatic and germinal elements. In fishes they form irrespective of the urinary system. Ovaries and testes arise from the same group of cells, corresponding to the corium of the peritoneal wall, from which the germinal layer is formed. The timing of gonadal development and

the anatomy of the gonads in fishes vary greatly. In some fish species sex differentiation occurs already during embryonic development or shortly after hatching – as in yellow perch *Perca flavescens* (Mitchill) (MALISON *et al.* 1986). In other cases the process usually takes a few weeks or months after hatching, as in common carp *Cyprinus carpio* L. (DAVIES & TAKASHIMA 1980). There are also species with differentiation occurring after a few years from hatching, e.g. the great sturgeon *Huso huso* (L.) or European eel *Anguilla anguilla* (L.) (COLOMBO & GRANDI 1996; YOUSEFIAN 2006). Sex determination consists of anatomical differentiation processes, i.e. the forming of macroscopic structures of testes/ovaries and cytological differentiation, and the development of the cells of the male or female sex line (DEVLIN & NAGAHAMA 2002). Gonadogenesis occurs in various periods depending on sex. Gonadal differentiation resulting in females often occurs earlier than male differentiation (HUNTER & DONALDSON 1983).

It is known that several biotic factors such as temperature and photoperiod (PATIÑO *et al.* 1996; MYLONAS *et al.* 2003), as well as abiotic factors – mainly steroid/hormonal substances, influence morphological sex formation (MALISON *et al.* 1986; DEVLIN & NAGAHAMA 2002). Skillful applica-

tion of hormonal treatment just before or during sex differentiation allows the formation of monosex populations of males or females (BEARDMORE *et al.* 2001; PIFERRER 2001).

The sex differentiation process has been described for only a few fish species, particularly those of great economic importance (COLOMBO & GRANDI 1996; KATO *et al.* 2003). Not much attention has been paid to representatives of the genus *Leuciscus*, characterized by a silvery, elongate body, a concave posterior margin of the anal fin, a complete lateral line and 7-8½ branched dorsal rays. The chub previously placed in *Leuciscus* sp. is now in the genus *Squalius*, characterized by the absence of a scaleless midventral keel in front of the anus, usually a terminal or subterminal mouth, two rows of pharyngeal teeth and 7-9½ branched dorsal rays (KOTTELAT & FREYHOF 2007). The available literature contains no data on the development of the genital system in this species or anatomical and cytological descriptions of the processes occurring in its gonads.

The aim of the study was to analyze the sex differentiation process of chub, i.e. to determine the manner and time of gonadal macroscopic structure differentiation as well as cytological differentiation in male and female sex line cells. An in-depth examination of the process provides essential information that may be used practically in breeding to amplify/enhance the spawning stock, as well as

in works concerning genomic and hormonal manipulations.

Material and Methods

Larvae of chub used for experimental rearing originated from artificial reproduction of spawners caught using electrofishing equipment IUP-12, in April 2002 in the Lyna River. In the laboratory, mature fish were kept in two separate sex groups in tanks (1 m³ of volume) with monitored temperature and photoperiod. A total of 15 individuals, six females and nine males, were used for artificial reproduction:

(♀) mean body fork length (L_F) 25.2 ± 2.7 cm
(range 21.0 to 29.0)

mean body weight 367.2 ± 119.2 g
(range 221.0 to 538.0)

(♂) mean body fork length (L_F) 21.5 ± 0.8 cm
(range 20.7 to 23.1)

mean body weight 249.3 ± 29.9 g
(range 212.0 to 288.0)

During all manipulations, spawners were anaesthetized by immersion in 2-phenoxyethanol (0.5 ml l⁻¹; Sigma Ltd.). Artificial spawning of chub was induced by injection of Ovopel, including an analogue of GnRH and dopamine inhibitor – metopramide (HORVÁTH *et al.* 1997) in doses:

Table 1

Size of chub larvae/juveniles (total length and body weight) during experimental rearing

Date (fish age in days)	L_T (cm)		B_W (g)	
	Min.-Max.	Mean (SD)	Min.-Max.	Mean (SD)
10 June 2002 (14)	1.0-1.2	1.1 (0.1)	0.016-0.080	0.011 (0.003)
17 June 2002 (21)	1.4-1.6	1.6 (0.1)	0.031-0.042	0.038 (0.004)
24 June 2002 (28)	1.3-2.1	1.6 (0.3)	0.020-0.096	0.043 (0.028)
01 July 2002 (35)	2.4-2.6	2.5 (0.1)	0.085-0.132	0.104 (0.018)
08 July 2002 (42)	2.4-2.7	2.5 (0.2)	0.087-0.174	0.113 (0.029)
15 July 2002 (49)	2.4-3.1	2.8 (0.3)	0.137-0.280	0.201 (0.054)
22 July 2002 (56)	2.7-2.9	2.8 (0.1)	0.190-0.252	0.219 (0.024)
29 July 2002 (63)	2.8-3.4	3.1 (0.2)	0.181-0.325	0.233 (0.048)
05 August 2002 (70)	3.1-3.6	3.3 (0.2)	0.214-0.367	0.281 (0.064)
19 August 2002 (84)	2.9-4.1	3.8 (0.4)	0.205-0.574	0.420 (0.115)
02 September 2002 (98)	3.7-4.8	4.4 (0.3)	0.483-1.014	0.755 (0.156)
16 September 2002 (112)	3.8-5.3	4.7 (0.5)	0.412-1.209	0.686 (0.255)
30 September 2002 (126)	4.2-5.4	4.8 (0.4)	0.724-1.476	1.053 (0.226)
14 October 2002 (140)	4.6-6.1	5.4 (0.5)	0.776-2.034	1.330 (0.391)
28 October 2002 (154)	4.9-5.7	5.6 (0.3)	1.178-2.051	1.686 (0.319)
11 November 2002 (168)	5.6-6.8	6.5 (0.4)	1.340-3.110	2.494 (0.559)
02 December 2002 (190)	6.4-7.7	7.1 (0.5)	2.041-3.940	2.905 (0.759)

L_T – total length; B_W – body weight; SD – standard deviation

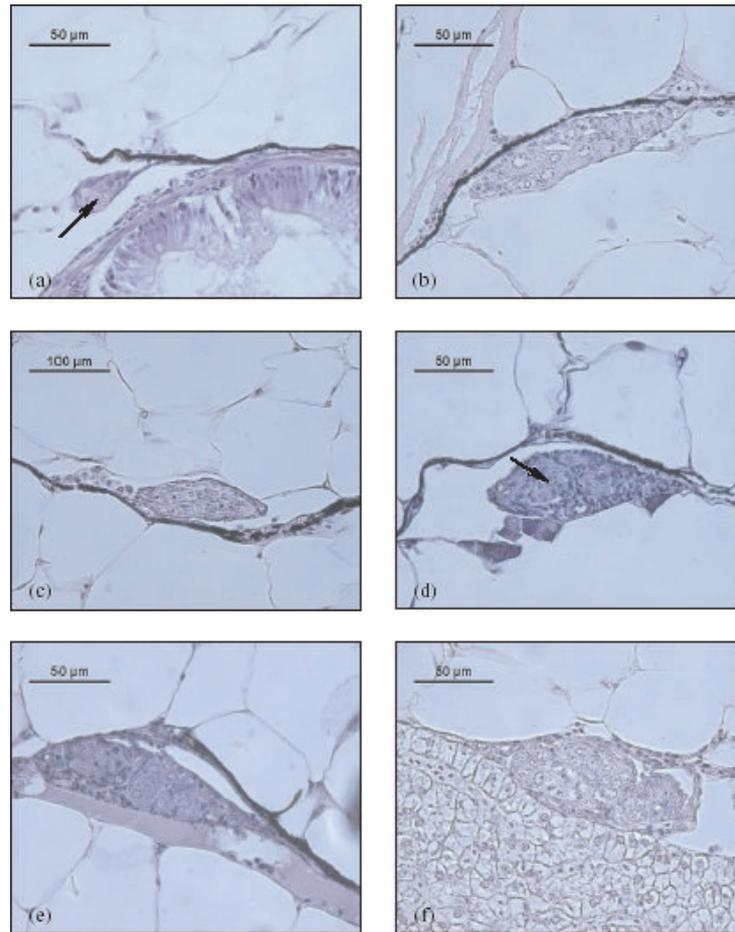


Fig. 1. Histological cross-sections of chub gonads: (a) undifferentiated gonadal anlage of fish at 35 days after hatching with one primordial germ cell (arrow); (b) spindle shaped gonad with few PGCs, attached on both sides to peritoneum – 98th day of postembryonal development; (c) pear shaped gonad attached by a single mesentery string to peritoneum – 98th day of postembryonal development; (d) gonad with PGCs (arrow) and single gonocytes 126 days after hatching; (e) ovary on the 140th day of postembryonal development – gonocytes and single oogonia are depicted; (f) testes with PGCs during mitotic divisions and gonocytes.

– double for females (first, 1/7 pellet and second, 1 pellet per kg of body weight, with a 12 hour interval between the first and the second injection),

– single for males (1 pellet per kg of body weight).

Fertilization was carried out using the standard “dry method” and incubation of eggs took place in a Weiss apparatus, at mean temperature $19 \pm 0.5^\circ\text{C}$. Larvae of chub were reared in controlled conditions in 12 aquaria (volume about 18 l^{-1}) set in a recirculation system at mean temperature $25 \pm 0.5^\circ\text{C}$ and fed *ad libitum* with naupli *Artemia* sp. The initial density of larvae in tanks was 25 individuals per l^{-1} .

Histological analyses were conducted from 10 June 2002 to 2 December 2002. Seven individuals were sampled at random at 7 or 14 days. Larvae/juveniles of chub were measured (total length - L_T) ($\pm 1.0 \text{ mm}$) and weighed (body weight - B_W) ($\pm 1.0 \text{ mg}$) (Table 1). The material for histological analysis was fixed in Bouin’s solution. The protocol of HUMASON (1970) for dehydration, immersion and embedding was followed. Slices 4-5 μm thick were cut using a ro-

tational microtome model RM 2155 (LEICA Microsystems, Wetzlar, Germany), stained with the haematoxyline and eosin topographic method and the Mallory method. Histological analyses of cross-sections for the shape, size and the type of germ cells present in gonads were conducted by light microscope ECOTONE with classical micro image computer analysis software MultiScanBase version 8.0 for WINDOWS (Computer Scanning Systems Ltd.).

Results

After 190 days the fish reached a 2.905 g average body weight and mean 7.1 cm total length (Table 1). Between the 14th and 28th day after hatching, no essential differences in anatomical or cytological structures of the gonads were observed. At the 35th day after hatching, only a large quantity of somatic cells and single primordial germ cells (PGCs) were observed (Fig. 1a). PGCs were round

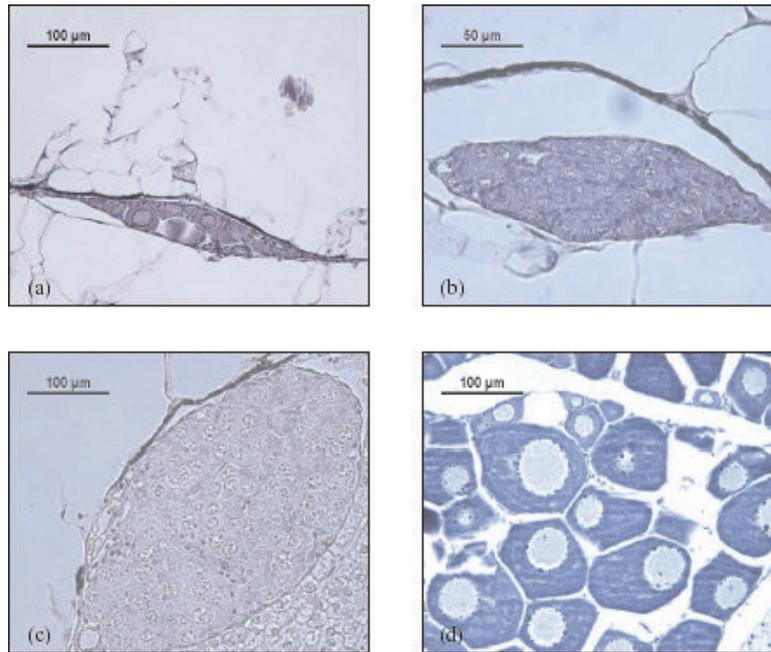


Fig. 2. Histological cross-sections of chub gonads: a – spindle shaped ovary with single previtellogenic oocytes 154 days after hatching; b – testes with spermatogonia on the 154th day of postembryonal development; c – seminal vesicles of testes filled with spermatogonia – 190 days after hatching; d – ovary with ovarian lamellae filled with previtellogenic oocytes on the 190th day of postembryonal development.

shaped and were considerably larger than somatic cells (average diameter $18.3 \pm 3.0 \mu\text{m}$). They were characterized by a marked border between the cytoplasm and somewhat eccentrically located nuclei. The nuclei of these cells were stained by haematoxyline more intensively than the cytoplasm. The beginning of anatomical differentiation took place at the 98th day after hatching when fish weighed 0.755 g and their mean length was 4.4 cm (Table 1). At the same time two morphological types of gonads were observed. The first type was spindle-shaped and attached at both sides by the mesentery to the peritoneum – probably future ovaries (Fig. 1b), the second type was pear-shaped and attached to a single mesentery string – probably future testis (Fig. 1c). The first signs of cytological differentiation of female gonads were observed at the 126th day after hatching. At this time, single multiplied gonocytes after mitotic divisions appeared in one specimen (Fig. 1d). Proper cytological differentiation of female gonads took place after 140 days post hatching, when the fish reached 1.33 g body weight and 5.4 cm overall length. In ovaries, further divisions of the gonocytes as well as individual oogonia were observed (Fig. 1e; Table 1). At the same time pear-shaped testes contained PGC's in mitotic division phase and gonocytes (Fig. 1f). At 154 days after hatching, when fish weighed from 1.178 to 2.051 g of body weight and an reached from 4.9 to 5.7 cm of total length (Table 1), previtellogenic oocytes were observed in ovaries, testifying to the end of

the differentiation process in female gonads. Oocytes were large with a broad band of homogeneous cytoplasm that was evenly stained with haematoxyline (Fig. 2a). At the same time the volume of the testes increased considerably and whole males gonads were filled with spermatogonia (Fig. 2b). At 190 days after hatching, both ovaries and testes had considerably larger volumes than previous testes. Testes were filled with spermatogonia, located in seminal vesicles (Fig. 2c) and ovaries were completely filled with the previtellogenic oocytes. Follicular cells were observed on the edges of the oocytes (Fig. 2d).

Discussion

The sex differentiation process occurs in different stages of fish ontogenesis. At present, complete data concerning sex differentiation in the cyprinids is sparse, but includes the common carp *C. carpio* (DAVIES & TAKASHIMA 1980), asp *Aspius aspius* (L.) (DEMSKA-ZAKĘŚ *et al.* 1998), vimba *V. vimba* (HLIWA *et al.* 2003) and grass carp *Ctenopharyngodon idella* (Val.) (JENSEN & SHELTON 1983).

The first steps towards the formation of functional ovaries and testes are the differentiation of the future germinal elements – primordial germ cells (PGCs) from the blastomeres during embryogenesis, the formation of the germinal ridge and migration of the primordial germ cells into these

ridges (STRÜSSMANN & NAKAMURA 2002). There are two theories on how PGCs migrate, i.e. actively (BRUSLÉ & BRUSLÉ 1978) or passively (SHINOMIYA *et al.* 2000), although the possibility of the existence of both theories cannot be excluded (YOSHIZAKI *et al.* 2002). In this study, the migration of PGCs into the chub gonad was not observed. The presence of PGCs in gonads of the chub was noted for the first time 35 days after hatching. In the asp, another rheophilic cyprinid fish, PGCs appeared 65 days after hatching (DEMSKA-ZAKEŚ *et al.* 1998).

In our study, clear anatomical differences in structure of the gonads between chub individuals at the 98th day after hatching were observed. Gonads differed from each other in shape (spindle-shaped in females and pear-like in males), size (ovaries were bigger), and means of attachment to peritoneum (double in females, single in males). The differences in the structure of gonads are determined by age or size and appear in different periods after hatching (DAVIES & TAKASHIMA 1980). In asp the first symptoms of anatomical sex differentiation were observed 65 days post hatching, but the final formation of the macroscopic structure of ovaries occurred much later than in chub, 245 days after hatching. During this period the ovaries changed their shape from spindle-shaped to sulcate, whereas seminal vesicles characteristic of testes appeared not before the 310th day after hatching (DEMSKA-ZAKEŚ *et al.* 1998). In vimba, differences in the structure of the gonads were observed on the 57th day after hatching (HLIWA *et al.* 2003). Similar to the chub, ovaries in vimba are spindle-shape and bigger than pear-shaped testes.

In most teleost fish species the rate of mitotic division of PGCs and gonocytes is higher in gonads differentiating in the “female direction”. As a result, fish of the same age, weight or body length have future ovaries of a larger volume than future testes. This increase in ovary size is consistent with the proliferation of germ cells and possibly also of somatic cells (germ cell mitosis and somatic cell rearrangement (STRÜSSMANN *et al.* 1996). According to HUNTER & DONALDSON (1983), the process of sex determination occurs much earlier in the homogametic than in the heterogametic gender. Therefore the chub females are probably the homogametic and males are the heterogametic sex.

The first symptoms of cytological differentiation in chub females were observed on the 98th day after hatching, but the end of sex differentiation in ovaries occurred much later, 140 days after hatching. In our study males could be identified cytologically about 154 days after hatching. The cytological differentiation of asp females took place on day 171, while in males not before the

268th day after hatching (DEMSKA-ZAKEŚ *et al.* 1998). In vimba females, cytological differentiation was observed 127 days after hatching, but in males much later, after about 228 days post hatching (HLIWA *et al.* 2003).

In comparisons of the course of sex differentiation in several species it should be underlined that the process was observed under various thermal conditions, usually close to the optimal level for a particular species. In our study chub larvae were reared just like vimba in 25°C, as an optimal temperature for the rearing conditions and the development of larval and juvenile stages of many cyprinid species (WOLNICKI 1996).

Producing material for the restocking of chub is currently of importance for aquaculturists (HARZEVILI *et al.* 2003). Chub as the most eurythermic fish among all rheophilic cyprinids, resistant to thermal pollution, could be a very interesting object for toxicological tests and for further studies of the biological effects of environmental pollution in aquatic ecosystems (FLAMMARION *et al.* 2000; POTTINGER *et al.* 2000).

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