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Gonadal sex differentiation in the bluegill sunfish *Lepomis macrochirus* and its relation to fish size and age

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ABSTRACT

Bluegill (*Lepomis macrochirus*) monosex culture holds considerable potential as a method to improve bluegill aquaculture production as males grow much faster than females. A detailed understanding of the time of gonadal development and differentiation is critical to control sex and optimize culture. In the present study, we systematically studied gonadal sex differentiation of the bluegill sunfish and its relation to fish size and age from hatching to 90 days post hatching (dph) using a slow-growing batch (SGB) and a fast-growing batch (FGB) of fish. The results indicated that the gonadal differentiation in bluegill was more related to body size than to age. In presumptive ovaries, germinal and somatic differentiation began between 13.2 and 16.0 mm (60 dph in SGB and 30 dph in FGB) in total length (TL). The outgrowths from the proximal and distal portions of the gonads and the fusion of the outgrowths to form the ovarian cavity occurred between 16.0 and 21.0 mm TL (80 dph in SGB and 50 dph in FGB) with germ cells undergoing meiosis. The gonads in the females larger than 25.5 mm TL always had peri-nucleolus oocytes. In presumptive testes, the efferent duct formed in the fish ranging from 25.4 to 28.0 mm TL (90 dph in SGB and 70 dph in FGB) with the onset of meiosis and testes contained spermatocytes exhibiting active meiosis in males larger than 33.0 mm TL. These findings indicate that bluegill is a differentiated gonochorist and sex differentiation occurs earlier in females than males. Based on our results, we suggest that the critical period of sex differentiation in bluegill occurs between 13.2 and 16.0 mm TL and histological sex differentiation is distinguishable in most fish larger than 21.0 mm TL.

1. Introduction

The bluegill sunfish *Lepomis macrochirus* is currently recognized as one of the most valuable North American recreational fishes. It has long been commercially cultured to support recreational fishery stocking needs throughout the middle south, and southeastern United States (Brunson and Robinette, 1986; Tidwell and Webster, 1993; Brunson and Morris, 2000). In the meantime, the bluegill sunfish has become increasingly important as a high-value species in aquaculture. In some states, like Ohio and Michigan in the United States, the bluegill sunfish has been listed as one of the top three culture species of fish (Lewis and Heidinger, 1978; McLarney, 1987; Ehlinger, 1989). Recently, a number of studies have shown that male bluegill sunfish appear to hold the greatest potential for the food market due to their more rapid growth capacity versus females (Hayward and Wang, 2006). Male bluegill sunfish outweighed female bluegill by 111.9% at the end of a 300-day experiment (Hayward and Wang, 2006). These findings suggest that monosex (all-male) culture will hold considerable potential as a method to increase the efficiency and profitability of bluegill sunfish aquaculture by improving growth rates.

Careful histological observations of the gonadal morphogenetic process are of primary importance for a precise understanding of the mechanisms of gonadal sex differentiation (Nakamura et al., 1998). Determination of gonadal sex differentiation mechanisms could help to develop efficient methods of directing sexual development in aquaculture species, since they can provide guidance for determining the hormone-sensitive period in cases of sex manipulation by exogenous steroid treatment (Hunter and Donaldson, 1983; Foyle, 1993; Strüssmann et al., 1996). In general, sexually undifferentiated fish are much more sensitive to the effects of steroid treatment than sexually differentiated ones. Many fish would not respond to steroid treatments once sexually differentiated; at least, with the same effective doses that are used when they are sexually undifferentiated. During gonad development, there exists a period known as the labile period, which is defined as the period of time when the still sexually undifferentiated gonads are more responsive to the action of exogenous steroids (Nakamura and Takahashi,

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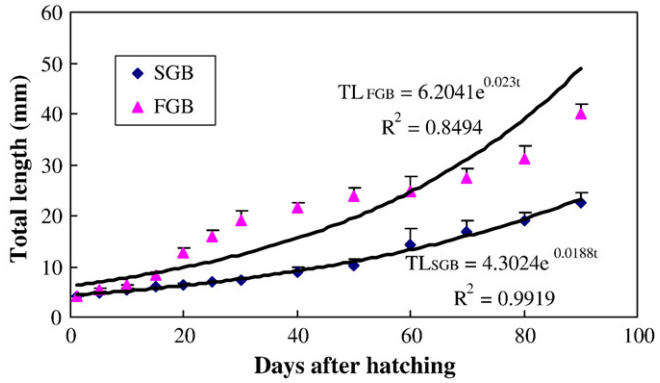


Fig. 1. Growth curves in total length (TL, mm) of the bluegill sunfish from the slow-growing batch and the fast-growing batch from 1 dph to 90 dph. *t*: time (days post hatching). Values are means (±SD).

1973; Hackmann and Reinboth, 1974; Piferrer, 2001). Thus, the period of gonadal differentiation is very important for hormonal sex reversal of fish.

According to Yamamoto (1969), two patterns of gonadal development exist among teleosts: differentiated and undifferentiated gonochorists. In the former type, an undifferentiated gonad develops directly into a testis or ovary. Some examples of fishes differentiating in this manner are the medaka (Yamamoto, 1958), lumpfish *Cyclopterus lumpus* (Martin-Robichaud, 1993) and Atlantic halibut *Hippoglossus hippoglossus* (Hendry et al., 2002). In the later type, the undifferentiated gonad first develops into an ovary-like gonad and then about one-half of the individuals become males and the other half females, such as the minnow *Phoxinus phoxinus* (Bullough, 1940), zebrafish *Danio rerio* (Takahashi, 1977; Maack and Segner, 2003) and protandrous anemonefish *Amphiprion clarkia* (Miura et al., 2008). Spontaneous intersexes occur mostly in undifferentiated gonochorists, while in the differentiated ones the occurrence of intersex is rarely or never seen in natural conditions (Yamamoto, 1969).

Since the demonstration in the late 1930s and early 1940s that the gonadal sex of fish can be influenced by administration of hormones, there have been several studies on the bluegill sunfish sex reversal. Androgens were used for the direct masculinization of fish (Chew and Stanley, 1973; Al-Ablani and Phelps, 2002). The indirect masculinization process uses estrogens to feminize genetic males (Al-Ablani, 1997; Arslan and Phelps, 2004; Piferrer, 2001), which are in turn mated with regular genetic males to produce homogametic males. These homogametic males can be used as broodstock to produce a 100% male population by mating with regular females. In addition, temperature as a main environmental factor influencing the sex ratio in fish (i.e. temperature dependent sex determination — TSD, Devlin and Nagahama, 2002) has been investigated in bluegill sunfish. However, rearing fry at 24.5 ± 1.6 °C or at a temperature close to the upper extreme (33.3 ± 3.5 °C) from 25 dph to 70 dph had no significant effects on the sex ratios of bluegill sunfish (Al-Ablani, 1997). Although these studies reported sex reversal of bluegill sunfish, information on the gonad formation and sex differentiation processes of the bluegill sunfish during the juvenile stages has not yet been clarified in detail. Whether administered through the oral route or short-term periodic immersions, all of these studies began to use hormones in 27-day-old fry or 30-day-old fry. Based on their experimental results, Arslan and Phelps (2004) indicated that gonadal differentiation was labile to the influence of exogenous estrogens in 27-day-old and 13.8-mm bluegill sunfish fry.

In this study, we followed the process of ovarian and testicular development histologically, with the aim to clarify gonadal sex differentiation of juvenile bluegill sunfish and its relation to fish size and age.

2. Materials and methods

2.1. Fry production

Two batches of experimental fish were generated for the study. For each batch, eight male and four female bluegill sunfish were selected from the broodstocks housed in the wet laboratory of the Ohio Center for Aquaculture Research and Development (OCARD) in 2007, tagged with PIT tags and stocked into two tanks with flow-through well water at a ratio of two females to four males per tank. To induce spawning out of the spawning season in November, water temperature and photoperiod were manipulated to match its natural spawning-season cycle. In October, the temperature in each tank was gradually decreased from 23 °C to 17–18 °C at the rate of 1 °C every two days, and photoperiod was decreased to 8 h light/day (d) from 16 h light/d within two weeks. A water temperature of 17–18 °C and photoperiod of 8 h light/d were kept for four weeks. Then temperature was gradually increased to 25 °C and photoperiod to 16 h light/d over two weeks. When water temperature and photoperiod reached 25 °C and 16 h light: 8 h dark, two artificial spawning nests were placed in each tank, and were checked twice daily. Fish in each tank were fed 1.5% body weight at first using a high protein feed (Silver cup, 45% crude protein, 16% crude fat) daily using an automated belt feeder, then 3% body weight when temperature reached 25 °C. Nests with eggs were placed in the bottom of aerated 30-L tanks with flow-through well water for incubation. Eggs hatched in 24–36 h at 24–26 °C; and newly hatched larvae and fry were reared in 30-L round tanks with flow-through well water.

2.2. Rearing of fish

To evaluate the effects of size and age on the gonadal sex differentiation, two batches of fry were cultured in different conditions to obtain the slow-growing fish and the fast-growing fish.

Slow-growing batch (SGB): fry were raised in relatively high density to limit their growth rates. Beginning at 1 day post hatching

Table 1
Summary of morphological events of gonadal development and sex differentiation in the slow-growing batch of bluegill sunfish.

Age (dph)	No. of fish	TL (mm)	Gonadal stage	Sex		
				U	F	M
5	10	4.6–5.2	PGCs present	10	0	0
25	10	6.6–7.2	A pair of gonads under the dorsal celomic epithelium	10	0	0
30	10	7.0–8.8	A pair of gonads under the abdominal cavity	10	0	0
50	8	9.5–13.2	Active germ cell mitosis present in half of the individuals	8	0	0
60	5	14.0–19.2	Increases in the number of germ cells and somatic cells in the presumptive ovary	3	2	0
70	5	16.2–21.0	Elongated aggregations of somatic cells and germ cells undergoing meiosis in the ovary, increases in the number of germ cells and stromal cells in the presumptive testis	0	2	3
80	6	18.2–23.0	Complete ovarian cavity in the ovary, efferent duct anlage in the testis	0	3	3
90	7	20.0–26.0	Peri-nucleolus oocytes and fusion of anterior part of gonadal tissues in the ovary, evident efferent duct and meiosis in the testis	0	4	3

U, undifferentiated; F, female; M, male; dph, days post hatching.

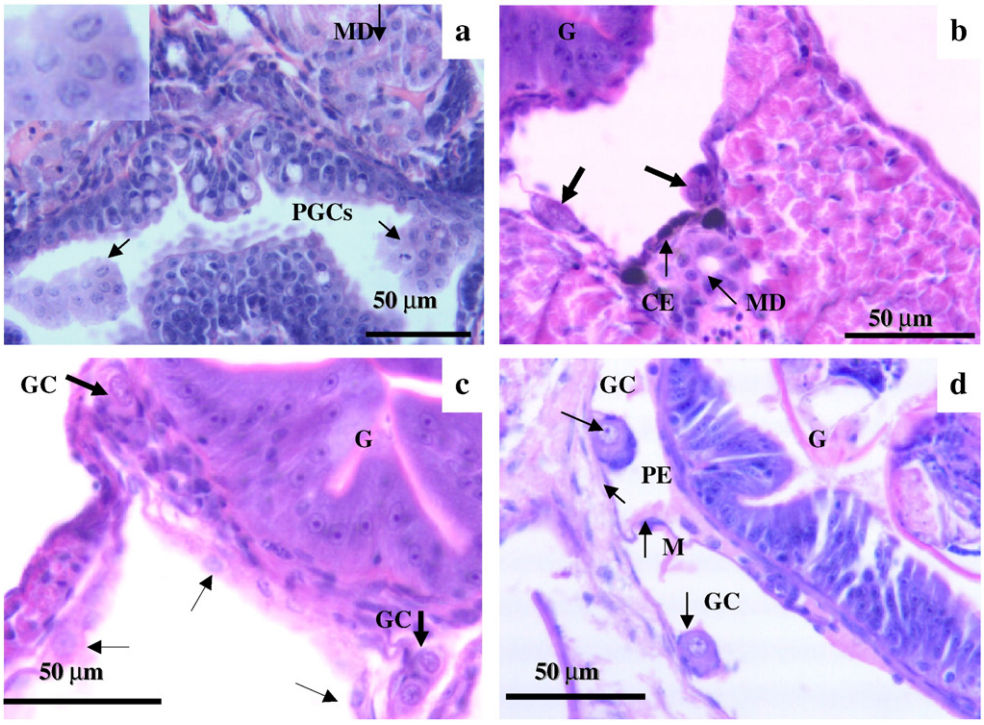


Fig. 2. Undifferentiated gonads of the bluegill sunfish at 5–40 dph in the slow-growing batch. (a) Clusters of primordial germ cells at 5 dph. Higher magnification of primordial germ cells (inset). (b) Primordial gonads at 25 dph. A pair of gonadal primordial is indicated by the thick arrows. (c) Primordial gonads at 30 dph. The migratory germ cells are indicated by the arrows and a pair of gonadal primordial is indicated by the thick arrows. (d) Undifferentiated gonads at 40 dph. Abbreviations: CE, celomic epithelium; G, gut; GC, germ cells; M, mesentery; MD, mesonephric duct; PE, peritoneal epithelium; PGCs, primordial germ cells.

(dph), the fry were fed four times daily with brine shrimp (Bio-Marine, INC., USA). At 10 dph, the commercial larval AP-100 micro-feed (Zeigler Bros., INC., USA) was gradually added to the diet and then AP-200 micro-feed was gradually added at 20 dph. From 30 dph to 90 dph, the fry were fed AP-200 micro-feed exclusively and were fed five times daily.

Fast-growing batch (FGB): fry were cultured in relatively low density to enhance their growth rate and the same rearing protocol was used as for the slow-growing fish from 1 dph to 30 dph. However, the fish were still fed brine shrimp twice daily (at 10:00 am and 7:00 pm) during the 30 dph to 90 dph to further increase their growth rates.

During the experiment, daily mean (±SD) water temperature and dissolved oxygen concentrations were 24.5 ± 1.5 °C and 6.1 ± 0.7 mg L^{-1}, respectively.

2.3. Histological analysis

For the SGB, five to ten fry were collected every 5 days between 0 and 30 dph, and every 10 days between 30 dph and 90 dph. For the FGB, five to seven fry were collected every 10 days between 30 dph and 90 dph. After anesthetization with MS-222 (100 mg L^{-1}), the total length (TL) and body weight (BW) of each fish were measured to the nearest 0.1 mm and 0.01 g. For the fish smaller than or equal to 18 mm TL, the whole body was fixed with Prefer solution at room temperature for at least 48 h, washed in 50% ethanol, and stored in 70% ethanol until histological processing. For specimens larger than 18 mm TL, the abdominal segments were fixed. Then the tissues were dehydrated in a series of alcohol, clarified in benzene, and finally embedded in paraffin. Cross-sections were cut every 5–7 μm and were stained with Mayer's hematoxylin and eosin phloxine B solution following conventional histological procedures (Park et al., 1998). A thorough examination of gonads was performed by cutting the fish's whole body or abdominal segment into serial sections. The use of serial sections was found to be important to determine the developmental stage of the gonads.

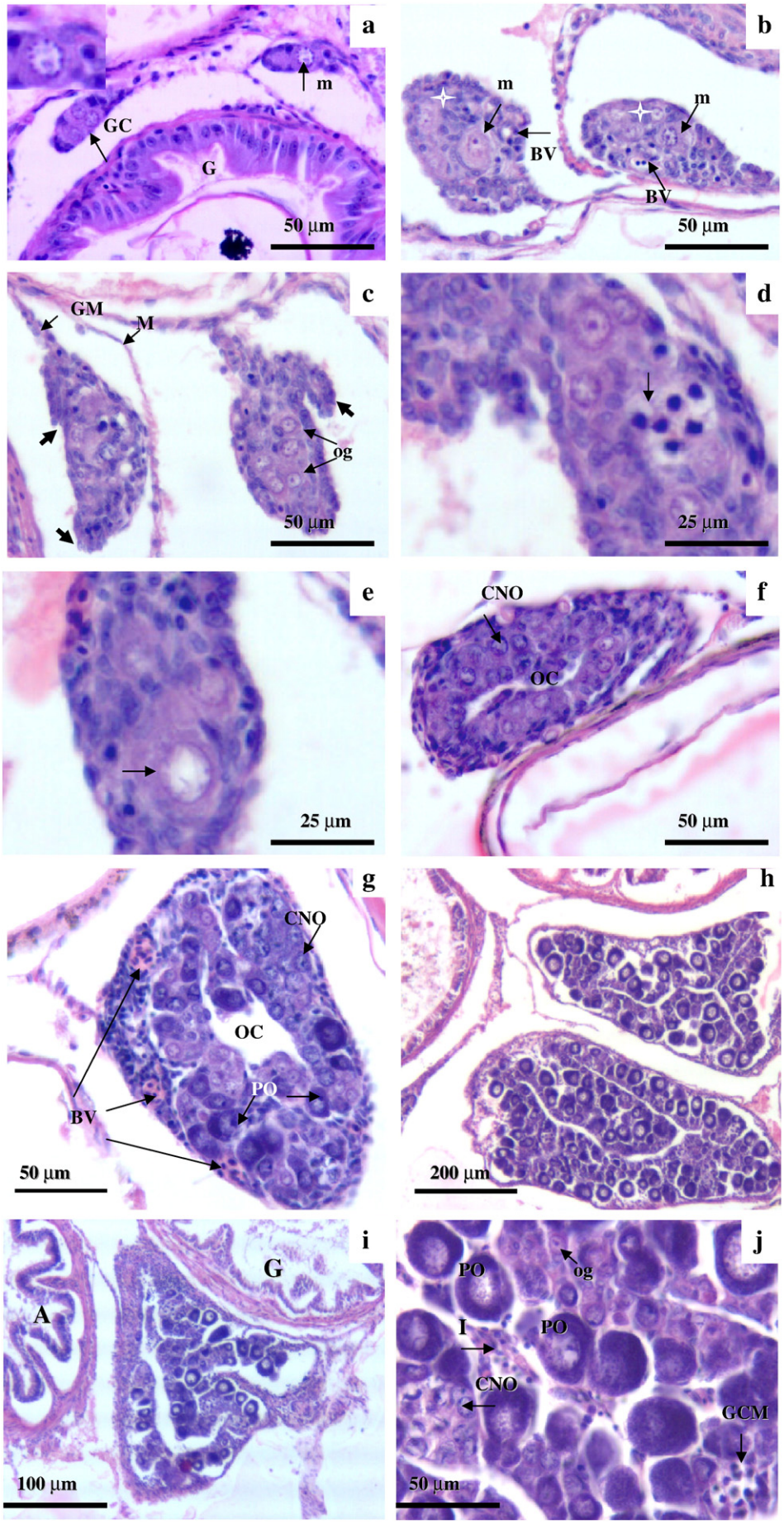
The slides were examined and photographed under light microscope with a video camera linked to computer image analysis software (Olympus MicroSuite™ FIVE). Measurements were also carried out by this software. Stages of gonad development were determined in relation to fish age, fish size and morphological features. Cellular identification was based on descriptions and photographs from established literature (Lebrun et al., 1982; Foyle, 1993; Hendry et al., 2002; Arezo et al., 2007; Uguz, 2008).

3. Results

3.1. Growth of the experimental fish

From hatching to the 90 dph, the growth of fry in FGB was significantly faster than that in SGB ($P<0.05$) (Fig. 1). An increase in

Fig. 3. Ovarian differentiation in the bluegill sunfish at 50–90 dph in the slow-growing batch. (a) Presumptive ovary at 50 dph showing gonadal type I in which germ cells are undergoing early mitosis (m). Higher magnification of mitotic germ cells (inset). (b) Presumptive ovary at 60 dph, showing germ cells multiplied in number, and blood vessel. The numerous somatic cells are indicated by stars. (c) Initial ovary at 70 dph, showing somatic elongations. Two somatic elongations forming the initial ovarian cavity formation are indicated by the thick arrows. (d) Initial ovary at 70 dph, showing germ cell nests with zygotene (bouquet) stage of oocyte meiosis (arrow). (e) Initial ovary at 70 dph, showing oocyte undergoing meiosis at pachytene stage (arrow). (f) Ovary at 80 dph, showing the ovary cavity (OC) and oocytes at chromatin-nucleolus stage (arrow). (g) Ovary at 90 dph, showing some ocytes at peri-nucleolus stage (PO). (h) Ovary at 90 dph, showing many peri-nucleous oocytes. (i) Ovary at 90 dph, showing the fusion in the anterior part of two gonadal tissues. (j) Ovarian tissue at 90 dph. Abbreviations: m, meiotic germ cell; A, anus; BV, blood vessel; CNO, chromatin-nucleolus oocyte; G, gut; GC, germ cells; GCM, germ cells undergoing meiosis at zygotene stage; GM, gonadal mesentery; I, interstitial or stromal tissue; M, mesentery; OC, ovarian cavity; og, oogonium; PO, peri-nucleolus oocyte.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total length (TL) of fish indicated continuous growth, described by the growth expression TL_t (mm) = $4.3024e^{0.0188t}$ ($R^2 = 0.99$) in SGB and TL_t (mm) = $6.2041e^{0.023t}$ ($R^2 = 0.85$) (t, days post hatching) in FGB.

3.2. Gonadal differentiation and development

Based on histological observation, the ovaries and testes of bluegill sunfish were found to develop directly from undifferentiated gonadal tissue, and bluegill sunfish can be classified as a differentiated gonochorist. The key morphological events during gonadal sex differentiation in SGB are summarized in Table 1 and described in detail below.

Undifferentiated gonads: At 5 dph, primordial germ cells (PGCs) were observed under the mesonephric duct (Fig. 2a). The PGCs were arranged in groups of 10–20 cells. Morphologically, the round to slightly oval PGCs were distinguished from somatic cells by their relatively large diameter (6–8 μm) and their histological features. Their cytoplasm was weakly stained by hematoxylin and they contained two or three round nuclei 1–2 μm in diameter. At 25 dph, a pair of gonads appeared under the dorsal celomic epithelium (Fig. 2b). Some germ cells can be found to project into the abdominal cavity within a cord-like gonadal tissue from the dorsal celomic epithelium at 30 dph (Fig. 2c) and a pair of gonads was present under the abdominal cavity. The nuclei of germ cells were polymorphic, often bilobed, with one or two large nucleoli and dispersed chromatin in the form of an irregular meshwork in which numerous small chromatin masses were suspended. Subsequently, at 40 dph, the gonads were attached at both sides of the mesentery by a mesogonadium and wrapped by the monolayered peritoneal epithelium (Fig. 2d). One pear-shaped gonad always contained one or two primordial germ cells per cross section. Nuclear contours of germ cells became round to ovoid and chromatin varied from dispersed concretions of various sizes to distinct threads arrayed in a fine peripheral meshwork. The gonads of the fish collected at 40 dph did not show any morphological characteristics indicative of a differentiating ovary or testis.

Ovarian differentiation: At 50 dph, two kinds of gonadal tissue were observed in different individuals. Gonadal tissue type I showed fewer cells and all of them had characteristics similar to those observed in the undifferentiated stage. Gonadal tissue type II consisted of two different populations of germ cells. One type of cell exhibited morphological characteristics resembling undifferentiated germ cells as described in the previous stage whereas the other type was undergoing mitosis (Fig. 3a). Germ cells undergoing active mitosis were present with different features such as smaller size, mottled nuclei with variable amounts of clumped chromatin around periphery or single prominent nucleoli. At 60 dph, in type II gonadal tissue, germ cells gradually multiplied in number by active mitosis and the number of somatic cells increased together with blood vessels appearing in the lateral region (Fig. 3b). Somatic reorganization of the presumptive ovary began. Gonads were present in a triangular or kidney-shape when observed in sections. Gonad size and the number of germ cells increased dramatically between 60 dph and 70 dph. At 70 dph, the initial ovarian cavity formation was indicated by the presence of two elongated aggregations of somatic cells in the proximal and distal portions of the gonads (Fig. 3c). The two elongating sheets of somatic tissue developed both upward and downwards to form a groove lateral to the gonad proper, the downward elongation from the proximal region being more conspicuous than the upward elongation from the distal region. In addition, germ cell nests with zygotene (bouquet) stage of oocyte meiosis (Fig. 3d) and oocytes undergoing meiosis at pachytene stage (Fig. 3e) were observed, signifying early oogenesis. Subsequently, the outgrowths of the somatic cell

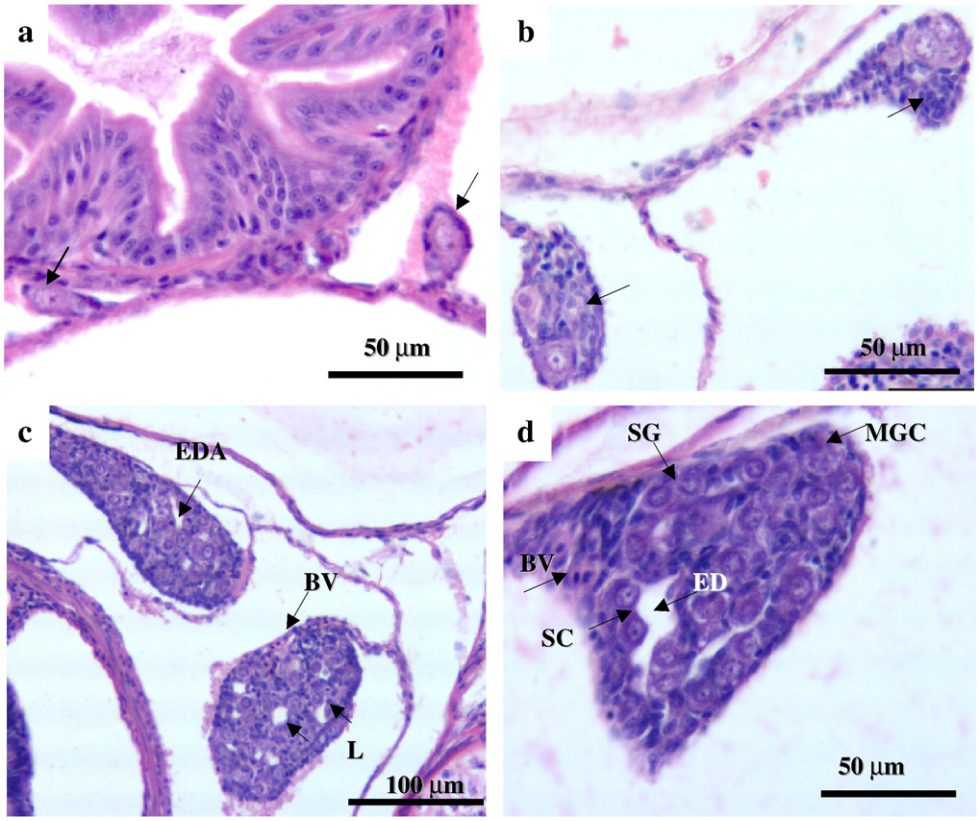

Fig. 4. Testicular differentiation of the bluegill sunfish at 50–90 dph in the slow-growing batch. (a) Fry at 50 dph showing gonadal type II tissue (arrows); (b) Presumptive testis at 70 dph. The aggregations of stromal cells are indicated by arrows. (c) Testis at 80 dph, showing the efferent duct anlage, lobule and blood vessel. (d) Testis at 90 dph, showing evident efferent duct, spermatogonia undergoing mitotic divisions to become spermatocytes, and the onset of meiosis. Abbreviations: BV, blood vessel; EDA, efferent duct anlage; ED, efferent duct; L, lobule; MGC, meiotic germ cells; SG, spermatogonium; SC, spermatocytes.

Table 2
Summary of morphological events of gonadal development and sex differentiation in the fast-growing batch of bluegill sunfish.

Age (dph)	No. of fish	TL (mm)	Gonadal stage	Sex		
				U	F	M
30	5	14.2–19.6	Elongated aggregations of somatic cells in the ovary	4	1	0
50	5	21.0–25.5	Complete ovarian cavity in the ovary	3	2	0
60	5	22.0–27.5	Peri-nucleolus oocytes in the ovary and increases in the number of germ cells and stromal cells in the presumptive testis	0	3	2
70	5	23.5–29.5	Efferent duct anlage in the testis	0	3	2
80	6	27.5–3.40	Numerous peri-nucleolus oocytes and fusion of anterior part of gonadal tissues in the ovary, evident efferent duct and meiotic activity in the testis	0	3	3
90	7	32.0–40.0	Ovary and testis became bigger	0	4	3

U, undifferentiated; F, female; M, male. dph, days post hatching.

aggregations had fused together to form the ovarian cavity. The ovarian cavity was completely formed by day 80 dph and oocytes at the chromatin nucleolus phase were present at this time of development (Fig. 3f). At 90 dph, most of the ovarian gonads observed contained a few oocytes at the peri-nucleolus stage together with a somatic layer including blood vessels, and the ovarian cavity was clearly observed in the central part of the ovary (Fig. 3g). Numerous peri-nucleolus oocytes were found in one fish and the anterior part of two gonadal tissues that attached at both sides of the mesentery had fused together, with the gonads coming near the anus (Fig. 3h–j).

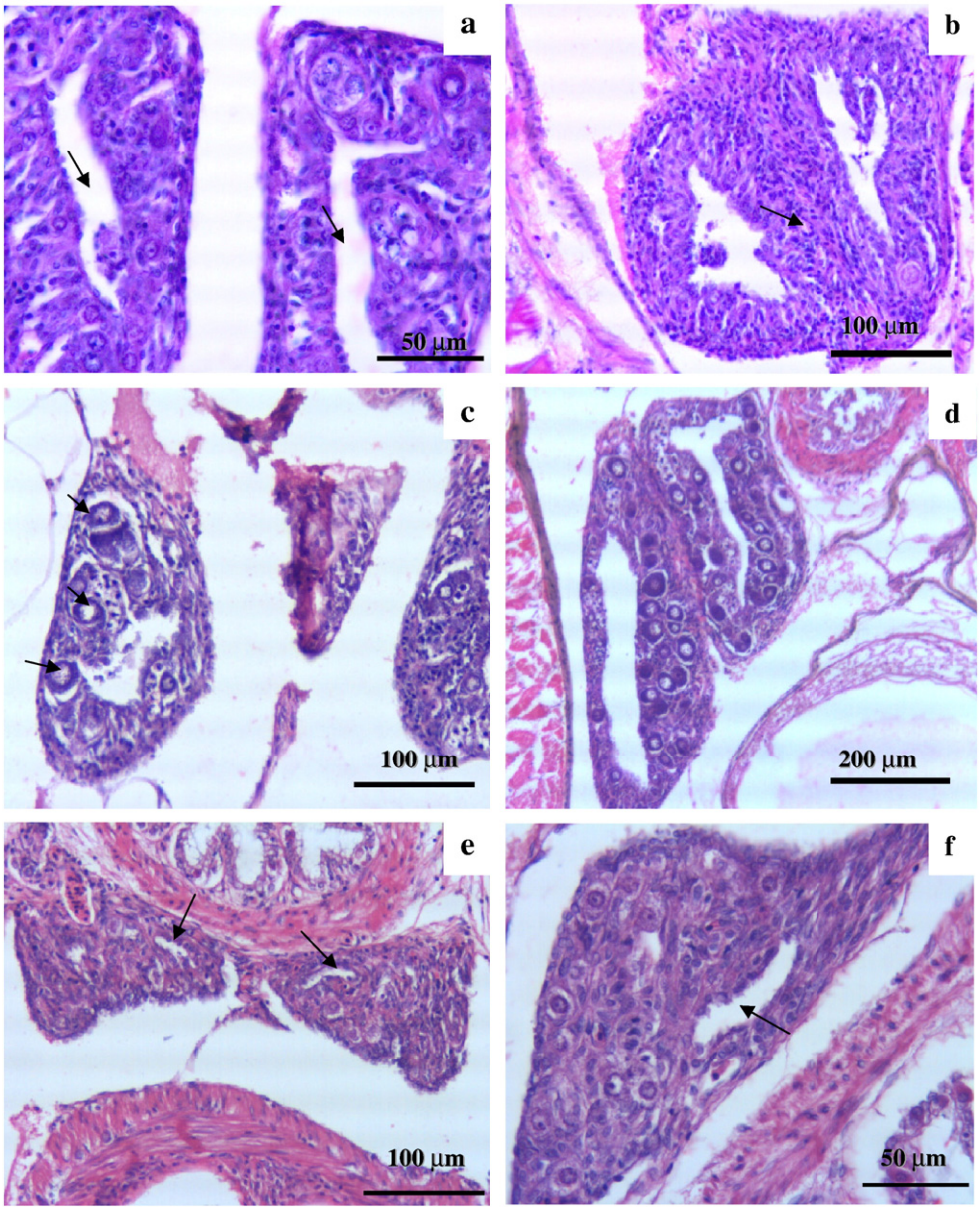

Fig. 5. Gonadal development of the bluegill sunfish in the fast-growing batch. (a) Ovary at 50 dph, showing the ovarian cavity; (b) Ovary at 50 dph, showing the beginning of fusion; (c) Ovary at 60 dph, showing the peri-nucleolus oocytes; (d) Ovary at 80 dph, showing the numerous peri-nucleolus oocytes; (e) Testis at 70 dph, showing the efferent duct anlage; (f) Testis at 80 dph, showing the evident efferent duct.

Testicular differentiation: In contrast to ovarian development, signs of histological differentiation were not observed in the presumptive testis until day 70. The overall appearance of spermatogonia was similar to that of undifferentiated germ cells at 50 dph, referred to previously as the gonadal tissue type I (Fig. 4a). At 70 dph, they retained the original pear-like shape of indifferent gonads and were much smaller than ovaries of the same developmental stage. The most characteristic features were the presence of germ cells undergoing mitosis and the aggregation of stromal cells (Fig. 4b). At 80 dph, blood vessels became evident in the dorsal region of the testis (Fig. 4c). Moreover, a central space that became recognizable as the efferent duct anlage was present in some sections of the testes and the unrestricted lobular organization of the testis can be distinguished. At 90 dph, some spermatogonia undergoing mitotic divisions became spermatocytes (Fig. 4d). The onset of meiosis was noticed in the gonads of males at this developmental stage. In contrast to ovarian development, no part of the two gonads attached at both sides of the mesentery was found to fuse together in the testes as that in the ovary by this time.

The key morphological events during gonadal sex differentiation in FGB are summarized in Table 2. Generally, the time of sex differentiation in FGB was earlier than that in the slow-growing batch. For the ovarian differentiation, the elongated aggregations of somatic cells were present at 30 dph. The ovarian cavity was completely formed at 50 dph and the anterior part of gonadal tissues attached at both sides of the mesentery began to fuse at this developmental stage (Fig. 5a–b), with the basophilic oocytes at the stage of meiosis present. The peri-nucleolus oocytes were observed at 60 dph (Fig. 5c). Subsequently, numerous peri-nucleolus oocytes were found at 80 dph (Fig. 5d). At 90 dph, the oocytes were still in peri-nucleolus stage while the gonad became bigger. As to the testicular differentiation, the efferent ducts were present at 70 dph (Fig. 5e) and became evident at 80 dph (Fig. 5f). The early meiotic activity occurred at 80 dph and became distinct at 90 dph. The anterior part of two testis tissues fused together in some fish at 90 dph.

3.3. Relationship of gonadal differentiation with fish size and age

Gonadal differentiation of bluegill appeared to be related to size more than to the age. In general, the proliferation of germ cells and somatic cells happened in putative females between 13.2 and 16.0 mm TL (at 60 dph in SGB and 30 dph in FGB) and the ovarian cavity was completely formed in all the female fish larger than 21.0 mm TL (at 80 dph in SGB and 50 dph in FGB). While, in putative males, the increases in the number of germ cells and stromal cells appeared in the fish between 19.0 and 22.5 mm (at 70 dph in SGB and 50 dph in FGB) and the efferent ducts were present in all the males larger than 28.0 mm (at 90 dph in SGB and 70 dph in FGB).

4. Discussion

Gonadal differentiation has not previously been reported for the bluegill sunfish. In this study, we described the pattern of sexual development in bluegill sunfish based on sex-specific histological criteria. Previous studies concerning the criteria for morphologically judging the initial differentiation of ovaries and testes have been made

Table 3
Summary of studies on bluegill sunfish sex reversal.

Hormone	C	Initial TL/mm (age)	Final TL/mm (age)	Females (%)	Males (%)	Inter-sex (%)	Sterile (%)	References
Feminize								
E_2 (Di)	100 mg/kg	15 ± 0.60 (30 days)	(60 days)	100	0			Al-Ablani (1997)
E_2 (Di)	200 mg/kg	13.8 ± 0.60 (27 days)	(72 days)	99.3 ± 1.2	0	0.7		Arslan and Phelps (2004)
E_2 (Di)	50 mg/kg	13.9 ± 1.3 (30 days)	(90 days)	80.0	0	20		Wang et al. (2008)
E_2 (Di)	100 mg/kg	13.9 ± 1.3 (30 days)	1.61 ± 0.14 (90 days)	93.4	0	6.6		Wang et al. (2008)
E_2 (Di)	150 mg/kg	13.9 ± 1.3 (30 days)	1.63 ± 0.20 (90 days)	100	0	0		Wang et al. (2008)
E_2 (Di)	200 mg/kg	13.9 ± 1.3 (30 days)	1.52 ± 0.15 (90 days)	100	0	0		Wang et al. (2008)
E_2 (Im)	1 mg/L	13.8 ± 0.60 (27 days)	(37 days)[A]	76.9 ± 3.5	20.4	2.7		Arslan and Phelps (2004)
DES (Im)	1 mg/L	16 ± 0.43 (34 days)	(37 days)[B]	43 ± 1.7	57.0			Al-Ablani (1997)
	1 mg/L	16 ± 0.43 (34 days)	(40 days)[C]	59 ± 6.7	41.0			Al-Ablani (1997)
Masculinize								
MT (Di)	10 mg/kg	(0 day)	(60 days)	40*	60.0*			Chew and Stanley (1973)
	30 mg/kg	(0 day)	(60 days)	100*	0*			Chew and Stanley (1973)
	50 mg/kg	(0 day)	(60 days)	75*	25.0*			Chew and Stanley (1973)
	60 mg/kg	11.54 ± 0.31 (28 days)	23.5 ± 0.62 (58 days)	0	5.0	95		Al-Ablani (1997)
	15 mg/kg	14 ± 0.73 (28 days)	21.0 ± 2.1 (58 days)	25.7 ± 4.5	17.7	56.7		Al-Ablani and Phelps (2002)
	30 mg/kg	14 ± 0.73 (28 days)	21.0 ± 1.9 (58 days)	24.7 ± 4.7	12.7	62.7		Al-Ablani and Phelps (2002)
	60 mg/kg	14 ± 0.73 (28 days)	20.0 ± 2.3 (58 days)	20.3 ± 5.7	11.7	68.0		Al-Ablani and Phelps (2002)
	60 mg/kg	14 ± 0.73 (28 days)	22.0 ± 3.2 (73 days)	10.7 ± 5.7	0	69.7	19.7	Al-Ablani and Phelps (2002)
	60 mg/kg	14 ± 0.73 (28 days)	21.0 ± 2.7 (88 days)	8.7 ± 1.1	0	46.0	45.3	Al-Ablani and Phelps (2002)
TBA (Di)	50 mg/kg	11.54 ± 0.31 (28 days)	23.5 ± 0.60 (58 days)	0.3 ± 0.6	4.0	95.7		Al-Ablani (1997)
	12.5 mg/kg	15 ± 0.42 (28 days)	20.0 ± 2.8 (58 days)	17.0 ± 5.0	43.3	39.7		Al-Ablani and Phelps (2002)
	25 mg/kg	15 ± 0.42 (28 days)	19.0 ± 2.8 (58 days)	17.3 ± 5.5	42.7	40.0		Al-Ablani and Phelps (2002)
	50 mg/kg	15 ± 0.42 (28 days)	19.0 ± 2.9 (58 days)	23.7 ± 1.5	26.7	49.7		Al-Ablani and Phelps (2002)
	50 mg/kg	18 ± 0.26 (28 days)	31.0 ± 5.2 (58 days)	24.3 ± 5.5	27.7	48.0		Al-Ablani and Phelps (2002)
	75 mg/kg	18 ± 0.26 (28 days)	33.0 ± 3.8 (58 days)	20.7 ± 11.8	6.3	68.7	4.3	Al-Ablani and Phelps (2002)
	100 mg/kg	18 ± 0.26 (28 days)	31.0 ± 4.4 (58 days)	19.7 ± 4.5	3.0	67.7	9.3	Al-Ablani and Phelps (2002)
	50 mg/kg	18 ± 0.26 (28 days)	31.0 ± 4.3 (88 days)	10.7 ± 0.6	0	57.7	32.3	Al-Ablani and Phelps (2002)
TBA (Im)	250 μg/L	16 ± 0.43 (34 days)	(37 days)[B]	15 ± 1.2	85.0			Al-Ablani (1997)
	500 μg/L	16 ± 0.43 (34 days)	(37 days)[B]	10 ± 0.6	90.0			Al-Ablani (1997)
	500 μg/L	16 ± 0.43 (34 days)	(40 days)[C]	6 ± 0.6	94.0			Al-Ablani (1997)
	750 μg/L	16 ± 0.43 (34 days)	(37 days)[B]	11 ± 2.1	89.0			Al-Ablani (1997)
	1000 μg/L	16 ± 0.43 (34 days)	(37 days)[B]	9 ± 2.5	91.0			Al-Ablani (1997)
	1000 μg/L	16 ± 0.43 (34 days)	(40 days)[C]	7 ± 1.0	93.0			Al-Ablani (1997)

Superscript *means the number of survivors in the experiment was insufficient for adequate statistical testing. Superscript A means fry were immersed in a 1 mg/L E_2 solution on three occasions for 5 h a day with 5-day intervals. Superscript B means fry were immersed in the hormone solution for 5 h a day on either days 34 and 37 (two-day exposure). Superscript C means fry were immersed in the hormone solution for 5 h a day on either days 34, 37 and 40 (three-day exposure). Abbreviations: Di, diet; Im, immersion; E_2, estradiol-17β; MT, 17α-methyltestosterone; TBA, trenbolone acetate; DES, diethylstilbestrol.

for many species of teleost fish. These studies showed that the presence of gonadal structures (i.e., formation of ovarian cavity and efferent duct), difference in germ cells number, and female earlier meiosis are valid criteria to determine gonadal sex at early phases in development. However, which is the most reliable criterion varies depending on the studied species. Generally, for differentiated gonochorists in teleost fish, the occurrence of meiotic germ cells for oogenesis subsequent to the increase of germ cells is now accepted as the criterion of initial ovarian differentiation, such as in the medaka *Oryzial latipes* (Yamamoto, 1958), stickleback *Gasterosteus aculeatus* (Shimizu and Takahashi, 1980), rainbow trout *Oncorhynchus mykiss* (Lebrun et al., 1982), pejerrey *Odontesthes bonariensis* (Strüssmann et al., 1996) and annual fish *Austrolebias charrua* (Arezo et al., 2007). While for undifferentiated gonochorists, which exhibit juvenile hermaphroditism, the appearance of pre-meiotic oocytes is not always a reliable criterion by which to judge differentiation in these species, such as in zebrafish *Danio rerio* (Takahashi, 1977; Maack and Segner, 2003). Therefore, in these fishes, histological evidence indicating the formation of the ovarian cavity may be a reliable criterion for identifying the gonad as an ovary. In our present study, stromal elongations of the gonad for the formation of the ovarian cavity were observed to occur coincidentally with the appearance of meiotic germ cells. But the rapid proliferation of germ cells by mitosis was observed before these elongations occurred. Since the bluegill sunfish is a differentiated gonochorist, we suggest the germ cell number can be accepted as the criterion of initial ovarian differentiation.

Sex differentiation has been shown to occur at different post-hatching times in most gonochoristic teleosts examined to date and always begins earlier in ovaries than in testes (Devlin and Nagahama, 2002; Strüssman and Nakamura, 2002; Arezo et al., 2007). In the bluegill sunfish, germ cell mitosis started simultaneously with somatic differentiation in males, a pattern shared with females, but meiosis occurred later in males. This feature preceded the formation of the sperm duct in the bluegill sunfish. As the formation of the efferent ducts being the criteria by which testicular differentiation can be recognized, this characteristic was also found in the bluegill sunfish.

Previous studies to examine the process of gonadal differentiation and development were based on the age or the size of fish. The growth in individual body length is variable, even when they are cultured under the same conditions, let alone under different conditions or from different batches. Colombo et al. (1984) reported that gonadal differentiation in European eel *Anguilla anguilla* was more related to the body size than to the age. In order to know whether the onset of sex differentiation in bluegill depends on age or on growth rate, we examined fish from two different batches with different growth rates and took the size of fish into consideration. Our results indicated the gonadal differentiation of the bluegill sunfish was more related to fish size than the age, as evidenced in other teleost species (Colombo et al., 1984; Malison et al., 1986; Grandi and Colombo, 1997; Blázquez et al., 2001). The ovarian cavity was completely formed at 80 dph in SGB and 50 dph in FGB with fish of approximately 21.0 mm TL. The efferent ducts became evident in all the males larger than 26.0 mm at 90 dph in SGB and 70 dph in FGB.

In Table 3, we summarized the published reports about the bluegill sunfish sex-reversal. These studies indicated all the initial attempts to feminize by oral administration of estradiol-17β (E_2) were quite successful. In contrast, most attempts to masculinize by oral administration or immersion of androgens were unsuccessful. The criteria established by Yamamoto (1969) for completing sex reversal required the administration of the hormone during the period from the undifferentiated gonad through sexual differentiation, and that the hormone be administered at an effective dose. Therefore, the most effective treatment period must be before sex differentiation. As testicular differentiation happened later than ovarian differentiation in the bluegill sunfish, all the treatments by oral administration of E_2 for feminization conducted before testicular differentiation could successfully induce genetic male bluegill to phenotypic females (Al-Ablani, 1997; Arslan and Phelps, 2004; Wang et al., 2008). However, androgen oral administration commonly resulted in a high percentage of intersex fish in previous bluegill sex-reversal studies (Table 3). Based on present results for the bluegill sunfish sex differentiation, the high frequency of intersex fish might be due to the later treatment timing (18 ± 0.26 mm TL) when some fish had begun the ovarian differentiation. Piferrer (2001) defined the labile period as the period of time when the still sexually undifferentiated gonads are more responsive to the action of exogenous steroids. Based on our findings from the present study on sex differentiation and other previous successful or unsuccessful studies on sex reversal of the bluegill sunfish, we suggest the labile period for the bluegill sunfish sex differentiation is between 13.2 and 16.0 mm TL.

Our present study described the development and differentiation of the gonad in juvenile bluegill sunfish, which is more related to body size than to age. Based on our results, we suggest that the critical period of sex differentiation in bluegill occurs between 13.2 and 16.0 mm TL when sexually undifferentiated gonads are more responsive to the action of exogenous steroids. These findings are critical to the sex-reversal studies of the bluegill sunfish in order to produce monosex (all-male) populations and accumulate some basic data for sex differentiation in fish.

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