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# Correlations between Plasma Levels of Sex Steroids and Spermatogenesis during the Sexual Cycle of the Chub, *Leuciscus cephalus* L. (Pisces: Cyprinidae)

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**Giulia Guerriero, Rosaria Ferro, and Gaetano Ciarcia (2005)** Correlations between plasma levels of sex steroids and spermatogenesis during the sexual cycle of the chub, *Leuciscus cephalus* L. (Pisces, Cyprinidae). *Zoological Studies* **44**(2): 228-233. Analyses of biometric data, plasma steroids levels, and gonadal morphology allowed us to define the sexual cycle of the cyprinid fish, the chub, *Leuciscus cephalus*, which is widely distributed in European rivers. Four physiological gonadal phases were defined: 1) stasis (Dec.~Feb.); 2) recovery (Mar.~Apr.); 3) spawning (May~June); and 4) post-spawning (July~Nov.). Specific changes in plasma sex hormone levels were found to occur during the different phases of gonadal development. Plasma androgen levels presented a biphasic profile, with peaks corresponding to the end of the stasis phase (Feb.) and during the spawning phase (June). High levels of estradiol-17β were detectable from the early recovery phase (Apr.) to the late spawning phase (June). Their possible roles in gonadal activity are discussed and compared with observations on reproduction in the chub in other areas of their European distribution.
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**Key words:** Reproduction, Gonadal histology, Steroid, *Leuciscus cephalus*, Cyprinidae.

Reproductive biology of fish has long been a widely investigated field (Orlando et al. 2003, Sisneros et al. 2004). Recently, the exploitation of several valuable cyprinid species for commercial purposes has made their investigation particularly relevant (Ronnback et al. 2002, Berry 2003). Cyprinids are an evolutionarily interesting family, and are often used as tools for genetic and physiological investigations (Tsigenopoulos et al. 2002). The genus *Leuciscus* occurs commonly throughout the Palearctic region, as reported by Howes (1990). In particular, numerous aspects of the biology of *Leuciscus cephalus*, a species widely distributed in Europe, have been well studied, and various and interesting aspects of the endocrine system have been examined (Pottinger et al. 2000). However, nothing is known about correlations between plasma levels of sex steroids and spermatogenesis during the major sexual cycle of this species.

Most fish living in the temperate zone exhibit an annual reproductive cycle (Nash 1999). Reproduction occurs when food is available for the offspring in the wild. Therefore, reproduction is closely related to the environment which directly acts on gametogenesis and spawning (Wen and Lin 2001, Tollefsen et al. 2002).

Seasonal changes in the concentrations of circulating sex hormones and their importance for reproduction have been reported for several species of teleosts (Fostier et al. 1983, Okuzawa et al. 1989, Borg, 1994, Rinchard and Kestemont 1996, Nash 1999, Barannikova et al. 2002, Consten et al. 2002).

It is known that the role of sex steroids in controlling the maturation cycle in teleosts especially during spawning times is altered by environmental or hormonal manipulation, and this has both theo-

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retical and practical relevance (Flammarion 2000). In the present study, monthly determinations were made of the plasma concentrations of androgens and estrogens in males of the cyprinid, *L. cephalus*. These determinations were correlated with the testicular/body ratio and the annual pattern of spermatogenesis as assessed by histological examination of the testes.

## MATERIALS AND METHODS

### Animals and sampling procedures

Mature male fish specimens of *L. cephalus* were collected from a large outdoor pond of the Tevere River (Central Italy) in different months of 2001, 2002, and 2003. The Tevere River is characterized by large seasonal fluctuations in discharge and water level, temperature, and nutrients. During the rainy season (Nov.~Dec.), the river floods, while during the summer there is a large decrease in discharge. Fish were collected using a backpack-mounted electric shocker. Experimental procedures were conducted in accordance with the *Guidelines for the Use of Animals in Research* 1998. Twenty fish specimens were collected at each sampled point and time. Only eight of these adult male were utilized monthly. The size range for all individuals caught was between 85 and 130 mm total length, and according to Encina and Granado-Lorencio (1991), we only included adult fish in the analyses. Each fish was weighed (wet weight, $W_T$ ± 0.1 g), measured (total length), and dissected to obtain the testes which were weighed ($W_G$ ± 0.01 g). The gonadosomatic index (GSI) was calculated as follows GSI = $W_G/W_T$ x 100%. Blood samples from each fish, after anesthetization using tricaine methanesulfonate (MS-222) (Sigma, St. Louis, MO), were taken using a caudal puncture. The plasma was separated and stored at - 20°C until analysis. The gonads were kept in Bouin's solution and were embedded in paraffin-celloidin, after which sections (7 μm) were stained with hematoxylin and eosin.

### Radioimmunoassay (RIA)

Sex steroid (testosterone and estradiol-17β) concentrations in plasma samples were assayed using an RIA method (D'Istria et al. 1974, Polzonetti et al. 1983), whose reliability for fish plasma was assessed and reported by Guerriero et al. (1998).

The sensitivity of testosterone was 7 pg (intra-assay, 7%; interassay, 13%), and that of estradiol-17β was 5 pg (intra-assay, 9%; interassay, 13%). The antibody used for testosterone determinations cross-reacted with dihydrotestosterone, and therefore the data are reported as "androgens".

The antiserum was provided by G. Bolelli (Physiopathology of Reproduction Service, Univ. of Bologna, Bologna, Italy). Tritium-labeled steroids were purchased from Amhersham International (Buckinghamshire, UK), and authentic steroids were obtained from Sigma.

### Statistical analysis

Data were analyzed by one-way analysis of variance (ANOVA). Duncan's multiple range test was applied to determine the significance of the differences among means.

## RESULTS

Specific changes in the gonadosomatic index, and in the androgen and estradiol-17β plasma levels occurred in adult males of *L. cephalus* throughout the year (Fig. 1A-C).

Here, we report the statistical examination done on data from Jan. to Dec. collected over a 3-yr period ($n$ = 8 males/mo). *Leuciscus cephalus* showed an annual spermatogenetic cycle with large changes in the number and type of germinal cells in the seminiferous tubules over the course of the year. These caused changes in the size and weight of the testes. Characteristic patterns of testis development are shown in fig. 2A-D. The gonadosomatic index (Fig. 1A) achieved a maximum early in Apr. (2.77% ± 0.04%), while the seminiferous tubules of the testes (Fig. 2B) were evidently filled with spermatocytes and spermatids. In the same period, plasma androgens (0.13 ± 0.05 ng/ml) and estradiol-17β (0.05 ± 0.01 ng/ml) were relatively low (Fig. 1B, C).

The sex steroids reached a peak level in June (Fig. 1B, C) with a mean value of 0.72 ± 0.09 ng/ml for androgens and 0.33 ± 0.05 ng/ml for estradiol-17β. In May and June, when courtship and mating occur, spermatogenesis in the gonads is fully active, and the lobules are filled with spermatozoa (0.43 ± 0.07 and 0.37 ± 0.04 g, respectively) (Fig. 2C).

From July to Nov. (Fig. 2D), only germinal cells were evident in the testes, and the sex

steroids rapidly decreased (from 0.37 ± 0.07 to 0.18 ± 0.02 g) ($p < 0.05$).

During the winter months (Dec.~Feb.), androgen levels increased and showed a new peak in Feb. (0.62 ± 0.09 ng/ml) (Fig. 1B), coinciding with an increase in the GSI (Fig. 1A); in the testes (0.17 ± 0.08 g), there was a resumption of spermatogenesis (Fig. 2A). The estradiol-17β levels remained low (Fig. 1C). Baseline values (< 0.1 ng/ml) were reached for all successively reported periods in June (0.12 ± 0.04 ng/ml).

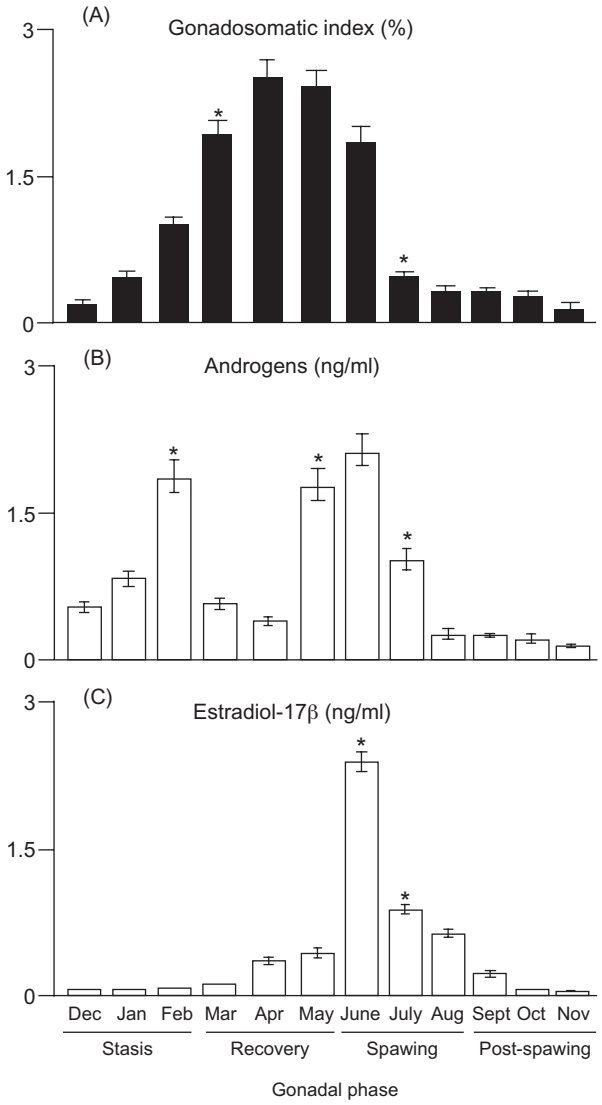


**Fig. 1.** Monthly changes in the gonadosomatic index (A), and plasma levels of androgens (B) and estradiol-17β (C) in males of the cyprinid, *Leuciscus cephalus*. Each value represents the mean of 8 determinations monthly over 3 yr ± S.D. * Levels of significance versus the mean values observed in the preceding month ($p < 0.05$).

## DISCUSSION

In this investigation, histological analysis of the testes, and determinations of the plasma sex steroid levels and GSI were employed to describe the sexual cycle and spawning season of males of the cyprinid, *L. cephalus*.

The histological examination of the testes of *L. cephalus* over the 3-yr period allowed us to define 4 phases: 1) a stasis phase (Dec.~Feb.), characterized by the presence of spermatogonia in the testicular lobules; 2) a recovery or pre-spawning phase (Mar.~Apr.), when the cysts were filled with spermatocytes and spermatids; 3) a spawning phase (May~June), when the cysts contained spermatozoa; and 4) a post-spawning phase (July~Nov.), when the gonads appeared less organized and only spermatogonia were seen.

As far as we know, only a few publications have dealt with the biochemical and histological data of the entire reproductive cycle in cyprinids (Degani et al. 1998, Guerriero et al. 1998), whereas, the role of specific steroids, such as 11-ketotestosterone, is very clear in male teleost gametogenesis (Borg 1994).

In general, a relatively high temperature is required for spawning to take place, and in agreement with that, we registered temperatures ranging from 28°C to 32°C in the Tevere River during the spawning season of *L. cephalus*.

As seen in some reptiles (see the review in Ciarcia et al. 1986) and fish (see the review in Prat et al. 1990), in males of *L. cephalus*, androgens showed a bimodal pattern of fluctuation throughout the year. The 1st increase occurred during the stasis phase at the beginning of testicular recovery. Such a trend seems to fit well with the proposed dependence of spermatogonial mitosis and the transformation of spermatocytes into spermatids on the presence of the pituitary (Zirkin 1998, Oliveira et al. 2002). Indeed, in vertebrates the pituitary-gonadal axis plays an important role in regulating gametogenesis. In most cases, gonadotropins act through the biosynthesis of gonadal steroid hormones which in turn mediate various stages of gametogenesis (Fostier et al. 1983 1988). High levels of gonadotropins are associated with spermiation in cyprinids as in other vertebrates (Zirkin 1998, Schulz et al. 2001, Kandel-Kfir et al. 2002). Negative feedback exerted by androgens on gonadotropin secretion can be hypothesized for *L. cephalus* as already reported in mammalian species by Kandel-Kfir et al. (2002). This hypothesis may also explain the 2nd increase

in androgens, registered during the spawning period, when males of *L. cephalus* go through spermiation; this is related to the reproductive behavior. Although androgens are necessary for sustaining spermatogenesis in fish, the relationship between plasma levels of androgens and spermatogenesis is difficult to interpret as demonstrated by the observation that dihydrotestosterone stimulates spermiation in hypophysectomized goldfish (Billard et al. 1978). It seems that intratesticular androgen levels and the androgen receptor play more-important roles, and they do not always follow androgen plasma levels (Zirkin 1998).

Low levels of estradiol-17β were found in males of *L. cephalus* throughout the year. Fluctuations in estradiol-17β paralleled those of androgens, suggesting a close relationship between these 2 steroids. In vitro experiments have shown that androgens are a substrate for estradiol-17β production in both male and female teleosts (Kagawa et al. 1983, Borg 1994, Barannikova et al. 2002). In this species, the plasma estradiol concentration progressively rises in spring as breeding proceeds and usually peaks in summer when reproduction is completed. This steroid may act as a systemic negative feedback mechanism on the hypothalamic-pituitary axis, resulting in an inhibitory effect on gonadotropin secretion and/or a local action on endocrine tissues by the inhibitory regulation of some enzymes involved in steroid biosynthesis. We, furthermore, suspect that estradiol reduction after June may be related to gonadal regression and gametogenesis blockage as reported for the refractoriness in birds and reptiles (Ciarcia et al. 1986).

Although estradiol-17β is present in the plasma of a variety of male teleosts (Fostier et al. 1983), a role for this steroid has not yet been found, although in *Dasyatis sabina*, the Atlantic stingray, estrogen was associated with testicular development (Snelson et al. 1997, Schulz and Miura 2002).

The GSI of *L. cephalus* showed a peak in Apr. with average values of about 2.77 ± 0.04. This suggests that Apr. can be considered the month in which the fish prepares for the spawning season by emptying its cysts followed by a gametogenesis blockade and cyst regression. This thus confirms the histological data more than it does the endocrine patterns. After spawning (May~June) when spermatogenesis regresses, testicular weight, the GSI, and steroid levels are also

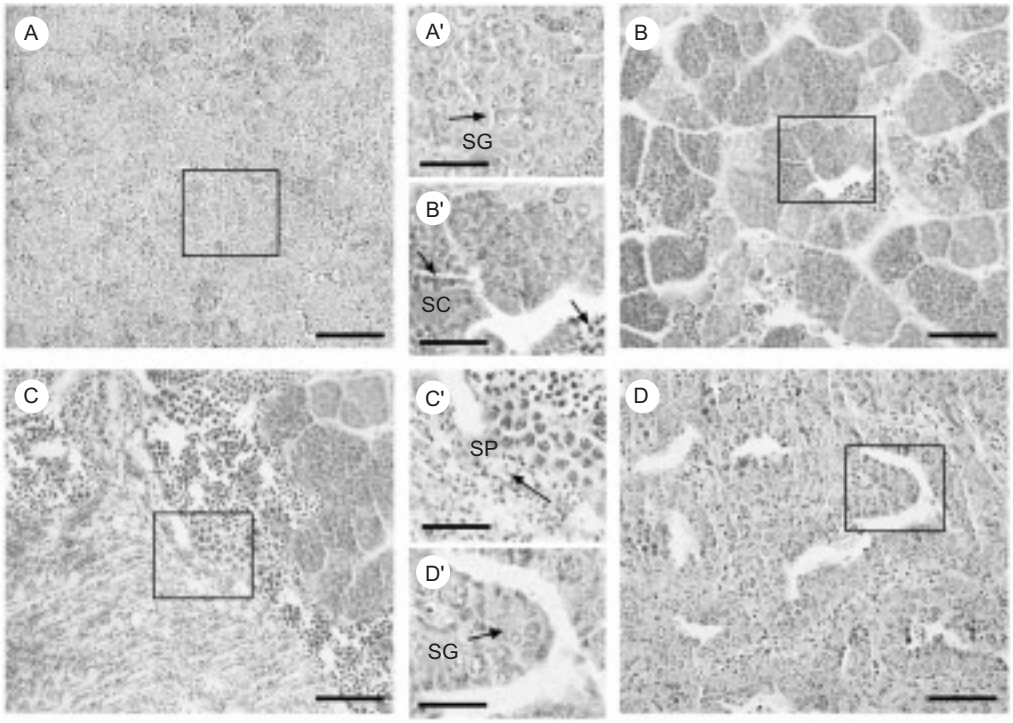


**Fig. 2.** Sections (A-D) and their magnifications (A'-D') of male gonads of *Leuciscus cephalus*. (A) Immature testis in Dec. showing spermatogonia (SG); (B) spermatogenetic activity in Apr. with spermatocytes (SC), spermatids (ST), and spermatogonia (SG); (C) spermatogenesis in June showing sperm (SP); (D) post-reproductive testis in Aug. mainly characterized by spermatogonia (SG). Stained with hematoxylin and eosin. Scale bar: A~D = 20 μm; A'~D' = 60 μm.

reduced by the influence of lower blood flow.

Furthermore, the GSI profile found in *L. cephalus* in the Tevere River of Italy is in accordance with the spawning season reported for this species in the Retina River in Greece, which occurs at the end of Apr. and the beginning of May (Unlu and Balci 1993). A slightly different spawning period has been reported for other populations of *L. cephalus* from Great Britain, Belgium, Spain, and Turkey. In these geographical areas, the spawning period begins a little later than in Greece and Italy (present data) and extends longer in time (Unlu and Balci 1993). These variations may be due to different water temperatures (Wen and Lin 2001).

In the Tevere River, the spawning period of *L. cephalus* is in agreement with the spawning period reported for other cyprinids by Rinchard and Kestemont (1996).

In conclusion, our data define the reproductive biology of the male chub, *Leuciscus cephalus*. Histological examination of the testes and sex steroid fluctuations in the plasma enabled us to divide the reproductive cycle into 4 stages identified by a characteristic population of germ cells and to determine the spawning period. Sex steroid fluctuations in the plasma correlated well with the maturation stage of the gonads and were in general agreement with what is known from other teleosts.

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## REFERENCES

Barannikova IA, VP Diubin, LV Baiunova, TB Semenkova. 2000. Steroids in the reproductive function regulation in fish. Rossiiskii Fiziologicheskii Zhurnal Imeni. I.M. Sechenova **86:** 968-978.

Berry C. 2003. Fishy business. Q. J. M.-Monthly. J. Assoc. Physician **96:** 83-84.

Billard R, B Breton, A Fostier, B Jalabert, C Weilk. 1978. Endocrine control of the teleost reproductive cycle and its relation to external factors: salmonid and cyprinid models. *In* PJ Galard, HH Beer, eds. Comparative endocrinology. Amsterdam: Elsevier, pp. 37-48.

Borg B. 1994. Androgens in teleost fishes. Comp. Biochem. Phys. C. **109:** 219-245.

Ciarcia G, F Angelini, A Polzonetti, M Zerani, V Botte. 1986. Hormones and reproduction in the lizard *Podarcis s. sicula* Raf. *In* I Assenmacher,and J Boissin, eds. Endocrine regulations and adaptive mechanisms to the environment. Paris: Centre National Recherche Scientifique, pp. 95-100.

Consten D, JG Lambert, H Komen, HJ Goos. 2002. Corticosteroids affect the testicular androgen production in male common carp (*Cyprinus carpio* L.). Biol. Reprod. **66:** 106-111.

Degani G, R Boker, K Jackson. 1998. Growth hormone, sexual maturity and steroids in male carp (*Cyprinus carpio*). Comp. Biochem. Physiol. C **120:** 433-440.

D'Istria M, G Delrio, V Botte, G Chieffi. 1974. Radioimmunoassay of testosterone, 17β-oestradiol and oestrone in the male and female plasma of *Rana esculenta* during sexual cycle. Steroids Lipids Res. **5:** 42-48.

Encina L, C Granado-Lorencio. 1991. Diet and diel feeding chronology of three Iberian fish species. Ecol. Int. Bull. **19:** 43-64.

Flammarion P. 2000. Induction of fish vitellogenin in testicular structure: preliminary results of estrogenic effects in chub. Ecotoxicology **9:** 127-135.

Fostier A, B Jalbert, R Billard, B Breton, Y Zohar. 1983. The gonadal steroids. *In* WS Hoar, DJ Randall, EM Donaldson, eds. Fish physiology. Vol. 9, Part B. New York, London: Academic Press, pp. 117-170.

Fostier A, F Le Gac, M Loir. 1988. Steroids in male reproduction. *In* DR Idler, LW Crim, JM Walsh, eds. Proceedings of the Third International Symposium on the Reproductive Physiology of fish. *Toronto*, Canada: Memorial Univ. of Newfoundland, pp. 239-245.

Guerriero G, M Paolucci, V Botte, G Ciarcia. 1998. The reproductive cycle of the cyprinid *Alburnus albidus*: morphological changes of the male gonads and plasma sex steroid fluctuations. Ital. J. Zool. **65:** 223-226.

Guidelines for the treatment of animals in behavioural research and teaching. 1998. Animal Behaviour **55:** 251-257.

Howes GJ. 1990. Systematic and biogeography: an overview. *In* IJ Winfield, JS Nelson, eds. Systematics, biology and exploitation, cyprinid fishes. London: Chapman and Hall, pp. 1-33.

Kagawa H, G Young, Y Nagahama. 1983. Changes in plasma steroid hormone levels during gonadal maturation in goldfish *Carassius auratus*. Bull. Jpn. Soc. Sci. Fish. **49:** 1783-1787.

Kandel-Kfir M, G Gur, P Melamed, Y Zilberstein, Y Cohen, N Zmora, M Kobayashi, A Elizur, Z Yaron. 2002. Gonadotropin response to GnRH during sexual ontogeny in the common carp, *Cyprinus carpio*. Comp. Biochem. Physiol. B **132:** 17-26.

Nash JP. 1999. Seasonal reproduction, fish. *In* E Knobil, JP Neill, eds. Encyclopedia of reproduction. Vol. 4. San Diego, London, Boston, New York, Sidney, Tokyo, Toronto: Academic Press, pp. 329-340.

Okuzawa K, K Furukawa, K Aida, I Hanyu. 1989. Effects of photoperiod and temperature on gonadal maturation, and plasma steroid and gonadotropin levels in a cyprinid fish, the honmoroko *Gnathopogon caerulescens*. Gen. Comp. Endocr. **75:** 139-147.

Oliveira RF, K Hirschenhauser, LA Carneiro, AV Canario. 2002. Social modulation of androgen levels in male teleost fish. Comp. Biochem. Physiol. B **132:** 203-215.

Orlando FE, GA Binczik, P Thomas, LJ Guillette Jr. 2003. Reproductive seasonality of the male Florida gar, *Lepisosteus platyrhincus*. Gen. Comp. Endocr. **131:** 365-371.

Polzonetti A, L Bellini, A Gobetti, V Botte, A Crasto. 1983. Plasma sex hormones and post-reproductive period in the green frog *Rana esculenta* complex. Gen. Comp. Endocrinol. **54:** 372-377.

Pottinger TG, TR Carrick, A Appleby, WE Yeomans. 2000.

High blood cortisol levels and low cortisol receptor affinity: Is the chub, *Leuciscus cephalus*, a cortisol-resistant teleost? Gen. Comp. Endocr. **120:** 108-117.

Prat F, S Zanuy, M Carrillo, A de Mones, A Fostier. 1990. Seasonal changes in plasma levels of gonadal steroids of sea bass, *Dicentrarchus labrax* L. Gen. Comp. Endocr. **78:** 361-373.

Rinchard J, P Kestemont. 1996. Comparative study of reproductive biology in single-and multiple-spawner cyprinid fish. I. Morphological and histological features. J. Fish Biol. **49:** 883-894.

Ronnback P, I Bryceson, N Kautsky. 2002. Coastal aquaculture development in eastern Africa and the western Indian Ocean: prospects and problems for food security and local economies. Ambio **31:** 537-542.

Schulz RW, HF Vischer, JEB Cavaco, EM Santos, CR Tyler, HJ Th Goos, J Bogerd. 2001. Gonadotropins, their receptors, and the regulation of testicular functions in fish. Comp. Biochem. Physiol. B **129:** 407-417.

Schulz RW, T Miura. 2002. Spermatogenesis and its endocrine regulation. Fish Physiol. Biochem. **26:** 43-56.

Sisneros JA, PM Forlano, R Knapp, AH Bass. 2004. Seasonal variation of steroid hormone levels in an intertidal-nesting fish, the vocal plainfin midshipman. Gen. Comp. Endocr. **136:** 101-116.

Snelson FF Jr, LE Rasmussen, MR Johnson, DL Hess. 1997. Serum concentrations of steroid hormones during reproduction in the Atlantic stingray, *Dasyatis sabina*. Gen. Comp. Endocr. **108:** 67-79.

Tollefsen KE, JF Meys, J Frydenlund, J Stenersen. 2002. Environmental estrogens interact with and modulate the properties of plasma sex steroid-binding proteins in juvenile Atlantic salmon (*Salmo salar*). Mar. Environ. Res. **54:** 697-701.

Tsigenopoulos CS, P Rab, D Naran, P Berrebi. 2002. Multiple origins of polyploidy in the phylogeny of southern *African barbs* (Cyprinidae) as inferred from mtDNA markers. Heredity **88:** 466-473.

Unlu E, K Balci. 1993. Observation on the reproduction of *Leuciscus cephalus orientalis* (Cyprinidae) in Savur Stream (Turkey). Cibium **17:** 241-250.

Wen H, H Lin. 2001. Effect of environmental factors on gonadal maturation as well as its ovulation and spawning in teleosts. Ying Yong Sheng Tai Xue Bao **12:** 151-155.

Zirkin BR. 1998. Spermatogenesis: its regulation by testosterone and FSH [Review]. Semin. Cell Dev. Biol. **9:** 417-421.