Journal of Fish Biology (2007) **70,** 83–99
doi:10.1111/j.1095-8649.2006.01257.x, available online at http://www.blackwell-synergy.com

Germ cell proliferation and apoptosis during different phases of swordfish (*Xiphias gladius* L.) spermatogenetic cycle

A. Corriero*, S. Desantis*, C. R. Bridges†, D. E. Kime‡, P. Megalofonou§, N. Santamaria*, F. Cirillo*, G. Ventriglia*, A. Di Summa*, M. Deflorio*, F. Campobasso‖ and G. De Metrio*¶

**Department of Animal Health and Welfare, University of Bari, S.P. per Casamassima km. 3, I-70010, Valenzano (BA), Italy*, *†Institut für Zoophysiologie, Lehrstuhl für Stoffwechselphysiologie, Heinrich Heine Universität, D-40225 Düsseldorf, Germany*, *‡Department of Animal and Plant Sciences, The University of Sheffield, Sheffield S10 2TN, U.K.*, *§Department of Zoology, Marine Biology, University of Athens, Panepistimiopolis GR 15784 Athens, Greece and ‖Department of Statistical Sciences, University of Bari, via Camillo Rosalba 53, 70124, Bari, Italy*

(Received 12 October 2005, Accepted 31 July 2006)

Seasonal changes of testicular activity of the swordfish *Xiphias gladius* and correlations of plasma levels of testosterone (T) and 11-ketotestosterone (11-KT) with proliferation and apoptosis of germ cells, determined, respectively, with monoclonal antibodies against proliferating cell nuclear antigen and terminal deoxynucleotidyl transferase-mediated d'UTP nick end labelling, are described. Three phases of the reproductive cycle were found: active spermatogenic (May), spawning (June to July) and spent (August to September) stages. Germ cell proliferating activity was highest in May, decreased during June to July and remained stable during August to September. Apoptotic germ cells, primary spermatocytes and spermatogonia, were present in all the specimens analysed and were more abundant in May. The levels of 11-KT in plasma were always higher than T and were highest in May, in concomitance with the maximum proliferation and apoptosis rate of germ cells.

Key words: apoptosis; germ cells; proliferation; spermatogenetic cycle; swordfish; testis.

INTRODUCTION

Spermatogenesis is a complex process in which spermatogonia become mature spermatozoa through a series of events involving mitosis, meiosis and cellular differentiation (Sharpe, 1994). In seasonally breeding mammals, testicular activity includes transitions between total arrest and recrudescence of spermatogenesis (Blottner *et al.*, 1995, 1999). During the transition from breeding to non-breeding state the testes undergo a remarkable atrophy due both to reduction of cell size and cell death (Young & Nelson, 2001).

¶Author to whom correspondence should be addressed. Tel.: +39 080 5443907; fax: +39 080 5443908; email: g.demetrio@veterinaria.uniba.it

Proliferating cell nuclear antigen (PCNA) is a polymerase delta accessory protein that is synthesized in late G1 and S phases of the cell cycle and is, therefore, correlated with the cell proliferating state (Woods *et al.*, 1991). The existence of PCNA has been used in numerous immunohistochemical studies as a biomarker of cell proliferation in a variety of normal and pathological tissues, the testis included (Woods *et al.*, 1991; Yu *et al.*, 1991; Štrbenc *et al.*, 2003).

Apoptosis is a form of cell death that plays a major role during the normal development and homeostasis of multicellular organisms (Sinha Hikim & Swerdloff, 1999). Germ cell apoptosis occurs during spermatogenesis of various mammalian species, and plays an important role in determining sperm output (Sharpe, 1994). In mammalian spermatogenesis, apoptosis occurs mainly at the level of spermatogonia (Dunkel *et al.*, 1997; Rodriguez *et al.*, 1997) or spermatocytes (Young *et al.*, 1999; Štrbenc *et al.*, 2003). Testosterone (T) withdrawal has been correlated with apoptosis of spermatocytes in the Syrian hamster (Nonclercq *et al.*, 1996) and of spermatocytes and spermatids in the rat (Woolveridge *et al.*, 1999), and an inverse correlation between proliferation and apoptosis of male germ cells has been observed in different seasonally breeding mammals (Blottner *et al.*, 1995; Young & Nelson, 2001; Štrbenc *et al.*, 2003).

The swordfish *Xiphias gladius* L. is a gonochoristic teleost of worldwide distribution. In the Mediterranean Sea, this top predator shows a reproductive cycle with a spawning period limited to summer months (Megalofonou *et al.*, 1995; Corriero *et al.*, 2004). In the teleost testis, germ cells and their associated Sertoli cells are organized in cysts (spermatocysts), each one containing a clone of germ cells at the same development stage (Grier *et al.*, 1980; Billard *et al.*, 1982). Two types of testicular organization have been described in teleosts (Grier *et al.*, 1980): unrestricted spermatogonial type (typical of Salmoniformes, Perciformes and Cypriniformes) and restricted spermatogonial type (typical of Atheriniformes). In the former, spermatogonia are found along the entire length of testicular tubules, while in the latter, spermatogonia are restricted to the distal end of the tubules.

In teleosts, the predominant androgens which stimulate spermatogenesis and testis growth are 11-ketotestosterone (11-KT) and, to a lesser extent, T (Scott *et al.*, 1980; Billard *et al.*, 1982; Kime & Manning, 1982; Borg, 1994; Weltzien *et al.*, 2002). Studies on the role of apoptosis in the regulation of fish spermatogenesis are scarce. Few investigations have been carried out on cartilaginous fishes such as the shark *Squalus achantias* L., where apoptosis has been documented at the level of spermatogonia and preleptotene spermatocytes (Callard *et al.*, 1995), and the spotted ray *Torpedo marmorata* Risso in which apoptosis involved spermatocytes, spermatids and Sertoli cells (Prisco *et al.*, 2003).

The seasonal changes of testicular activity in swordfish from the Mediterranean Sea were examined in the present study and correlations of plasma androgen levels with proliferation and apoptosis of germ cells were investigated.

MATERIALS AND METHODS

SAMPLING

Blood and testis samples from 22 swordfish with lower-jaw fork length (L_{LJF}) $\geq$110 cm were obtained during May ($n = 7$), June to July ($n = 9$) and August to September

($n = 6$) 2000 aboard commercial fishing vessels operating in the North Ionian Sea (Gulf of Taranto), using long lines or drift nets. Soaking times for both fishing gears ranged between 0 and 6 h. After fish capture, L_{LJF} was measured to the nearest cm, the testes were removed and the testis mass (M_G) measured to the nearest g. Slices of testes (1 cm thick) were fixed in Bouin's solution or neutral 10% formalin for light microscopy studies. At landing, the eviscerated body mass (M_B) was weighted to the nearest 100 g.

For T and 11-KT measurements, blood was collected from the heart with heparinized syringes. Syringes were rinsed in advance with a solution containing 200 mM NaCl, 8·6 mM KCl, 8000 IU ml^{-1} sodium heparin (Fluka Chemie, Buchs, Switzerland) and 1 mM phenylmethylsulphonylfluoride (Sigma-Aldrich, Milan, Italy), pH 7·3. Blood was kept on ice after sampling at sea and then centrifuged at 5000 *g* for 15 min. Plasma was collected using a plastic pipette and stored at −20° C until analysis for hormones.

BASIC HISTOLOGY

Fixed gonad samples were dehydrated in ethanol and embedded in paraffin wax. Tissue sections (5 μm thick) were mounted on slides pre-coated with poly-L-lysine (Menzel-Glaser, Braunschweig, Germany). The sections were de-paraffinized in xylene, re-hydrated through graded ethanol solutions and either stained with haematoxylin–eosin to study the seasonal changes of testicular activity, or processed immunohistochemically for the detection of PCNA and apoptotic cells [terminal deoxynucleotidyl transferase-mediated d'UTP nick end labelling (TUNEL) method].

IMMUNOHISTOCHEMICAL DETECTION OF PCNA

For the immunohistochemical detection of PCNA, sections were pre-treated for 30 min with 0·3% H_2O_2 in methanol to inhibit endogenous peroxidase activity. They were then incubated for 30 min in normal horse serum (NHS) (Vector, Burlingame, CA, U.S.A.) diluted 1:50 in phosphate buffered saline (PBS) (0·01 M phosphate buffer pH 7·4 containing 0·15 M NaCl) to block non-specific binding sites for immunoglobulins. The sections were then incubated overnight at 4° C, in a moist chamber with monoclonal antibodies to PCNA (ICN Pharmaceuticals, Milan, Italy) diluted 1:25 in PBS containing 0·1% BSA. After rinsing for 10 min in PBS, the immunohistochemical visualization was carried out using the Vectastain Universal Elite Kit (Vector). This method utilized the avidin–biotin–peroxidase complex procedure. Peroxidase activity was visualized by incubating for 10 min with Vector DAB Peroxidase Substrate Kit (Vector), which produces a brown precipitate.

To confirm the specificity of the immunostaining, the following control staining procedures were carried out: (1) replacement of primary antibody with NHS and (2) omission of the primary antibody.

DEMONSTRATION OF APOPTOTIC CELLS

Detection of apoptotic cells was performed using the TUNEL method with an In Situ Cell Death Detection Kit, AP (Roche Diagnostics, Mannheim, Germany) that was used in accordance with the manufacturer's instructions. Prior to incubation with the reaction mixture, the slides were incubated in a permeabilization solution of 0·1% Triton X-100 in 0·1% sodium citrate for 8 min at 37° C. Terminal deoxynucleotidyl transferase was diluted 1:5 in TUNEL Dilution Buffer (Roche Diagnostics). A ready to use solution of NBT/BCIP (Roche Diagnostics) was used as a substrate for the signal conversion.

TESTOSTERONE AND 11-KETOTESTOSTERONE MEASUREMENT

Two hundred μl of plasma were extracted three times with 5 ml dichloromethane, the extracts redissolved and 11-KT and T measured by ELISA using acetylcholinesterase as tracer (Cuisset *et al.*, 1994; Nash *et al.*, 2000).

MORPHOMETRY, GONADO-SOMATIC INDEX, QUANTIFICATION OF CELL PROLIFERATION AND APOPTOSIS, AND STATISTICAL ANALYSES

The diameter of germ cells was measured on at least 50 cells for each developmental stage. The diameter of seminiferous tubules was determined on at least 50 randomly chosen tubules per specimens. The relative testicular mass (gonado-somatic index, I_G) was calculated as $I_G = 100\ M_G M_B^{-1}$.

The percentage of anti-PCNA-positive spermatocysts, the percentage of cysts containing TUNEL-positive germ cells, and the mean number of apoptotic cells per spermatocysts were measured in 200 randomly selected spermatocysts per specimen. The apoptotic index was calculated by multiplying the percentage of cysts containing apoptotic germ cells by the number of apoptotic germ cells per cyst.

Significant differences among I_G, diameter of seminiferous tubules, percentage of anti-PCNA-positive spermatocysts, percentage of cysts containing apoptotic cells, number of apoptotic cells per spermatocysts, as well as plasma T and 11-KT levels during different periods were assessed using the Kruskal–Wallis one-way ANOVA and the Mann–Whitney *U*-test.

The Pearson coefficient (r) was used to evaluate the correlation between the percentage of anti-PCNA-positive spermatocysts and the percentage of cysts containing apoptotic cells. As the data did not show a reliable bivariate normality of the two percentages in the entire population, the Spearman coefficient (r_s) was also used for the same purpose in the eventuality of a non-parametric inference.

Finally, in order to assess the androgen influence on proliferation and apoptosis of male germ cells, T and 11-KT plasma levels were used as explanatory variables in a logit model, whereas the explained variable was either the percentage of anti-PCNA-positive spermatocysts or the percentage of cysts containing apoptotic cells. Statistical significance was assessed by Wald χ^2 test.

In all analyses, statistical significance was accepted at $P < 0{\cdot}05$ and results are presented as mean ± S.E.

RESULTS

SEASONAL CHANGES OF TESTICULAR ACTIVITY

The swordfish testis consisted of seminiferous tubules, interspersed in the connective stroma, radiating from the longitudinal main sperm duct towards the testicular periphery [Fig. 1(a)]. Within seminiferous tubules, germ cells developed in groups called germinal cysts or spermatocysts, each of them consisting of isogenic germ cells enveloped by Sertoli cell processes [Fig. 1(b)].

Type A spermatogonia, with diameter 10·4 ± 0·4 μm and high nucleocytoplasmic ratio, were single cells distributed all along the germinal epithelium and showed a roundish eucromatic nucleus with an eccentric basophilic nucleolus and a slightly acidophilic cytoplasm. Type B spermatogonia were found in small groups. They had a smaller diameter (6·4 ± 0·4 μm) and a lower nucleocytoplasmic ratio than type A spermatogonia, and displayed a nucleus with chromatin strands and one or more nucleoli and a weakly acidophilic cytoplasm. Primary spermatocytes and secondary spermatocytes measured 6·2 ± 0·3 μm and 4·4 ± 0·3 μm, respectively, and were characterized by a heterochromatic nucleus whose appearance varied according to the different phases of the meiosis. Spermatids (2·6 ± 0·1 μm in diameter) were characterized by a strongly basophilic nucleus, which condensed progressively and became indented.

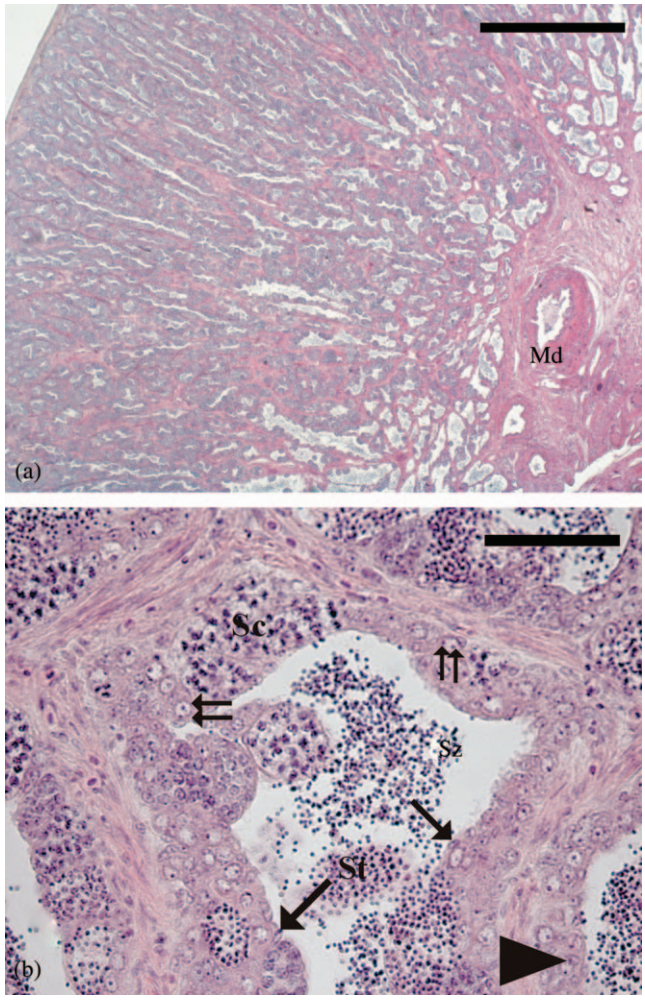

FIG. 1. Micrographs of testes from swordfish caught in May. (a) Testis showing numerous seminiferous tubules radiating from the main sperm duct towards the testicular periphery. Haematoxylin–eosin staining (bar = 1000 μm). (b) Higher magnification of a seminiferous tubule showing spermatocysts containing germ cells at all the spermatogenic stages. Spermatids and mature spermatozoa can be observed in the lumen. Haematoxylin–eosin staining (bar = 40 μm). →, Sertoli cell nucleus; ⇉, spermatogonium A; ►, spermatogonium B; Md, main sperm duct; Sc, spermatocytes; St, spermatids; Sz, spermatozoa.

Spermatozoa were flagellated cells with an oval head intensely stained with haematoxylin. Mature spermatozoa were released into the tubule lumen after the breakdown of cysts. Immature germ cells, mostly spermatocytes and spermatids, were often observed in the tubule lumen.

Testis activity showed seasonal changes, thus allowing for definition of three different phases. In May, when active spermatogenesis occurred, testes showed a heterogeneous histological appearance with both seminiferous tubules in early and late spermatogenesis. Seminiferous tubules at early spermatogenic stage

[Fig. 2(a)] showed mainly spermatogonial cysts, few cysts with spermatocytes, rare cysts containing spermatids and few spermatozoa in the lumen. The diameter of seminiferous tubules was 100·6 ± 4·5 μm. The lumen of seminiferous tubules was restricted or absent. In seminiferous tubules at late spermatogenic stage [Fig. 2(b)], most of the germinal epithelium surface was occupied by cysts containing spermatocytes and spermatids. The diameter of seminiferous tubules increased significantly to 104·0 ± 9·5 μm. Cysts containing spermatozoa and luminal spermatozoa were also observed in most of seminiferous tubules.

In June to July (spawning stage) the germinal epithelium consisted mainly of cysts containing spermatids and spermatozoa. The tubule diameter rose significantly to 115·2 ± 8·7 μm. The lumen of seminiferous tubules was filled with spermatozoa [Fig. 2(c)]. In August to September (spent stage) the germinal epithelium often consisted exclusively of spermatogonia, and only few residual spermatocysts were visible, mainly with spermatids or spermatozoa. The diameter of seminiferous tubules decreased significantly to 107·7 ± 4·6 μm. Luminal residual spermatozoa could still be observed [Fig. 2(d)].

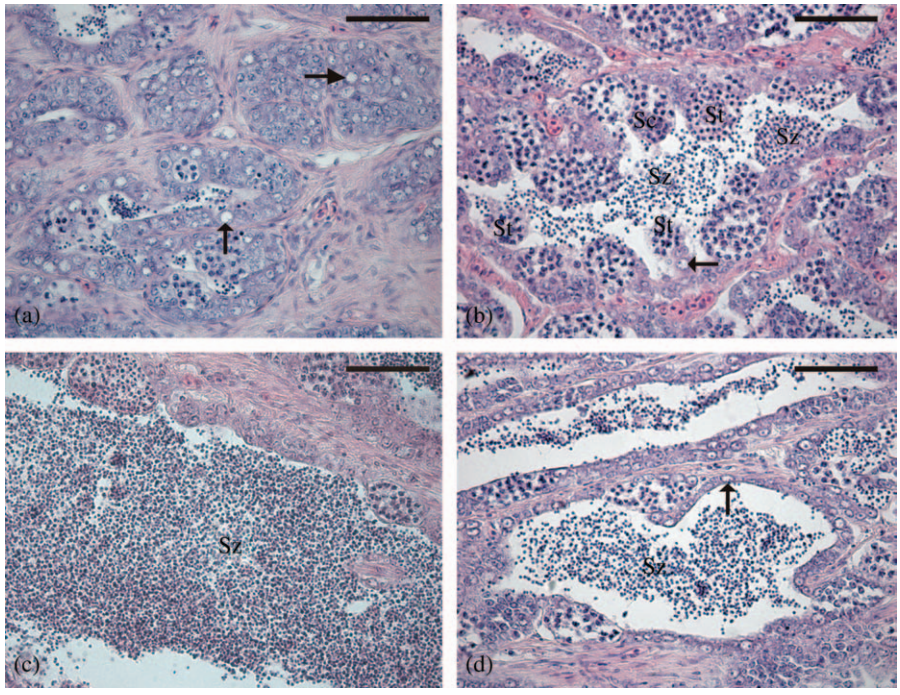

FIG. 2. Micrographs of swordfish testes. (a) Seminiferous tubules at early spermatogenic stage in the testis from a specimen caught in May. Few spermatozoa are present in the lumen. Haematoxylin–eosin staining. (b) Seminiferous tubules at late spermatogenic stage in the testis from a specimen caught in May. Haematoxylin–eosin staining. (c) Detail of seminiferous tubules from a specimen caught in July. The lumen is largely filled with spermatozoa. Haematoxylin–eosin staining. (d) Seminiferous tubules in the testis from a specimen caught in August. Residual spermatozoa are present in the lumen. Haematoxylin–eosin staining. Bars in (a), (b), (c), (d) = 50 μm. →, spermatogonium; Sc, spermatocytes; St, spermatids; Sz, spermatozoa.

Mean I_G values changed significantly from each period to the following one (Fig. 3), increasing from 0·21 ± 0·04 in May to 0·34 ± 0·02 in June to July and decreased to 0·18 ± 0·03 in August to September.

DETECTION OF PROLIFERATING AND APOPTOTIC GERM CELLS

Anti-PCNA-positive cysts were present in all the specimens analysed. The immunoreaction was observed in spermatogonia and in primary spermatocytes (Fig. 4). Germ cell proliferating activity was highest in May when 68·5 ± 3·5% of the cysts were anti-PCNA immunoreactive. The percentage of cysts containing immunopositive germ cells decreased significantly during June to July (62·3 ± 2·0%) and remained unchanged in August to September [Fig. 5(a)].

Spermatocysts containing TUNEL-positive germ cells were observed in all the specimens analysed and occasional luminal apoptotic cells were also observed (Fig. 6). TUNEL reaction involved mainly primary spermatocytes, but also type B spermatogonia. The percentage of cysts containing apoptotic cells was highest in May (11·8 ± 1·1%), decreased significantly in June to July (7·3 ± 0·5%) and then remained stable during August to September [Fig. 5(b)]. The average number of apoptotic cells per germinal cyst [Fig. 5(c)] was highest in May (2·3 ± 0·2), decreased significantly in June to July (1·7 ± 0·2) and reached the minimum value during August to September (1·2 ± 0·1). The same trend was shown by the apoptotic index (Fig. 5).

SEASONAL CHANGES IN T AND 11-KT AND CORRELATION WITH GERM CELL PROLIFERATION AND APOPTOSIS

Plasma T levels increased from 0·44 ± 0·14 ng ml^{-1} in May to a peak level in June to July (1·69 ± 0·14 ng ml^{-1}) and decreased to 0·38 ± 0·08 ng ml^{-1} in August to September (Fig. 7). Plasma 11-KT levels showed wider changes than T ones. The levels were highest in May (5·90 ± 2·20 ng ml^{-1}), decreased during June to July (3·13 ± 0·86 ng ml^{-1}) and then fell significantly during August to September (0·60 ± 0·19 ng ml^{-1}).

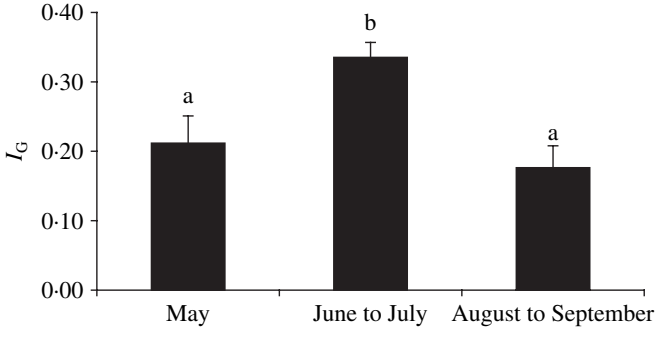

FIG. 3. Seasonal changes in swordfish gonado-somatic index. Identical lowercase letters represent statistical non-significance ($P > 0·05$).

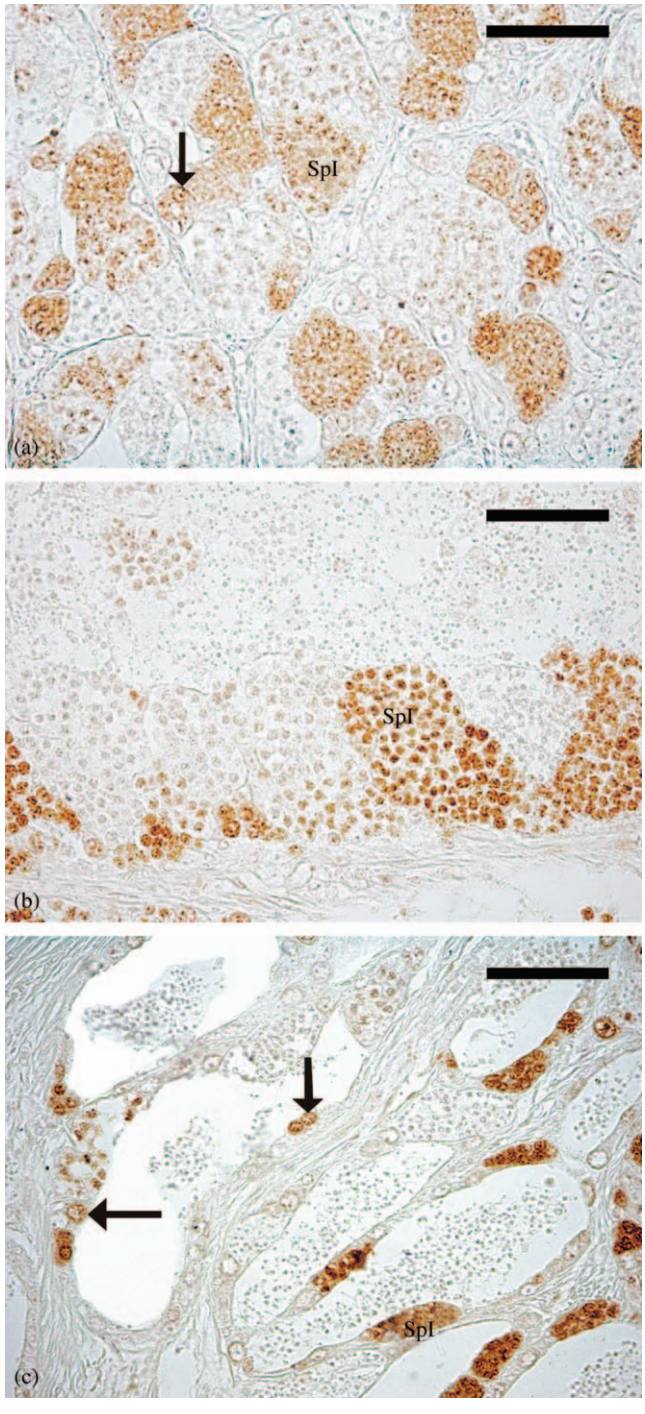

FIG. 4. Anti-PCNA immunopositive germ cells in testes from swordfish caught in different periods: (a) May, (b) June and (c) August. Bars in (a), (b), (c) = 50 μm. →, Spermatogonium; SpI, primary spermatocytes.

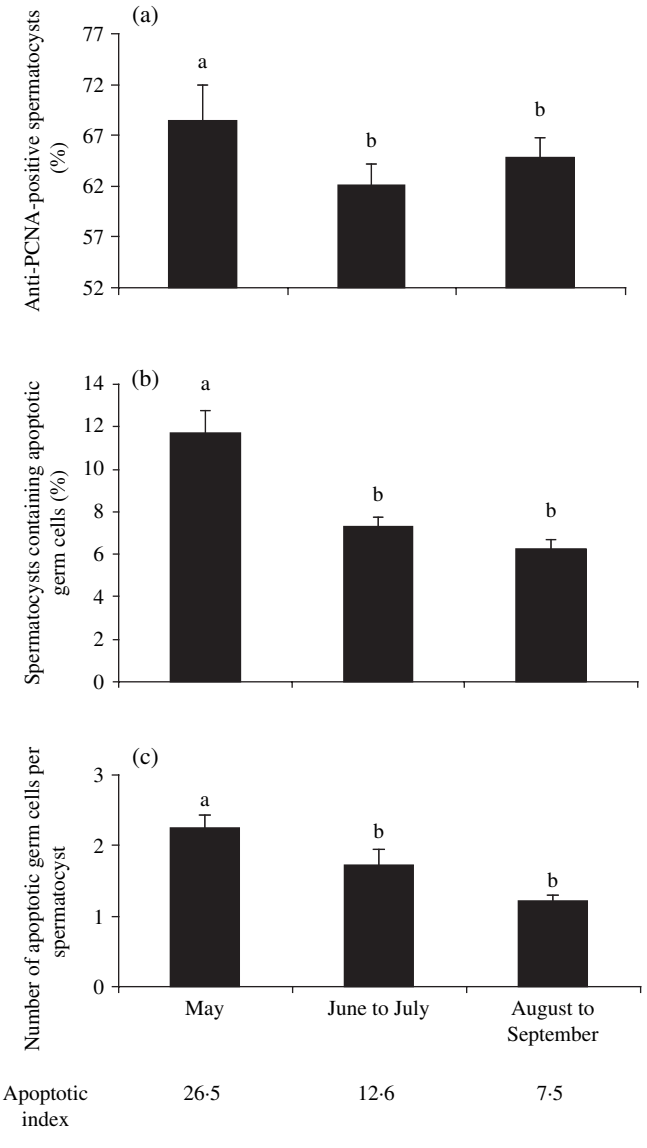

FIG. 5. Seasonal changes in proliferation and apoptosis of swordfish male germ cells. (a) Changes in the percentage of spermatocysts containing anti-PCNA-positive germ cells. (b) Changes in the percentage of spermatocysts containing TUNEL-positive germ cells. (c) Changes in the number of apoptotic germ cell per spermatocysts. Identical lowercase letters represent statistical non-significance ($P > 0{\cdot}05$). Values of the apoptotic index are also given.

A low positive, although not significant, correlation between the percentage of PCNA-positive spermatocysts and the percentage of cysts containing apoptotic cells was shown by r and r_s (0·270 and 0·354, respectively).

The two androgens appeared to be significantly correlated neither to the percentage of anti-PCNA-positive spermatocysts nor to the percentage of cysts containing apoptotic cells.

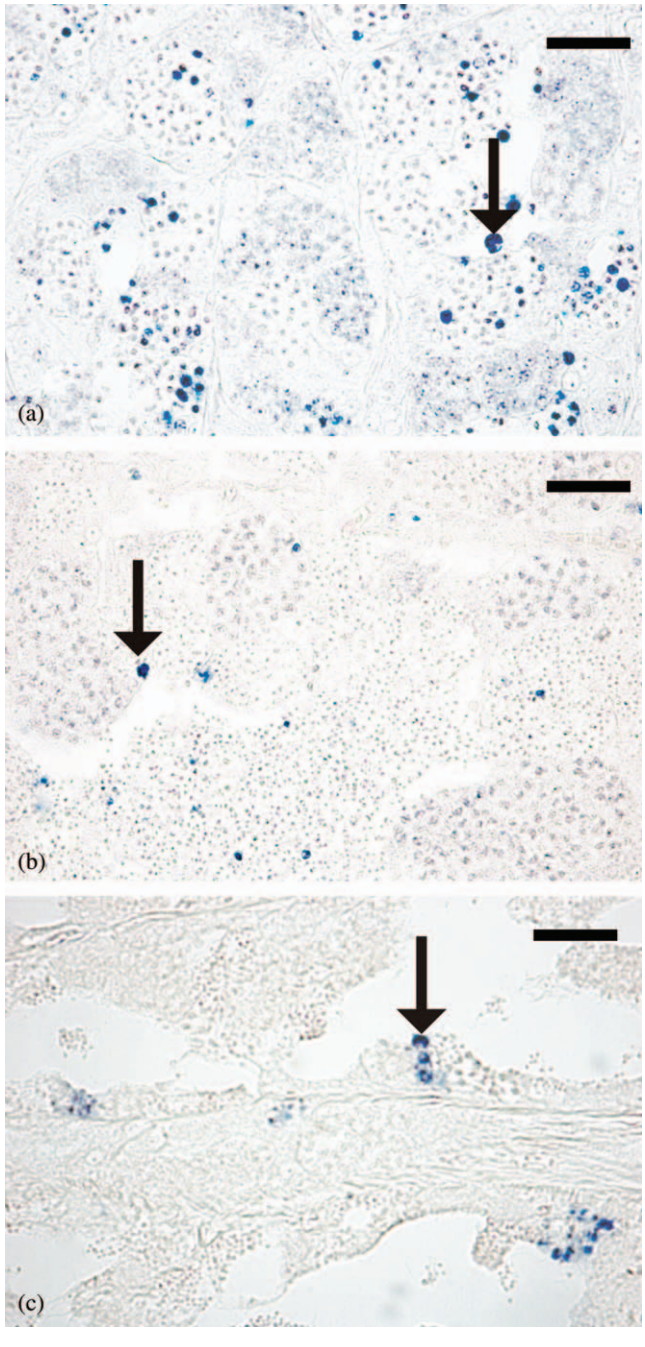

FIG. 6. Localization of TUNEL-labelled apoptotic germ cells in testes from swordfish caught in different periods: (a) May, (b) June and (c) August. Bars in (a), (b), (c) = 50 μm. →, Representative TUNEL-positive germ cells.

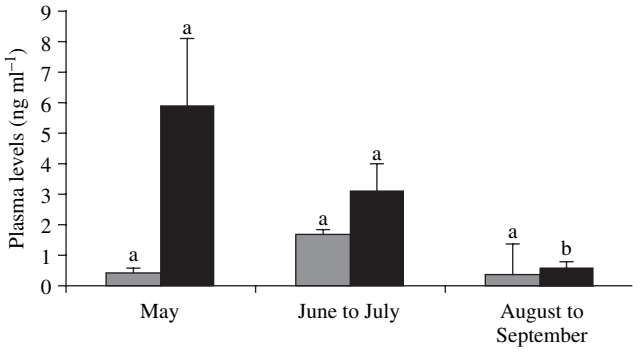

FIG. 7. Seasonal changes in mean ± S.E. testosterone (T; ▒) and 11-ketotestosterone (11-KT; ■) plasma levels. Identical lowercase letters represent statistical non-significance ($P > 0{\cdot}05$).

DISCUSSION

The testicular organization of the swordfish testis, as already described in histological (De Metrio *et al.*, 2003) and ultrastructural studies (De Metrio *et al.*, 1996; Cefali *et al.*, 1998; Minniti *et al.*, 2005), corresponds to the 'unrestricted spermatogonial type' of Grier *et al.* (1980). The walls of the seminiferous tubules contained Sertoli cells, spermatogonia, spermatocytes, spermatids and spermatozoa. Clusters of germ cells in the same developmental stage associated with Sertoli cells constituted true cysts. Immature germ cells were present within the tubule lumen. The presence of immature germ cells in the tubule lumen has been already observed in several other teleosts, such as members of the *Neoceratiidae* (Jespersen, 1984), blenniid fishes (Lahnsteiner & Patzner, 1990), *Ophidion* spp. (Mattei *et al.*, 1993) and *Lophiomus setigerus* (Vahl) (Yoneda *et al.*, 1998). Germ cells within cysts are linked by cytoplasmic bridges, which are necessary for their synchronous development (Cefali *et al.*, 1998; Minniti *et al.*, 2005). Once released in the lumen cytoplasmic bridges break down, possibly leading to an asynchronous differentiation (Mattei *et al.*, 1993). Mattei *et al.* (1993) proposed that testes with spermatogenesis not being completed entirely within cysts should be distinguished from the cystic type typical of most teleosts, and termed semi-cystic. This definition does not seem appropriate to the swordfish since in this species cysts with cells in all stages of spermatogenesis, spermatozoa included, were found in the seminiferous epithelium, although sometimes cysts could open prematurely releasing immature cells into the lumen.

The histological analysis of testes from swordfish caught at different periods showed seasonal changes in testicular activity. In May, all stages of spermatogenesis were present, in June to July testes were filled with spermatozoa and during August to September testes appeared regressed, having tubules mainly consisting of spermatogonia and residual spermatozoa. The seasonal trend of I_G well represented the changes of testicular activity. In fact, mean I_G values showed an increase from May to June to July, followed by a decrease in August to September. Swordfish incidentally captured, throughout the period not covered by the present study (October to April), by fishing vessels targeting other fish species, had quiescent testes with seminiferous tubules mainly constituted

by spermatogonia (unpubl. data). Several studies based on seasonal trend of I_G (Orsi Relini *et al.*, 1996; de la Serna *et al.*, 1996; De Metrio *et al.*, 1998; Tserpes *et al.*, 2001), larval findings (Potoschi *et al.*, 1994) or juvenile daily growth (Megalofonou *et al.*, 1995) indicated that swordfish reproduction in different spawning areas of the Mediterranean Sea occurs between June and September. A histological study carried out on swordfish ovaries in the North Ionian Sea (Corriero *et al.*, 2004) demonstrated the presence of hydrated oocytes and post-ovulatory follicles only in June and July. In the present study, testes filled with spermatozoa were found only in this last period. Taken together, these histological findings seem to indicate that the spawning period of this species in the North Ionian Sea is restricted to June and July.

In the present study, monoclonal antibodies to PCNA were used in order to follow germ cell proliferation during spermatogenesis in the swordfish. The highest percentage of PCNA-positive cysts was found in May, when an intense proliferating activity occurred. The number of cysts containing anti-PCNA-positive cells decreased significantly in the spawning stage (June to July), and they did not change significantly in the spent period (August to September), even if in this last period the immunopositivity was often localized at the level of cysts containing single spermatogonia.

Apoptosis is a form of cell death and it is morphologically identified by characteristic membrane blebbing, condensation of the nucleus and DNA condensation (Vaux & Strausser, 1996; LeGrand, 1997). In mammals, apoptosis is an integral component of normal testicular function and has been hypothesized to limit the germ cell population and prevent maturation of aberrant germ cells (Yin *et al.*, 1998). In the cartilaginous fish the spotted ray, Prisco *et al.* (2003) suggested a role of apoptosis in the regulation of the ratio of germ cells associated with Sertoli cell. In juvenile zebrafish *Danio rerio* (Hamilton) 30 days post-hatching, during transition from ovary-like undifferentiated gonadal tissue to testis, all the oocytes die by apoptosis and spermatocytes develop (Uchida *et al.*, 2002). An increase of apoptosis in the medaka *Oryzias latipes* (Temminck & Schlegel) spermatocytes has been experimentally induced by chronic aqueous exposure to nonylphenol (Weber *et al.*, 2002). In the present study, TUNEL-positive germ cells, presumptively type B spermatogonia and spermatocytes, were observed in all the specimens analysed. This finding is in agreement with the ultrastructural observations of Cinquetti & Dramis (2003), who described apoptotic degeneration of spermatogonia and spermatocytes during the phase of proliferation of secondary spermatogonia of the teleost *Padogobius martensi* (Günther). Similar findings were also reported in seasonal breeding mammals where TUNEL labelling data indicated that spermatocytes are the cell type more frequently involved in apoptosis (Young & Nelson, 2001).

As already reported for several teleosts (Billard *et al.*, 1972; Grier, 1993; Cinquetti & Dramis, 2003), degenerating germ cells may be phagocytized by Sertoli cells. In the swordfish, the presence of TUNEL-positive cells within the tubule lumen allow the hypothesis to be made that degenerating germ cells can simply be voided *via* the genital opening.

The maximum apoptotic rate of germ cells was found in May, in concomitance with the maximum proliferating activity of the same cells. The quantitative analysis of TUNEL-positive germ cells during the spermatogenetic cycle

showed no significant correlation between germ cell proliferation and apoptosis. In seasonal breeding mammals, increased rate of apoptosis occurs during testicular regression, while little testicular apoptosis is observed during recrudescence or the breeding season (Blottner *et al.*, 1995; Young & Nelson, 2001; Štrbenc *et al.*, 2003), although in mink the highest apoptotic rate of testicular cells was found during the breeding period (Blottner *et al.*, 1999).

Testosterone and 11-KT are the predominant androgens in teleosts (Borg, 1994). It is known that increases in plasma cortisol can affect steroid hormones, usually causing a reduction in their levels (Consten *et al.*, 2002). Acute stress in adult sockeye salmon *Oncorhynchus nerka* (Walbaum), however, did not appear to play an important role in controlling sex steroid levels (Patterson *et al.*, 2004), and no evidences of the effects of gear-type on steroid hormones were found in the cod *Gadus morhua* L. when comparing samples collected with different fishing gears in the same area on the same day (Comeau *et al.*, 2001). Since the swordfish specimens used for the present study remained at sea 1–6 h between capture and sampling, T and 11-KT levels could be affected by capture-induced acute stress. Therefore, the reported levels of steroids hormones are more useful for comparison between the different reproductive phases rather than absolute values.

Throughout the investigated period in the present study, 11-KT occurred in larger concentrations than T, with peak levels almost four-fold higher than those for T, indicating that 11-KT is the predominant androgen in this species. This is in agreement with the findings in other fishes such as the winter flounder *Pseudopleuronectes americanus* (Walbaum) (Harmin *et al.*, 1995), Pacific halibut *Hippoglossus stenolepis* Schmidt (Liu *et al.*, 1991), English sole *Pleuronectes vetulus* (Girard) (Sol *et al.*, 1998), Atlantic halibut *Hippoglossus hippoglossus* (L.) (Weltzien *et al.*, 2002) and Pacific herring *Clupea pallasii* Valenciennes (Koya *et al.*, 2002). In swordfish, plasma 11-KT levels were highest in the pre-reproductive period and decreased in the following periods. The same trend was found in the Pacific herring (Koya *et al.*, 2002), while in Atlantic halibut 11-KT was maintained at peak levels throughout the pre-reproductive and reproductive period (Weltzien *et al.*, 2002). *In vitro* experiments demonstrated that 11-KT induces all stage of spermatogenesis in the Japanese eel *Anguilla japonica* Temminck & Schlegel (Miura *et al.*, 1991).

In mammals, T is a cell survival factor for germ cells (Young & Nelson, 2001). Withdrawal of T induces apoptosis in the testis (Nandi *et al.*, 1999; Woolveridge *et al.*, 1999) and reintroduction of this steroid hormone can reduce apoptotic cell death (Nandi *et al.*, 1999). In the swordfish, a significant influence of T and 11-KT on apoptosis of germ cells could not be demonstrated. The presence of a high apoptosis rate during the maximum spermatogenic activity could be explained by a role of apoptosis in the quantitative control of germ cell population as well as in the prevention of the maturation of aberrant germ cells (Blanco-Rodriguez & Martinez-Garcia, 1996).

In conclusion, spermatogenesis in swordfish from the Mediterranean Sea begins in May, at a time when plasma 11-KT levels are maximal. Spermiation occurs in June to July, at which time plasma 11-KT levels decrease and I_G is highest. Germ cell proliferation and apoptosis occurs throughout the reproductive season, although they are maximal at the onset of spermatogenesis.

We wish to thank F. Angelini for his critical revision that helped to improve the manuscript. We wish also to thank A. Marinelli, V. Pesola, M. Cacucci and A. Disabato for their technical assistance. This work was partially supported by an EU project SIDS Contract Nr: QLRT-PL 1999-01567, and partially by 'Fondi di Ateneo 2006' from the University of Bari.

References

Billard, R., Jalabert, B. & Breton, B. (1972). Les cellules de Sertoli des poissons téléostéens. I. Étude ultrastructurale. *Annales de Biologie Animale, Biochimie et Biophysique* **12,** 19–32.

Billard, R., Fostier, A., Weil, C. & Breton, B. (1982). Endocrine control of spermatogenesis in teleost fish. *Canadian Journal of Fisheries and Aquatic Sciences* **39,** 65–79.

Blanco-Rodriguez, J. & Martinez-Garcia, C. (1996). Spontaneous germ cell death in the testis of the adult rat takes the form of apoptosis: re-evaluation of cell types that exhibit the ability to die during spermatogenesis. *Cell Proliferation* **29,** 13–31.

Blottner, S., Hingst, O. & Meyer, H. H. D. (1995). Inverse relationship between testicular proliferation and apoptosis in mammalian seasonal breeders. *Theriogenology* **44,** 320–328.

Blottner, S., Roelants, H., Wagner, A. & Wenzel, U. D. (1999). Testicular mitosis, meiosis and apoptosis in mink (*Mustela vison*) during breeding and non-breeding season. *Animal Reproduction Sciences* **57,** 237–249.

Borg, B. (1994). Androgens in teleost fishes. *Comparative Biochemistry and Physiology* **109C,** 219–245.

Callard, G. V., Jorgensen, J. C. & Redding, J. M. (1995). Biochemical analysis of programmed cell death during premeiotic stages of spermatogenesis in vivo and in vitro. *Developmental Genetics* **16,** 140–147.

Cefali, A., Bruno, R. & Minniti, F. (1998). Intercellular bridges in the immature swordfish (*Xiphias gladius* L. 1758) testis. *Biologia Marina Mediterranea* **5,** 670–673.

Cinquetti, R. & Dramis, L. (2003). Histological, histochemical and ultrastructural investigations of the testis of *Padogobius martensi* between annual breeding seasons. *Journal of Fish Biology* **63,** 1402–1428.

Comeau, L. A., Campana, S. E., Chouinard, G. A. & Hanson, J. (2001). Timing of Atlantic cod *Gadus morhua* seasonal migrations in relation to serum levels of gonadal and thyroidal hormones. *Marine Ecology Progress Series* **221,** 245–253.

Consten, D., Keuning, E. D., Bogerd, J., Zandbergen, M. A., Lambert, J. G. D., Komen, J. & Goos, H. J. Th. (2002). Sex steroids and their involvement in the cortisol-induced inhibition of pubertal development in male common carp, *Cyprinus carpio* L. *Biology of Reproduction* **67,** 465–472.

Corriero, A., Acone, F., Desantis, S., Zubani, D., Deflorio, M., Ventriglia, G., Bridges, C. R., Labate, M., Palmieri, G., McAllister, B. G., Kime, D. E. & De Metrio, G. (2004). Histological and immunohistochemical investigation on ovarian development and plasma estradiol levels in the swordfish (*Xiphias gladius* L.). *European Journal of Histochemistry* **48,** 413–422.

Cuisset, B., Pradelles, P., Kime, D. E., Kühn, E. R., Babin, P., Davail, S. & Le Menn, F. (1994). Enzyme immonoassay for 11-ketotestosterone using acetylcholinesterase as label: application to the measurement of 11-ketotestosterone in plasma of Siberian sturgeon. *Comparative Biochemistry and Physiology C* **108,** 229–241.

De Metrio, G., Desantis, S., Santamaria, N., Labate, M., Acone, F., Sanna, L. & Palmieri, G. (1996). Osservazioni in TEM sui tubuli seminiferi del testicolo di giovani di pesce spada (*Xhiphias gladius* L.). *Biologia Marina Mediterranea* **3,** 365–369.

De Metrio, G., Cacucci, M., Corriero, A., Santamaria, N. & Spedicato, D. (1998). Investigation on the fishing activities and on the biology of large pelagic fish (*Thunnus thynnus* L., *Thunnus alalunga* Bonn., *Sarda sarda* Bloch, *Xiphias gladius* L., *Auxis rochei* Risso) in the northern Ionian Sea from 1990 to 1997. *Biologia Marina Mediterranea* **5,** 215–228.

De Metrio, G., Corriero, A., Desantis, S., Zubani, D., Cirillo, F., Deflorio, M., Bridges, C. R., Eicker, J., de la Serna, J. M., Megalofonou, P. & Kime, D. E. (2003). Evidence of a high percentage of intersex in the Mediterranean swordfish. *Marine Pollution Bulletin* **46,** 358–361.

Dunkel, L., Hirvonen, V. & Erkkila, K. (1997). Clinical aspect of male germ cell apoptosis during testis development and spermatogenesis. *Cell Death and Differentiation* **4,** 171–179.

Grier, H. J. (1993). Comparative organization of Sertoli cells including the Sertoli cell barrier. In *The Sertoli Cell* (Russel, L. D. & Griswold, M. D., eds), pp. 704–739. Clearwater, FL: Chace River Press.

Grier, H. J., Linton, J. R., Leatherland, J. F. & DeVlaming, V. L. (1980). Structural evidence for two different testicular types in teleost fishes. *The American Journal of Anatomy* **159,** 331–345.

Harmin, S. A., Crim, L. W. & Wiegand, M. D. (1995). Isolation, partial characterization, induction, and the occurrence in plasma of the major vitelline envelope proteins in the Atlantic halibut (*Hippoglossus hippoglossus*) during sexual maturation. *Canadian Journal of Fisheries and Aquatic Sciences* **51,** 1700–1707.

Jespersen, Å. (1984). Spermatozoans from a parasitic dwarf male of *Neoceratias spinifer* Pappenheim, 1914. *Videnskabelige Meddelelser Dansk naturhistorisk Forening* **145,** 37–42.

Kime, D. A. & Manning, N. J. (1982). Seasonal patterns of free and conjugated androgens in the brown trout *Salmo trutta*. *General and Comparative Endocrinology* **48,** 222–231.

Koya, Y., Soyano, K., Yamamoto, K., Obana, H. & Matsubara, T. (2002). Testicular development and serum profiles of steroid hormone levels in captive male Pacific herring *Cuplea pallasii* during their first maturational cycle. *Fisheries Sciences* **68,** 1099–1105.

Lahnsteiner, F. & Patzner, R. A. (1990). Spermiogenesis and structure of mature spermatozoa in blenniid fishes (Pisces, Blenniidae). *Journal of Submicroscopic Cytology and Pathology* **22,** 565–576.

LeGrand, E. K. (1997). An adaptationist view of apoptosis. *Quarterly Review of Biology* **72,** 135–147.

Liu, H. W., Stickney, R. R. & Dickhoff, W. W. (1991). Changes in plasma concentrations of sex steroids in adult Pacific halibut, *Hippoglossus stenolepis*. *Journal of the World Aquaculture Society* **22,** 30–35.

Mattei, X., Siau, Y., Thiaw, O. T. & Thiam, D. (1993). Peculiarities in the organization of testis of *Ophiodon* sp. (Pisces Teleostei). Evidence for two types of spermatogenesis in teleost fish. *Journal of Fish Biology* **43,** 931–937.

Megalofonou, P., Dean, J. M., Metrio, G., Wilson, C. & Berkeley, S. (1995). Age and growth of juvenile swordfish, *Xiphias gladius* Linnaeus, from the Mediterranean Sea. *Journal of Experimental Marine Biology and Ecology* **188,** 79–88.

Minniti, F., Bruno, R., Labate, M., Desantis, S., De Metrio, G. & Mauceri, A. (2005). Ultrastructural features of germ cells in immature swordfish *Xiphias gladius* (Teleostei, Xiphidae). *Italian Journal of Zoology* **72,** 217–221.

Miura, T., Yamauchi, K., Takahashi, H. & Nagahama, Y. (1991). Hormonal induction of all stages of spermatogenesis in vitro in the male Japanese eel (*Anguilla japonica*). *Proceedings of the National Academy of Sciences USA* **88,** 5774–5778.

Nandi, S., Banerjee, P. P. & Zirkin, B. R. (1999). Germ cell apoptosis in the testes of Sprague Dawley rats following testosterone withdrawal by ethane 1,2-dimethanesulfonate administration: relationship to Fas? *Biology of Reproduction* **61,** 70–75.

Nash, J. P., Davail-Cuisset, B., Bhattacharyya, S., Suter, H., Le Menn, F. & Kime, D. E. (2000). An enzyme linked immunosorbant assay (ELISA) for testosterone, 17β-estradiol, and 17α,20β-dihydroxy-4-pregnen-3-one using acetylcholinesterase as tracer: application to measurement of diel patterns in rainbow trout (*Oncorhynchus mykiss*). *Fish Physiology and Biochemistry* **22,** 355–363.

Nonclercq, D., Reverse, D., Toubeau, G., Beckers, J. F., Sulon, J., Laurent, G., Zanen, J. & Heuson-Stiennon, J. A. (1996). In situ demonstration of germinal cell apoptosis

during diethylstilbestrol-induced testis regression in adult male Syrian hamsters. *Biology of Reproduction* **55,** 1368–1376.

Orsi Relini, L., Palandri, G., Garibaldi, F. & Cima, C. (1996). Swordfish maturity and growth. New observations in the Ligurian Sea. *Biologia Marina Mediterranea* **3,** 352–359.

Patterson, D. A., MacDonald, J. S., Hinch, S. G., Healey, M. C. & Farrell, A. P. (2004). The effect of exercise and captivity on energy partitioning, reproductive maturation and fertilization success in adult sockeye salmon. *Journal of Fish Biology* **64,** 1039–1059.

Potoschi, A., Cavallaro, G., Sturiale, P. & Lo Duca, G. (1994). Eggs and larvae of swordfish, tuna and albacore caught in the Ionian Sea. *Biologia Marina Mediterranea* **1,** 119–124.

Prisco, M., Liguoro, A., Comitato, R., Cardone, A., D'Onghia, B., Ricchiari, L., Angelini, F. & Andreucci, P. (2003). Apoptosis during spermatogenesis in the spotted ray *Torpedo marmorata*. *Molecular Reproduction and Development* **64,** 341–348.

Rodriguez, I., Ody, C., Araki, K., Garcia, I. & Vassalli, P. (1997). An early and massive wave of germinal cell apoptosis is required for the development of functional spermatogenesis. *EMBO Journal* **16,** 2262–2270.

Scott, A. P., Bye, V. J., Baynes, S. M. & Springate, J. C. R. (1980). Seasonal variation in plasma concentration of 11-ketotestosterone and testosterone in male rainbow trout, *Salmo gairdnerii* Richardson. *Journal of Fish Biology* **17,** 495–505.

de la Serna, J. M., Ortiz de Urbina, J. M. & Macias, D. (1996). Observations on sex-ratio, maturity and fecundity by length-class for swordfish (*Xiphias gladius*) captured with surface longline in the western Mediterranean. *ICCAT Collective Volume of Scientific Papers* **45,** 115–139.

Sharpe, R. M. (1994). Regulation of spermatogenesis. In *The Physiology of Reproduction* (Knobil, E. & Neill, J. D., eds), pp. 1363–1434. New York: Raven Press.

Sinha Hikim, A. P. & Swerdloff, R. S. (1999). Hormonal and genetic control of germ cell apoptosis in the testis. *Reviews of Reproduction* **4,** 38–47.

Sol, S. Y., Olson, O. P., Lomaz, D. P. & Johnson, L. L. (1998). Gonadal development and associated changes in plasma reproductive steroids in English sole, *Pleuronectes vetulus*, from Puget Sound, Washington. *Fisheries Bulletin* **96,** 859–870.

Štrbenc, M., Fazarinc, G., Bavdek, V. & Pogačnik, A. (2003). Apoptosis and proliferation during seasonal testis regression in the brown hare (*Lupus europaeus* L.). *Anatomia Histologia Embryologia* **32,** 48–53.

Tserpes, G., Peristeraki, P. & Somarakis, S. (2001). On the reproduction of swordfish (*Xiphias gladius* L.) in the eastern Mediterranean. *ICCAT Collective Volume of Scientific Papers* **52,** 740–744.

Uchida, D., Yamashita, M., Kitano, T. & Iguchi, T. (2002). Oocyte apoptosis during the transition from ovary-like tissue to testes during sex differentiation of juvenile zebrafish. *The Journal of Experimental Biology* **205,** 711–718.

Vaux, D. L. & Strausser, A. (1996). The molecular biology of apoptosis. *Proceedings of the National Academy of Sciences USA* **93,** 2239–2244.

Weber, L. P., Kiparissis, Y., Hwang, G. S., Niimi, A. J., Janz, D. M. & Metcalfe, C. D. (2002). Increased cellular apoptosis after chronic aqueous exposure to nonylphenol and quercetin in adult medaka (*Oryzias latipes*). *Comparative Biochemistry and Physiology* **131,** 51–59.

Weltzien, F.-A., Taranger, G. L., Karlsen, Ø. & Norberg, B. (2002). Spermatogenesis and related androgen levels in Atlantic halibut (*Hippoglossus hippoglossus* L.). *Comparative Biochemistry and Physiology A* **132,** 567–575.

Woods, A. L., Hall, P. A., Shepherd, N. A., Hanby, A. M., Waseem, N. H., Lane, D. P. & Levison, D. A. (1991). The assessment of proliferating cell nuclear antigen (PCNA) immunostaining in primary gastrointestinal lymphomas and its relationship to histological grade, S+G2+M phase fraction (flow cytometric analysis) and prognosis. *Histopathology* **19,** 21–27.

Woolveridge, I., de Boer-Brouwer, M., Taylor, F., Teerds, K. J., Wu, F. C. W. & Morris, I. D. (1999). Apoptosis in the rat spermatogenic epithelium following androgen withdrawal: changes in apoptosis-related genes. *Biology of Reproduction* **60,** 461–470.

Yin, Y., Stahl, B. C., DeWolf, W. C. & Morgentaler, A. (1998). p53-mediated germ cell quality control in spermatogenesis. *Developmental Biology* **204,** 165–171.

Young, K. A. & Nelson, R. J. (2001). Mediation of seasonal testicular regression by apoptosis. *Reproduction* **122,** 677–685.

Young, K. A., Zirkin, B. R. & Nelson, R. J. (1999). Short photoperiod induces testicular apoptosis in the white-footed mouse (*Peromyscus leucopus*). *Endocrinology* **140,** 1331–1339.

Yoneda, M., Tokimura, M., Fujita, H., Takeshita, N., Takeshita, K., Matsuyama, M. & Matsuura, S. (1998). Reproductive cycle and sexual maturity of the anglerfish *Lophiomus setigerus* in the East China Sea with a note on specialized spermatogenesis. *Journal of Fish Biology* **52,** 164–178.

Yu, C., Hall, P. A., Fletcher, C. D., Camplejohn, R. S., Waseem, N. H., Lane, D. P. & Levison, D. A. (1991). Haemangiopericytomas: the prognostic value of immunohistochemical staining with a monoclonal antibody to proliferating cell nuclear antigen (PCNA). *Histopathology* **19,** 29–33.