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SPECIAL SECTION: FISHERIES REPRODUCTIVE BIOLOGY

Assessment of Testis Development during Induced Spermatogenesis in the European Eel *Anguilla anguilla*

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Abstract

In a study of reproduction in male European eels *Anguilla anguilla*, we induced spermatogenesis through hormone injection and established a spermatogenic maturity index (SMI) as a novel quantification of testis development. Eels in the experiments were sacrificed weekly and testis tissue was sampled for histological analysis of spermatogenesis. Testis development was followed over 18 weeks, during which the males continued to develop spermatocytes and produce spermatozoa. The SMI describes testis development from estimation of the area fractions of various tissue categories characterized by progressive gamete development stages in histological sections of the testes. The index weighs the volume fractions of the different tissues (somatic cells and germ cell stages) and describes development on a scale of 0 to 1. The method improves the existing histological classification, providing a quantitative measure that reflects the spermatogenic process and can be correlated with morphological and physiological parameters. In this study, the SMI reacted immediately to the onset of spermatogenesis and increased linearly over time, tracking the development of spermatocysts and spermatozoa. In week 7, the SMI reached a stable level of around 0.75, where it remained, with limited fluctuations, until the end of the experiment. This reflected the composition of different germ cell stages in the testis tissue with a continuous generation of spermatocysts and production of spermatozoa. In comparison, the gonadosomatic index showed a delayed response to the onset of spermatogenesis and fluctuated substantially during the sperm production period. The properties of the SMI made it a useful index for describing spermatogenesis in male European eels during this experiment and a promising tool for quantifying testis development and describing male reproductive strategy in other fish species.

The population of European eels *Anguilla anguilla* has in recent decades declined to a level that raises major concerns for the species' long-term persistence (Dekker 2008; Freyhof and Kottelat 2008). To ensure survival of the species, conservation measures are needed, including captive breeding and production of fry for self-sustained aquaculture and potential stock enhancement. However, the life cycle of the European eel is complex, including a continental phase and an oceanic phase, and much of its reproductive biology remains an enigma (Tesch 2003; Van Ginneken and Maes 2005). The European eel spawning area in the Sargasso Sea was identified approximately a century ago through the prevalence of early larval stages (Schmidt 1922), and recent records from electronic tags have documented migratory stage silver eels migrating from European coasts towards the Sargasso Sea (Aarestrup et al. 2009). However, European eels with developed gonads or in spawning condition were never caught during their oceanic phase. When the yellow eels toward the end of the immature, continental stage start the process of silvering, they are preparing physiologically for the long spawning migration towards the Sargasso

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Sea as silver eels. During silvering, gonadal development becomes inhibited by complex hormonal control mechanisms at the hypothalamus and pituitary level (Dufour et al. 1988, 2005; Vidal et al. 2004; Schmitz et al. 2005; Pasqualini et al. 2009). This inhibition must be released to permit continued gonadal development when the migrating eels approach the spawning area; however, the timing and mechanisms responsible for the cessation of inhibition are uncertain.

In captivity, gametogenesis can be induced through hormonal treatment (Fontaine 1936; Fontaine et al. 1964; Boëtius and Boëtius 1967; Ishida and Ishii 1970; Ohta et al. 1997), forming the basis of experimental work on the captive breeding of European eels and Japanese eels *A. japonica* (e.g., Prokhorchik 1987; Tanaka 2003; Tomkiewicz and Jarlbæk 2008). In male eels, spermatogenesis can be induced by injection of human chorionic gonadotropin (hCG; Fontaine 1936; Boëtius and Boëtius 1967; Pérez et al. 2000; Ohta and Unuma 2003). In male European eels that are given weekly hCG injections, spermiation starts at around the fifth week of treatment (Pérez et al. 2000; Asturiano et al. 2005, 2006). Different treatment schemes have been tested (e.g., Boëtius and Boëtius 1967; Pérez et al. 2000; Asturiano et al. 2005, 2006), and a weekly dosage of 1.5 international units (IU) of hCG per gram of body weight (BW) combined with stripping 24 h after injection has been shown to optimize the proportion of spermiating males and sperm motility (Asturiano et al. 2005).

The morphological and histological changes that occur during hormonally induced development of the testes in European eels were first described by Boëtius and Boëtius (1967). Before treatment, the lobes of the dorsally attached testes were only slightly differentiated. The spermatogenic tissue was represented by clusters of spermatogonia (Sg) separated by interstitial tissue. Immediately after the first injection, testis tissue became organized as spermatic tubules with a bordering layer of connective tissue. Spermatogenesis of silvering eels during induced development has been further documented by Miura et al. (1991a), Walsh et al. (2003), Huertas and Cerdà (2006), and Peñaranda et al. (2010). The duration of spermatogenesis and the spermiation period depends on the treatment (i.e., hormone dosage and injection frequency; Boëtius and Boëtius 1967; Asturiano et al. 2005; Peñaranda et al. 2010). If treatment is suspended after the onset of spermiation, the testes continue to produce sperm for a few weeks, after which they regress (Boëtius and Boëtius 1967; Dollerup and Graver 1985).

The progression of spermatogenesis in fishes is often classified histologically into developmental phases defined by the most advanced germinal cells present (i.e., Sg, spermatocytes [Sc], spermatids [St], or spermatozoa [Sz]) and their prevalence (e.g., Grier and Taylor 1998; Brown-Peterson et al. 2002; Utoh et al. 2004; Grier and Uribe-Aranzábal 2009), and such classifications have been defined for eels *Anguilla* spp. (Miura et al. 1991a, 1991b; Huertas et al. 2006; Pérez et al. 2009; Peñaranda et al. 2010). Classification ranges from qualitative indices that grade testis development into categories or phases (e.g., immature, developing, spawning capable, regressing, and regenerating; Brown-Peterson et al. 2011, this special section) to the most advanced indices that use quantitative stereology to estimate the volume of different germ cells in the testes (Nielsen and Baatrup 2006). These indices reflect the progression from the onset of development to sperm production and spawning cessation, which depends on the reproductive strategy of the fish and the testicular organization (Grier 1993; Schulz and Miura 2002; Parenti and Grier 2004; Nóbrega et al. 2009; Schulz et al. 2010). The histological indices of development are useful in combination with physiological measurements and provide more detailed information than the gonadosomatic index (GSI), which is often used to quantify gonadal development in fishes.

For successful reproduction of European eels in captivity, adequate production of semen and high quality of sperm are crucial; this requires knowledge about the progression of spermatogenesis and the duration of the spermiation period. The objective of the present study was to assess testis development in European eels during induced spermatogenesis, including (1) a description of the testis development in captive male European eels given weekly hormonal injections and (2) the development of a method for quantitatively assessing the spermatogenetic progression. We applied histological information about the proliferation of the testes, and we estimated area fractions of different tissue types within the testes by using a point grid. The area fractions represent the volume fractions according to Delesse's principle (Weibel 1989) and provide an unbiased estimate of the prevalence of different cell types in the testes. A spermatogenic maturity index (SMI) was established on the basis of weighted area fractions of tissue types, providing a novel method of quantifying spermatogenic development. The SMI was compared with the GSI and was applied in analyses to determine the homogeneity of testes and to determine whether the plane of sectioning or level of magnification affected the SMI. The area fraction and SMI provide methods that can also be used for quantifying the progression of spermatogenesis and describing the male reproductive strategy in other fish species.

METHODS

Experimental animals, rearing conditions, and hormonal treatment.—Fifty-seven farmed male European eels were selected for the experiments (standard length [mean ± SD] = 38.6 ± 2.2 cm; BW = 103.8 ± 18.8 g). The males were transferred to one of four 300-L containers in a recirculation system and were gradually acclimated from freshwater to artificial seawater (Tropic Marin; Dr. Biener GmbH, Wartenberg, Germany) by increasing the salinity stepwise from 0‰ to 35‰ over a period of 10 d. The water temperature was maintained at about 20°C. Feed was withheld from the experimental animals during the experiments to approximate the natural conditions wherein the migrating silver eels do not feed (Aoyama and Miller 2003). At the onset of the hormonal treatment, the males were anesthetized with benzocaine dissolved in water and were tagged with a

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passive integrated transponder tag dorsally in the muscle. Once per week and for a period of up to 18 weeks, each male received an intramuscular injection of hCG (200 IU). Prior to injection, the health condition and production of semen were checked. After the first release of sperm, the males were stripped weekly 24 h after each injection and the volume of semen (mL) stripped per male was measured (Pérez et al. 2000).

Sampling and histological analyses.—Three males were sacrificed each week to follow testis development and morphological changes, including histological analyses of the testicular tissue. Sampling of males was randomized on the basis of passive integrated transponder tag numbers and was conducted prior to injection. Morphometric measures obtained from the sacrificed males included total BW (nearest 0.1 g) and testis weight (nearest 0.1 g). For histology, three testis lobes per male were sampled (i.e., from the anterior, middle, and posterior portions of the testes) by dissection at the site of attachment to the vas deferens. The lobes were preserved in a 4% solution of formalin buffered by NaH_2PO_4-H_2O and Na_2HPO_4-$2H_2O$.

For the general assessment of testis development, the middle testis lobe sample was analyzed for each male (series A1). Each sampled lobe was halved to obtain tissue sections along the axis from the base of the lobe to the edge (longitudinal section; Figure 1). The tissue samples were dehydrated, embedded in paraffin, and sectioned at 5 μm along the longitudinal axis. The sections were stained with hematoxylin and eosin (VWR International, Bie & Berntsen A/S, Herlev, Denmark). For a subset of males (i.e., the three males sampled during week 9), the histological analysis was extended with the purpose of ascertaining the homogeneity of tissues and development within the testes (series A2). For these males, all three sampled lobes (anterior, middle, and posterior) were processed by use of the same methods for preparation of sections as described above.

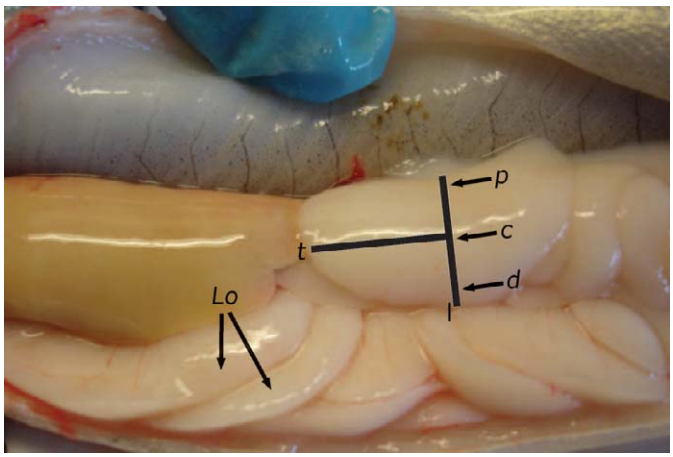


FIGURE 1. Testis in the abdominal cavity of a male European eel, showing lobes (*Lo*) and subsampling of preserved lobes for histology (t = transverse section; l = longitudinal section; arrows indicate photography points: p = proximal, c = central, d = distal). The thumb at the top of the photo indicates scale.

The influence of sectioning angle in relation to the lobe orientation on the appearance of the testicular tissue and analysis of testis development was investigated by using the tissue samples from a subset of males sampled in weeks 2, 4, and 7 (series B1). For this purpose, the tissue sample from the middle lobe of one male per week used in series A1 was re-embedded at a 90° angle and sectioned (5 μm) transversely (Figure 1). These sections were processed histologically as above for comparison with the longitudinal sections of the same lobes.

Micrographs for image analysis.—The histological sections were photographed with a digital camera (Model DP71; Olympus, Center Valley, Pennsylvania) at 200× magnification for identification of gamete development stages and tissue types. Testis tissues were categorized according to cell types: testicular somatic cells (Ts), which included Sertoli and Leydig cells; and germ cells (Sg, Sc, St, and Sz; modified from Miura et al. 1991a). Excluded areas were those with no tissue.

For the image analysis and estimation of area fraction per tissue type, an array of photomicrographs was sampled per section. For the general assessment of testis development (series A1), three photomicrographs were obtained from the middle lobe, covering the proximal, central, and distal parts of the tissue section (Figure 1). For the comparison of homogeneity among lobes sampled from the anterior, middle, and posterior portions of the testes, one additional photomicrograph was obtained from the central part of the anterior and posterior lobes from males sampled in week 9 (series A2). For the test of sectioning angle, three photomicrographs of different locations within the transverse sections of the re-embedded middle lobe were obtained from the males sampled in weeks 2, 4, and 7 (series B1). To test for potential effects of a difference in magnification level, three images at 400× magnification were obtained from the proximal, central, and distal locations of the longitudinal sections from males sampled in weeks 2, 4, and 7 (series B2).

Estimation of area fractions, SMI, and GSI.—The estimation of area fractions for the different tissue categories was carried out by placing a point grid (48 points) on the images (photomicrographs) with ImageJ software (National Institutes of Health, Bethesda, Maryland) as shown in Figure 2. The tissue type at each intersection of the grid lines (i.e., each point) was categorized by using the cell type present in the upper right corner of the intersection as an identifier (Table 1). The area fraction per tissue type was estimated as the sum of points identified per category divided by the total number of grid points that hit testis tissue in the photomicrograph. Table 1 provides an example of the calculation of area fraction based on the image in Figure 2. The ImageJ plug-in Analyze was applied, placing a grid over the micrographs (Grid function) and adjusting the number of points (Area per Point function). The categories were marked and counted by using the Cell Counter plug-in.

To assess the progression of spermatogenesis, we defined the SMI based on summarizing the area fractions per tissue category weighted by a factor (w = 0.0, 0.25, 0.5, 0.75, or 1.0)

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TABLE 1. Calculations of tissue area fractions (F) and the spermatogenic maturity index (SMI) based on the histological image of the European eel testis and point counts (n) per tissue category (i) in the 48-point grid illustrated in Figure 2. The SMI is the product of the weighting factor (w) and estimated F. The SMI of the testis sample in Figure 2 is 0.64. Cell types are testicular somatic cells (Ts), spermatogonia (Sg), spermatocytes (Sc), spermatids (St), and spermatozoa (Sz; Ea = excluded area).

			Area fraction calculation		SMI calculation	
Cell type	i	n_i	$n_i/(n_{\text{total}}-n_1)$	F	w	$F \times w$
Ea	1	2				
Ts	2	6	6/(48–2)	0.13	0.00	0.00
Sg	3	1	1/(48–2)	0.02	0.25	0.01
Sc	4	12	12/(48–2)	0.26	0.50	0.13
St	5	16	16/(48–2)	0.35	0.75	0.26
Sz	6	11	11/(48–2)	0.24	1.00	0.24
Total		48		1.00		0.64

that increases with progressing development:

$$\text{SMI} = 0.0F_{\text{Ts}} + 0.25F_{\text{Sg}} + 0.5F_{\text{Sc}} + 0.75F_{\text{St}} + 1.0F_{\text{Sz}}, \quad (1)$$

where F is the area fraction for the indicated cell type (Ts, Sg, Sc, St, or Sz). The index ranges from 0 when only Ts cells are present to 1.0 when all germinal cells have transformed into Sz. An example of SMI estimation is provided in Table 1.

The SMI was estimated for each of the testis images to assess the morphological development of the testis tissue (series A1). The weekly SMI (mean ± SD) for the 18-week period represents the average SMI of the nine images obtained from the three males sampled per week. The SMI as a quantitative measure of testis development was related to the GSI ([{testis weight, g}/{BW, g}] × 100) and semen production for the same males. The SMI and GSI were compared for individual males assigned to reproductive phases. Histological indicators, namely the presence of Sg, or Sc as the most developed gamete stage, identified the immature and developing phases, respectively (Brown-Petersen et al. 2011), while first release of sperm at stripping was applied as the criterion for the sperm production phase (i.e., males that were capable of spawning).

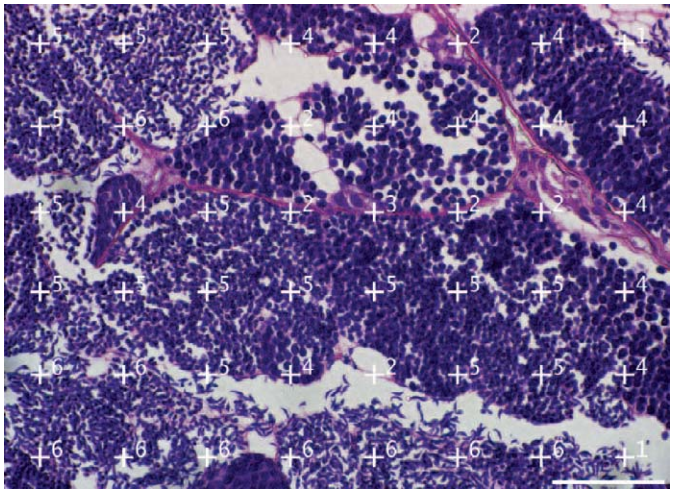


FIGURE 2. Photomicrograph (scale bar = 50 μm) of male European eel testicular tissue with a 48-point grid overlay generated by the Analyze plug-in of ImageJ software. The number next to each grid intersection (cross) identifies the testis tissue category observed in the upper right corner of the cross, which is used in obtaining counts per tissue category (categories are defined in Table 1). The categories were marked and counted by using the Cell Counter plug-in. The estimation of area fraction per testis tissue category is detailed in Table 1.

Statistical analyses.—For evaluating homogeneity of the testis tissue, two-way analyses of variance (ANOVAs) were applied to SMI estimates obtained from different parts of the testes. These analyses compared SMIs from different locations (proximal, central, and distal) within the middle lobe and SMIs from the central location of different lobes (anterior, middle, and posterior), respectively, for three males in the early sperm production phase that were sampled in week 9 (series A1 and A2).

Two-way ANOVAs were used to evaluate whether the plane of sectioning to the orientation of lobes or the level of magnification influenced the estimation of SMI within the lobes. The comparison included three different testis development stages: early developing (week 2), late developing (week 4), and early sperm producing (week 7). The test to evaluate the influence of the plane of sectioning included SMI estimates from 1 male/week for which the testis was sectioned longitudinally (series A1), re-embedded, and sectioned transversely (series B1). The test of magnification level compared SMIs from the same males as above by using the longitudinal sections and three micrographs per section (proximal, central, and distal) obtained at two magnification levels (i.e., 200×, series A1; 400×, series B2).

RESULTS

Progression and Dynamics of Spermatogenesis

All males responded successfully to the hCG treatment, and testes in different developmental phases are illustrated macroscopically in Figure 3 and microscopically in Figure 4. At

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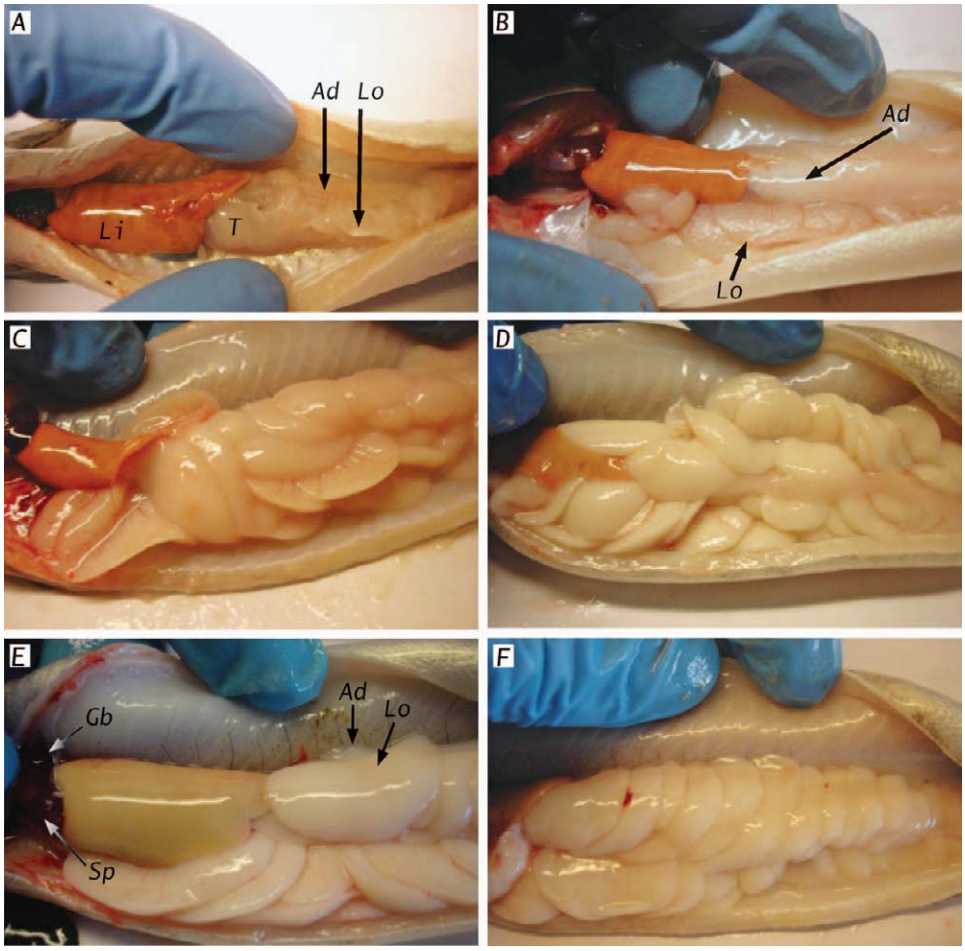


FIGURE 3. Photographs illustrating macroscopic changes in hormonally induced testis development in male European eels sampled in **(A)** week 0 (*T* = testis; *Lo* = testis lobes; *Ad* = adipose tissue; *Li* = liver), **(B)** week 3, **(C)** week 5, **(D)** week 9, **(E)** week 12 (*Gb* = gall bladder; *Sp* = spleen), and **(F)** week 18. The thumb in each photo indicates scale.

the onset of the experiments, lobes were small and insignificant (Figure 3A) and the tissue comprised Ts and Sg as the only germ cells, indicating that the testes were in the immature phase (Figure 4A). Spermatocytes occurred in the second week (Figure 4B, early developing phase), and St and Sz had developed by week 4 (Figure 4C, developing phase). Concurrent with the progression of spermatogenesis, the lobe size increased (Figure 3B, C). During the subsequent weeks, the abundance of Sz and the area of the germinal compartments increased (Figure 4D, E). Spermatocytes and St were present during the entire spermiation period when fish were in the sperm production phase, which continued until the end of the experiment (Figure 4F). The expansion of lobes and the continued spermatogenesis were also clearly visible macroscopically (Figure 3D, F).

Figure 5A shows the area fractions per tissue category to illustrate the progression of testis development during the 18-week experimental period. The area fractions document changes in the relative composition of the testis tissue based on the weekly samples of three males. The proliferation of Sg started immediately after the first injection, and Sg were present throughout the experimental period. Spermatocytes occurred in the second week, and the F_{Sc} dominated during weeks 3–6, followed by a decrease to a level around 15–20%, where it remained until week 18. Spermatids occurred during the third week, and the F_{St} fluctuated between 10% and 20% during the experimental period. In week 4, Sz appeared for the first time, and F_{Sz} increased gradually from week 4 to week 7. During weeks 8–18, the F_{Sz} fluctuated between 25% and 50%.

The SMI responded immediately to the increase in proportion of Sg and Sc during the first weeks of treatment and followed the gradual development of gametes through the occurrence of Sc, St, and Sz (Figure 5A). The SMI started at a value of 0.06 in week 0 and increased almost linearly to 0.76 in week 7. From week 7 to week 17, the SMI was stable at an average of 0.71 (SD = 0.08), which corresponded to a relatively stable composition of the tissue fractions. The samples in week 18 showed a slight decrease in the prevalence of Sz and thus a lower SMI value.

The comparable GSI (Figure 5B) remained at a low value during the first weeks, when the increase in testis size was limited (Figure 3); this was followed by a steep increase from

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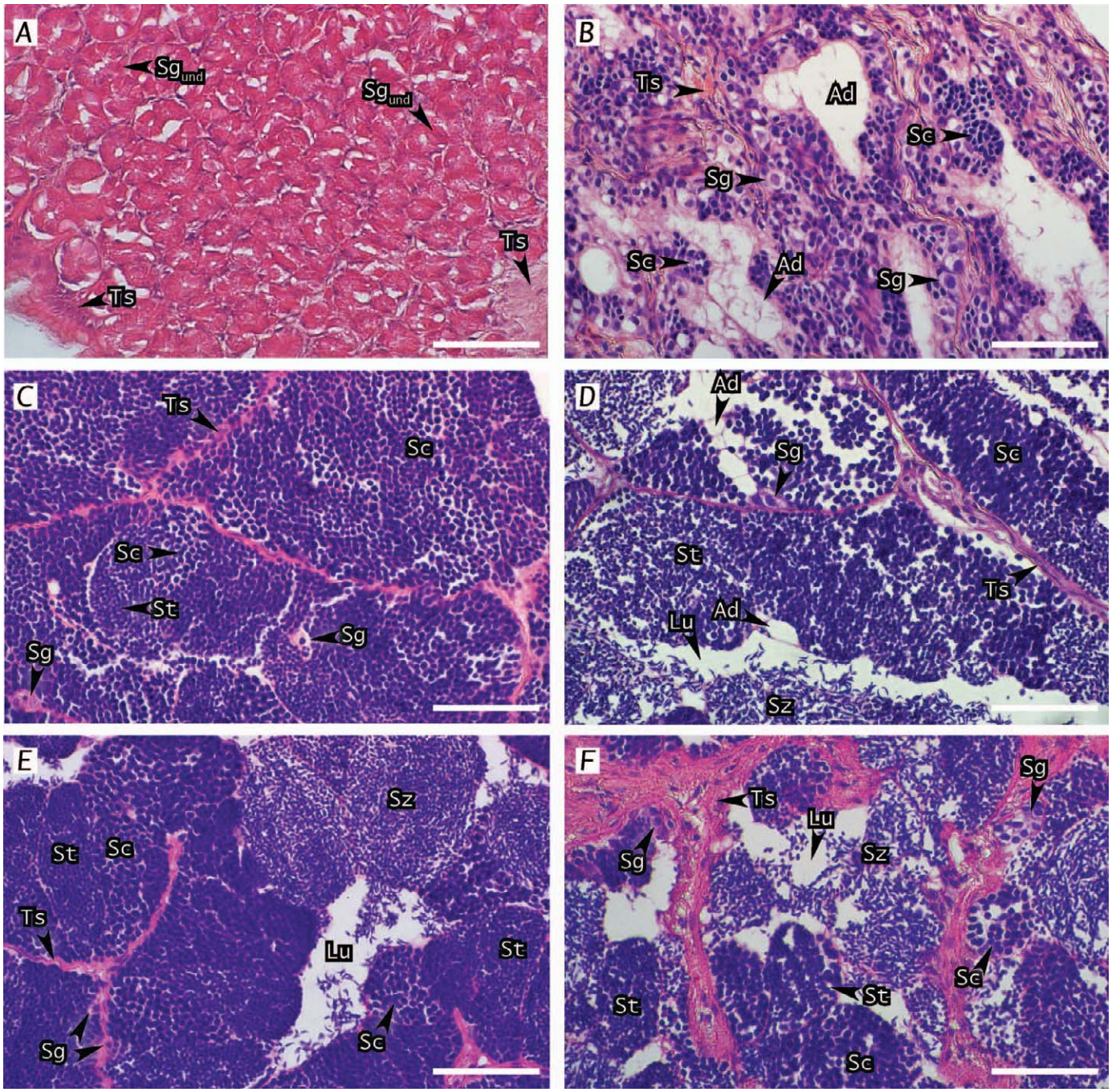


FIGURE 4. Photomicrographs (scale bar = 50 μm).of histological sections, illustrating characteristics of European eel testes at different stages of development during hormonally induced spermatogenesis: **(A)** sample week 0 (Sg_{und} = undifferentiated spermatogonia; Ts = testicular somatic cells), **(B)** week 2 (Sg = differentiated spermatogonia of types A and B; Sc = spermatocytes; Ad = adipocytes), **(C)** week 4 (St = spermatids), **(D)** week 7 (Sz = spermatozoa; Lu = lumen), **(E)** week 12, and **(F)** week 18.

week 3 to week 8. After week 8, the GSI fluctuated around an average value of 9 with considerable variation (GSI [mean ± SD] = 9.24 ± 2.82), declining to a low level in week 18.

Sperm production was checked in all males weekly, and although semen was already observed upon application of light pressure to the abdomen of some males in week 4, the majority of males showed the first signs of spawning readiness in week 5. Figure 5C shows the volume of semen produced by sampled males in the week prior to their sacrifice (e.g., the volume of semen stripped in week 4 is from the three males that were

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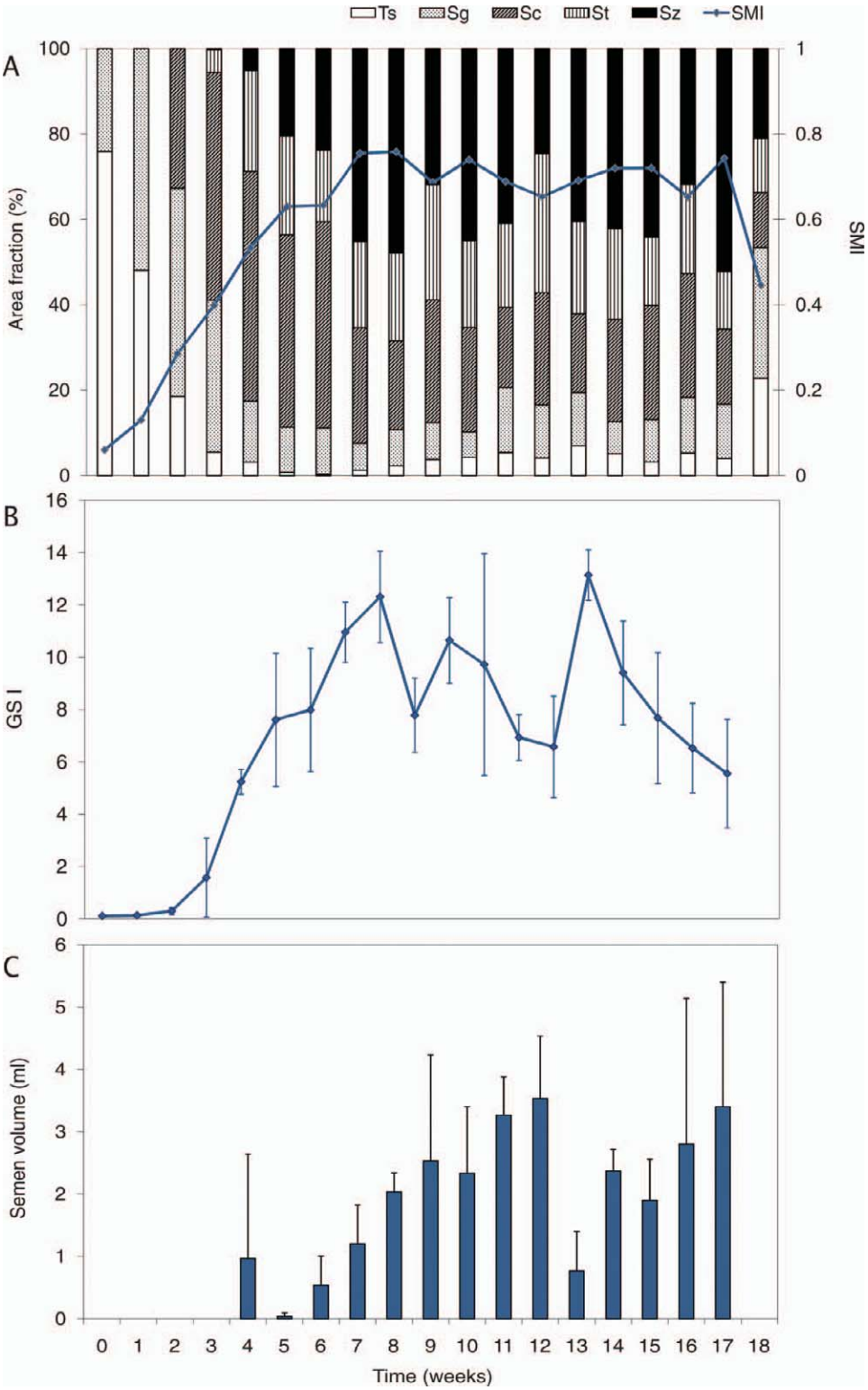


FIGURE 5. European eel testis development during induced maturation (weeks 0–18) based on the sampling of 3 males/week: **(A)** histological changes provided as the mean area fractions of different tissue types (Ts = testicular somatic cells; Sg = spermatogonia; Sc = spermatocytes; St = spermatids; Sz = spermatozoa) and spermatogenic maturity index (SMI; solid line); **(B)** corresponding gonadosomatic index (GSI; mean ± SD) for the same period; and **(C)** stripped semen volume (mean + SD; mL per 100 g of body weight) obtained from three males during the week prior to sacrifice.

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sacrificed in week 5). After the onset of semen production during weeks 4–6, the volume of semen increased and production continued throughout the experimental period; variation among individuals was substantial and tended to increase toward the end of the experiment. Considering that males were stripped weekly, the constant F_{Sz} in the testes during weeks 7–17 as illustrated in Figure 5A suggests a continuous production of gametes and Sz during the experimental period.

Statistical Analyses of Homogeneity and Methodology

The test of homogeneity was applied to a subset of males in the early sperm production phase (i.e., those with all tissue types present; week 9, series A1 and A2). Neither the SMIs estimated for the proximal, central, and distal photomicrographs of the middle lobe nor the SMIs estimated for the anterior, middle, and posterior lobes differed significantly (two-way ANOVAs with tissue part and individual as factors; photomicrograph location: $F_{2,4} = 0.417$, $P = 0.68$; sampled lobe: $F_{2,4} = 3.11$, $P = 0.15$). This indicated a homogeneous development throughout the testes, which confirmed the macroscopically uniform appearance of lobes within the testes (Figure 3).

Figure 6 shows images of the testis tissue in the middle lobes sectioned longitudinally and transversely for the three different developmental phases obtained in weeks 2, 4, and 7 (series A1 and B1). The appearance differed to some extent among images from the same males. However, the SMI estimates did not differ based on the plane of sectioning (Table 2). A two-way ANOVA with method (sectioning plane) and time as factors showed no significant difference among methods ($F_{1,12} = 1.11$, $P = 0.31$). Thus, the plane of sectioning did not seem to influence the assessment of testis development by the SMI. As expected, time was a highly significant factor ($F_{2,12} = 201$, $P < 0.0001$; Bonferroni post hoc test: $P < 0.0001$ for all pairs of dates), reflecting the steady increase in SMI from week 2 to week 7. The estimates of SMI from longitudinal sections at two different magnification levels did not significantly differ (Table 2). A two-way ANOVA with method (magnification level) and time as factors showed that the SMI for the males sampled in weeks 2, 4, and 7 (series A1 and B2) did not differ depending on the level of magnification used ($F_{1,12} = 1.30$, $P = 0.28$). Similar to the previous analysis, time was a significant factor ($F_{2,12} = 151$, $P < 0.0001$; Bonferroni post hoc test: $P < 0.001$ for all pairs of dates). The interaction terms in both analyses were not significant (plane of sectioning: $F_{2,12} = 0.56$, $P = 0.59$; magnification level: $F_{2,12} = 1.82$, $P = 0.21$).

DISCUSSION

Development of Testes

Testis development and production of sperm were successfully achieved in the present experiment by using weekly hormonal injection of hCG similar to the methods used by Ohta et al. (1997) and Ohta and Unuma (2003) for Japanese eels and by Pérez et al. (2000) and Asturiano et al. (2005, 2006) for European eels. Spermiation and first release of semen were achieved in weeks 4–5 by using an hCG injection of 200 IU/male or about 2 IU/g BW, which compares to the results of Pérez et al. (2000), Asturiano et al. (2005, 2006), and Peñaranda et al. (2010), who used 1.5 IU/g BW and larger males (i.e., ~200 IU/male). A continued weekly supply of hormone can sustain eel sperm production for a longer period and is required for an adequate volume of semen and an adequate quality of sperm (Ohta and Tanaka 1997; Pérez et al. 2000; Ohta and Unuma 2003; Asturiano et al. 2005, 2006). The course and full duration of the spermiation period under sustained treatment so far have received little study as most experiments end before the cessation of sperm and semen production. Pérez et al. (2000) reported sperm production of European eel males during weeks 4–16 and a decreased sperm density after week 13 but a continued high stripped semen volume, and Pérez et al. (2009) summarized that most testes were exhausted after 15–17 injections in their experiments. Experimental work by Palstra and van den Thillart (2009) showed that males were still spermiating after 19 weeks of hCG treatment; however, the testicular lobes of most males were greatly reduced and the efferent ducts were filled with Sz. Huertas et al. (2006) treated male European eels for about 20 weeks with a high dosage of hCG (8 IU/g BW) and observed males with signs of testis depletion at the end of the experimental period. In the present study, spermiation and production of semen continued until the end of the experimental period, but starting at week 16 the semen production and GSI of some males declined, although the germinal tissue was still not depleted.

Histologically, the progression of spermatogenesis followed the pattern described by Miura et al. (1991a, 1991b), Miura and Miura (2001), and Miura et al. (2003) for Japanese eels and by Huertas et al. (2006), Pérez et al. (2009), and Peñaranda et al. (2010) for European eels. Before treatment with hCG, the testes of male eels contain only Sg (undifferentiated, type A, or early type B; Miura et al. 1991a) arranged in cysts and surrounded by connective tissue (Huertas and Cerdà 2006; Pérez et al. 2009). Injection of hCG rapidly initiates the proliferation of Sg, and late type B Sg (which are smaller and have a dense nucleus) appear as a result of mitosis (Miura et al. 1991a; Pérez et al. 2009). The late type B Sg differentiate into primary Sc after 1–2 weeks of hCG treatment, and the number of germ cells per cyst increases (Miura et al. 1991a; Pérez et al. 2009). Two meiotic divisions follow within a short interval, making secondary Sc difficult to observe as they soon become St and transform into Sz through spermiogenesis (Miura et al. 1991a, 2003). In the present study, Sc were first observed during week 2, St appeared during week 3, and Sz appeared during weeks 4–5 (hCG injection of 200 IU/male; BW = 85–122 g). A faster progression in which Sz were obtained in week 3 was observed in the experiments by Miura et al. (1991a), who applied a single hCG injection of 5 IU/g BW (BW = 180–200 g), and by Peñaranda et al. (2010), who used weekly hCG treatments of 1.5 IU/g BW (BW = 112–137 g). Variation between experiments may be caused by an interactive effect of male size and

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TABLE 2. Comparison of the spermatogenic maturity index (SMI) among European eel testes sampled in weeks 2, 4, and 7; SMI was estimated based on either transverse (*t*) or longitudinal (*l*) histological sections of the same lobes or by using 200× or 400× magnification of longitudinal sections (series A1 and B1). See text for further details on the statistical analysis.

	SMI: *t*, 200×[ac]		SMI: *l*, 200×[abc]		SMI: *l*, 400×[bc]	
Sampling week	Mean	SD	Mean	SD	Mean	SD
2	0.29	0.006	0.24	0.008	0.33	0.037
4	0.57	0.035	0.55	0.019	0.56	0.016
7	0.79	0.041	0.79	0.095	0.78	0.061

[a]$P = 0.31$ for plane of sectioning comparison.
[b]$P = 0.28$ for magnification comparison.
[c]$P < 0.001$ across time for each comparison.

hormone dosage, but differences in male responsiveness can also influence development (Han et al. 2006).

The progression of spermatogenesis is often categorized into successive development classes and phases according to the most advanced germ cell type present (e.g., Miura et al. 1991a; Grier and Taylor 1998; Brown-Peterson et al. 2002; Utoh et al. 2004). Huertas et al. (2006) and Peñaranda et al. (2010) extended this classification for eels by considering the relative abundance of Sz and other germ cell categories, and they included new development stages to better describe the progression during the sperm production phase and spawning cessation. In the present study, we assessed testis development based on the area fractions of different tissue categories in combination with first sperm release and semen production. Application of a point grid to quantify the area fractions of the different compartments in the testes further elaborates the classification of Huertas et al. (2006) and Peñaranda et al. (2010). This method changes the estimation of abundance from one of judgment to one of measurement and includes information about the development of all tissue types during the progression of spermatogenesis and reproductive phases. This makes the present method useful in quantitative studies of testis development, as was also illustrated in stereological analyses of spermatogenesis in guppies *Poecilia reticulata* (Nielsen and Baatrup 2006).

The area fraction per tissue category in this study documented that Sg, Sc, St, and Sz persisted in the European eel testis tissue throughout the experimental period. The constant high F_{Sz} during weeks 7–17 suggests the occurrence of continual spermatogenesis since all males were stripped weekly to measure the semen volume. This continual gamete development in European eels agrees with the long period of semen production and the progression of spermatogenesis observed in experiments by Huertas et al. (2006), Pérez et al. (2009), and Peñaranda et al. (2010). As the area fractions illustrate the succession of germ cell types and tissue compartments during testis development, the method may prove useful for describing progressive development and reproductive phases. This information can be applied to distinguish reproductive strategies in male fish and to assess gonadal development and compare effects of treatments in experimental studies (Nielsen and Baatrup 2006).

Applicability of the Spermatogenic Maturity Index

The estimated area fractions provided a basis for establishment of the SMI, which captured the characteristics of the observed spermatogenic pattern, including the rapid proliferation of germ cells and the continuous development of gametes and sperm production. The average SMI of the males in this study increased linearly during the period of testis development and early sperm production until week 7, when it stabilized, reflecting ongoing spermatogenesis and sperm release. During the initial period, the SMI was found to be a more precise quantitative estimator of testis development than the GSI, which showed a delayed response to the germ cell progression. Additionally, the SMI remained stable during weeks 7–17, reflecting the continuous sperm production, while the GSI fluctuated substantially, as has also been observed in other experiments (e.g., Peñaranda et al. 2010). The cessation of spawning was not reached in the experiment; however, the decline in GSI through weeks 15–18 indicated a gradual depletion of testes, while the SMI remained high until week 17 due to continued spermiogenesis. The indices thus supplemented each other. The constant area fractions and hence SMI reflected the continued spermatogenesis, whereas the GSI reflected the relative weight reduction and depletion of the testes. In particular, the SMI can be a useful tool for assessing testis development experimentally in relation to sperm production, which is an important issue in controlled reproduction. Consideration of the relationship to the sperm density would add further information (Pérez et al. 2000, 2009).

The relationship between SMI and GSI is shown in Figure 7 by using individual males to illustrate the progression of testis development in relation to reproductive phases; the relative area fractions of testis tissue categories are also shown in the figure to document gamete development. The pronounced increase of SMI at the time of transition from the immature phase to the developing phase is characterized by the appearance of Sc and provides a better indicator of early development than the GSI, which remains low. Both the SMI and GSI increase during the developing phase. However, the SMI continued to increase as the males initiated sperm production, whereas the GSI reached a plateau early in the sperm production period. In this study,

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we used the first release of semen at stripping to signify entry into the spawning period, which we term the "sperm production phase." Although Sz appeared in the lobules and lumen during the late developing phase, these testicular Sz are not yet mature but acquire motility during their passage through the sperm duct (Miura et al. 1991a; Miura and Miura 2001). The increase in GSI during the transition from the developing phase to the sperm production phase reflects the milt hydration prior to sperm release. While the GSI reflects the milt production, the SMI tends to reflect the continuous presence of Sg, Sc, and St. The GSI of males that showed signs of depletion dropped rapidly, whereas the gradual decrease in SMI indicated that spermatogenesis

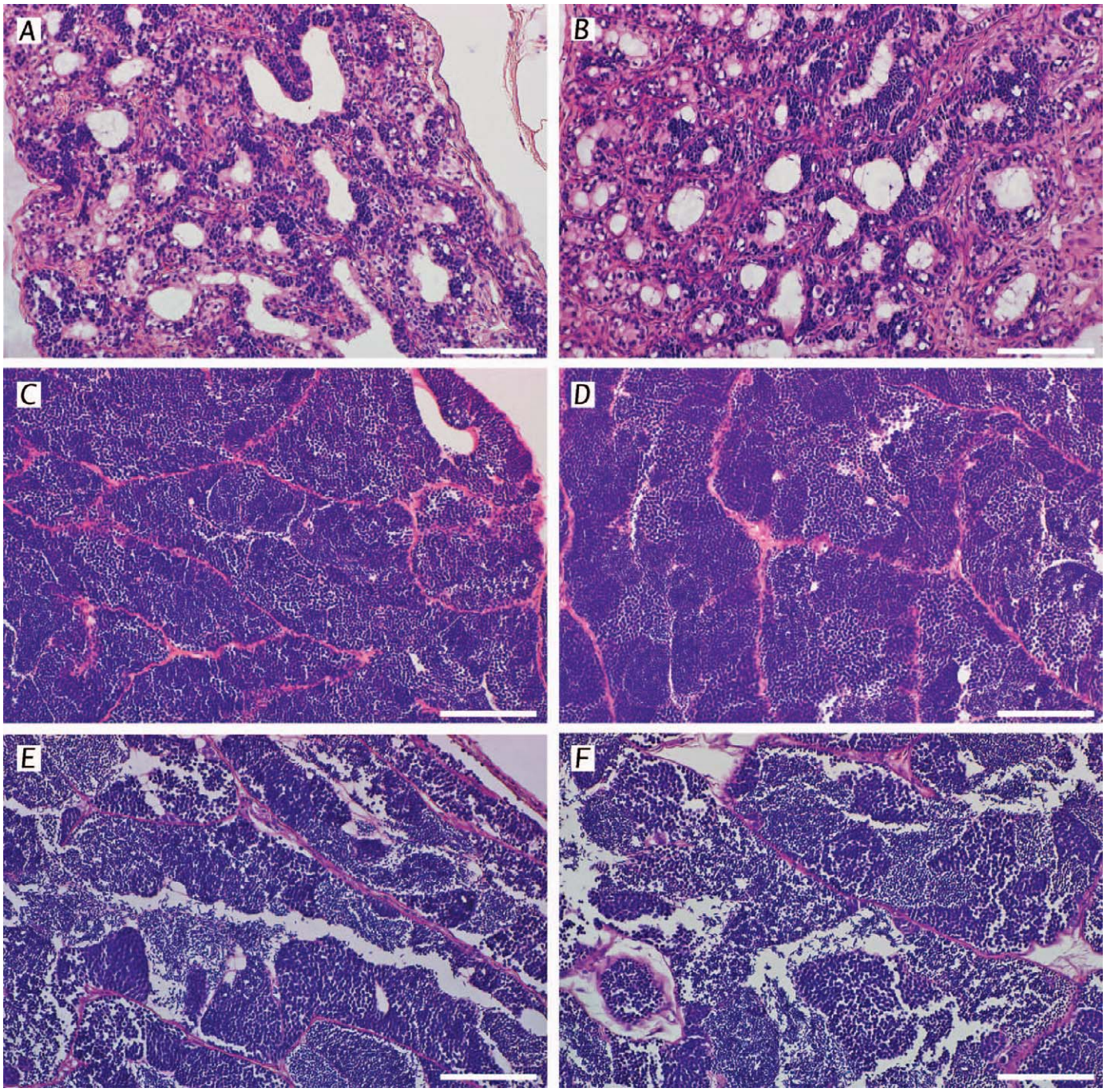


FIGURE 6. Photomicrographs (scale bar = 100 μm) of three histological samples of European eel testicular tissue representing various phases of development and sectioned at different angles to the lobe: **(A)** longitudinal section at week 2 (early developing), **(B)** transverse section at week 2, **(C)** longitudinal section at week 4 (late developing), **(D)** transverse section at week 4, **(E)** longitudinal section at week 7 (early sperm production), and **(F)** transverse section at week 7.

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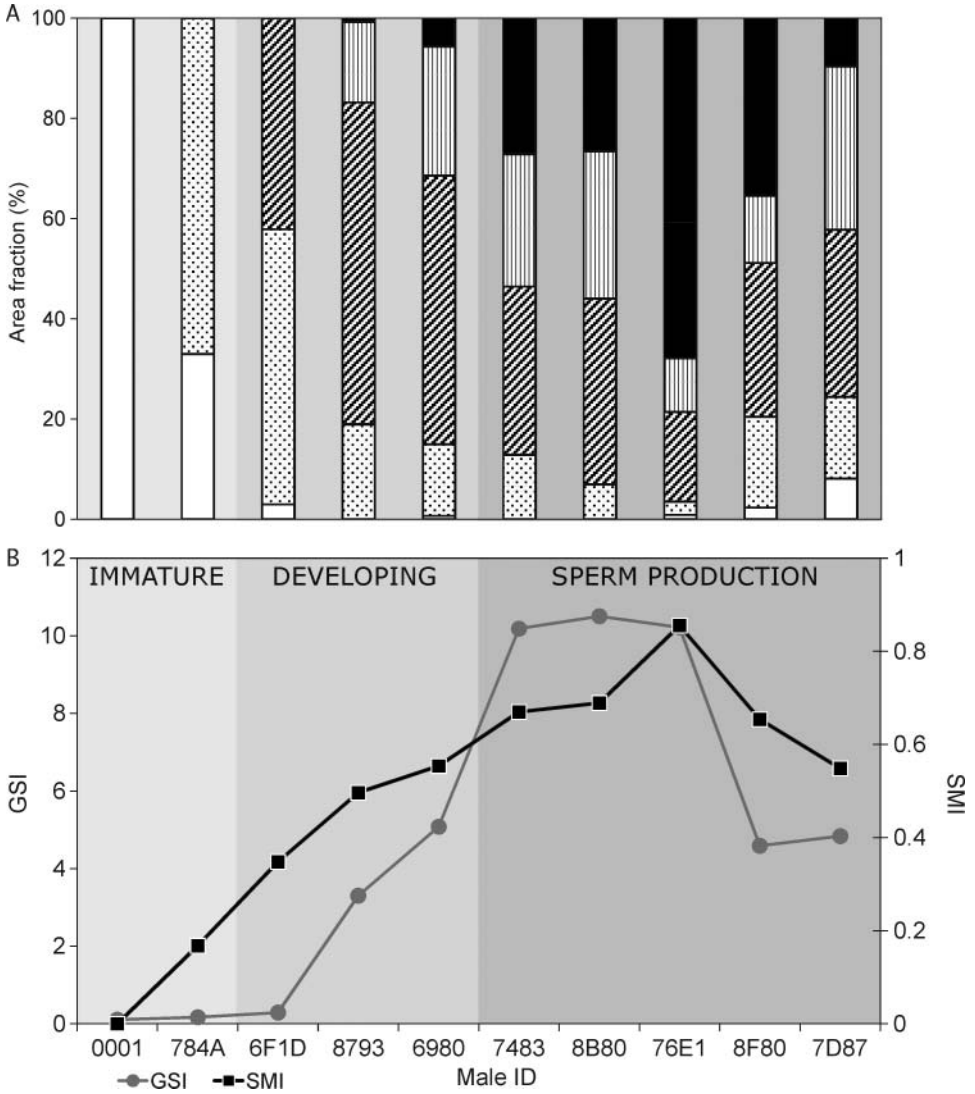


FIGURE 7. Progression of spermatogenesis in individual male European eels based on different quantitative methods as related to successive development phases (immature, developing, and sperm production): **(A)** mean area fraction per tissue type for individual males (tissue types and shading are as in Figure 5) and **(B)** corresponding mean spermatogenic maturity index (SMI) and gonadosomatic index (GSI) for each male. Male identification number (ID) 0001 is a theoretical starting point.

continued while sperm production and resources declined. In the present experiment, no males entered the regressing phase, although a gradual decrease of germinal tissue and an increase in the proportion of somatic tissue (primarily connective tissue and blood vessels) were reflected in the decreased SMI at week 18 (Figure 5a). In the present study, the SMI was useful for evaluating development in relation to reproductive phases by quantifying the amount of active spermatogenesis taking place and describing the variation among individual males.

The SMI provides an integrated index of spermatogenesis that can be correlated with other morphological and physiological parameters or can be used as a simple quantitative descriptor (e.g., in the statistical tests of testis tissue homogeneity, sectioning plane effect, and magnification effect). The SMI did not differ among samples obtained from different parts of the testes, confirming that germ cell development is fairly synchronous throughout the testes of European eels (Miura et al. 1991a; Miura and Miura 2001). The application of area fractions to estimate the prevalence of different categories of germ cells should eliminate potential bias that may arise from differences in the orientation of the tissue in histological sections (e.g., Chang et al. 2002; Neuenhagen et al. 2007). The independence of the SMI in relation to sectioning angle and magnification level is contrasted by the significant differences in SMI among weeks, indicating the capabilities of the method. The homogeneous structure and organization of the tissue as suggested by the limited difference among locations in the longitudinal and transverse sections indicate an organization of the

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germinal cells in anastomosing tubules rather than in lobules (Grier 1993; Schulz and Miura 2002; Schulz et al. 2010), which corresponds to the phylogenetic position of eels (Parenti and Grier 2004). Further improvement of the SMI as a quantitative index for assessment of testis development can be made by improving the sampling of tissue within testes through application of systematic uniform random sampling and considering differential shrinkage of tissues (e.g., Gundersen and Jensen 1987).

The SMI provides an opportunity to describe testis development quantitatively and to identify different male reproductive strategies in a manner similar to that used to describe female reproductive strategies (Murua and Saborido-Rey 2003). Knowledge of the progression and duration of gametogenesis is useful for understanding reproductive biology, including the functional and regulatory mechanisms of spermatogenesis in relation to reproductive strategy (Parenti and Grier 2004; Nóbrega et al. 2009), the latter of which is important to fisheries reproductive biology (Lowerre-Barbieri et al. 2011, this special section) and fish reproduction in aquaculture. Ovarian development and female reproductive strategies have received substantial attention due to the importance of fecundity and egg production in stock assessment and recruitment studies, while comparatively few studies have addressed male reproductive strategies and sperm production. In particular, applications of quantitative methods are rare (Trippel 2003). The present study represents a quantitative assessment of testis development that employs the area fraction per testis tissue category in combination with the SMI as a novel method to grade and compare development among fish in different reproductive phases and that is applicable to wild-caught fish and to cultured fish subjected to different treatments.

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