SCI. MAR., 66 (3): 273-282 SCIENTIA MARINA 2002

Group-synchronous ovarian development, ovulation and spermiation in the European sea bass (*Dicentrarchus labrax* L.) could be regulated by shifts in gonadal steroidogenesis*

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SUMMARY: European sea bass (*Dicentrarchus labrax* L.) is a species with group-synchronous ovarian development. A mechanism is required which enables maturation to occur in the first clutches of oocytes without inducing maturation in subsequent clutches. The present study examined the individual plasma variations of testosterone (T), 17,20β-dihydroxy-4-pregnen-3-one (17,20βP) and 17,20β,21-trihydroxy-4-pregnen-3-one (20βS) in both sexes, estradiol (E_2) and vitellogenin (VTG) in females (n=15) and 11-ketotestosterone (11KT) in males (n=21), in an effort to elucidate the hormonal control of the reproductive cycle in this species. A sample of oocytes was obtained at every sampling from each female and the stage of development of the most advanced clutch of oocytes was determined and related to the individual hormone plasma levels. Total expressible milt was collected from males at each sampling during the spawning period and variations in the sperm production were related to hormone plasma levels. Successive elevations of plasma T and E_2 levels were observed prior to peaks of progestagens, which resulted from the shift in gonadal steroidogenesis and coincided with the maturation-ovulation of the different clutches of oocytes or with increases in the sperm production. Following each progestagens wave, a new shift in gonadal steroidogenesis, resulting in a new elevation in plasma T and E_2, was observed. This hormonal pattern was repeated several times depending on the number of ovulations per female. Results from the present study suggest a mechanism, based on shifts in gonadal steroidogenesis, which may be responsible for regulation of group-synchronous ovarian development, ovulation and spermiation in this species. In addition, evidence is presented which supports a role for both 17,20βP and 20βS as the maturation-inducing steroids (MIS) in male and female European sea bass.

Key words: *Dicentrarchus labrax*; reproduction; hormones; maturation-inducing steroid, fish.

RESUMEN: EL DESARROLLO OVÁRICO SÍNCRONO POR GRUPOS, LA OVULACIÓN Y LA ESPERMIACIÓN DE LA LUBINA EUROPEA (*DICENTRARCHUS LABRAX* L.) PODRÍAN ESTAR REGULADOS POR CAMBIOS EN LA ESTEROIDEOGÉNESIS GONADAL. – La lubina europea (*Dicentrarchus labrax* L.) es una especie con un desarrollo ovárico síncrono por grupos. Por ello precisa de un mecanismo que permita la maduración de las primeras cohortes de oocitos sin inducir la maduración de las siguientes. El presente estudio examina las variaciones individuales de los niveles plasmáticos de testosterona (T), 17,20β-dihidroxi-4-pregnen-3-ona (17,20βP) y 17,20β,21-trihidroxi-4-pregnen-3-ona (20βS), en ambos sexos; así como de estradiol (E_2) y vitelogenina (VTG) en las hembras (n=15) y de 11-cetotestosterona (11KT) en los machos (n=21). En cada muestreo se obtuvo una alícuota de oocitos de cada hembra y se determinó el estado de desarrollo de la cohorte más avanzada, relacionándolo con los niveles plasmáticos de hormonas de cada ejemplar. Durante el periodo de espermiación, se extrajo la totalidad del

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*Received July 18, 2000. Accepted January 9, 2002.

esperma de cada macho y las variaciones en la producción de esperma se relacionaron con los correspondientes niveles plasmáticos de hormonas. Se observaron sucesivas elevaciones de los niveles plasmáticos de T y E_2 previas a los picos de progestágenos resultantes del cambio en la esteroidogénesis gonadal y coincidentes con la maduración-ovulación de las diferentes cohortes de oocitos, o con incrementos en la producción de esperma. Siguiendo a cada subida de progestágenos, se observó un nuevo cambio en la esteroidogénesis gonadal, que suponía una nueva elevación de los niveles plasmáticos de T y E_2. Este patrón hormonal se repitió varias veces dependiendo del número de ovulaciones por hembra. Los resultados del presente estudio sugieren la existencia de un mecanismo, basado en cambios en la esteroidogénesis gonadal, que podría ser responsable de la regulación del desarrollo ovárico síncrono por grupos, de la ovulación y de la espermiación en esta especie. Además, se aportan evidencias que apoyan el papel de 17,20βP y de 20βS como esteroides inductores de la maduración (MIS) en machos y hembras de lubina europea.

Palabras clave: *Dicentrarchus labrax*; reproducción; hormonas; esteroide inductor de la maduración, pez.

INTRODUCTION

European sea bass (*Dicentrarchus labrax* L.) has a group-synchronous ovarian development (Carrillo *et al.*, 1989; Mayer *et al.*, 1990; Alvariño *et al.*, 1992). Thus, during gonadal recrudescence, each ovary contains two or more clutches of oocytes in different stages of development that are successively ovulated. Recent studies have shown that one female of this species can have up to four ovulations during the natural reproductive period (Asturiano *et al*., 2000).

Several studies have described the seasonal variations of plasma levels of testosterone (T), 17β-estradiol (E_2) and vitellogenin (VTG) in the female European sea bass (Prat *et al*., 1990, 1999; Mañanós *et al*., 1994, 1997a, b; Zanuy *et al*., 1995; Navas *et al*., 1998) as well as the seasonal plasma variations of T and 11-ketotestosterone (11KT) levels in the males (Prat *et al*., 1990, 1999; Cerdá *et al*., 1997). However, these previous studies were based on monthly samplings and results were expressed as the mean hormonal plasma level from several fish. Due to the group synchronous nature of this species and the unpredictability of the multiple ovulations that occur during the reproductive season, large variations in cycling are evident both in males and females. Therefore, using monthly sampling method, rapid changes in hormonal levels may be missed. New studies, based on more intense strategies of sampling and an individualized study of the gonadal morphology are required to determine the precise relationship between plasma levels of T, 11KT, E_2 and VTG and the progression of the reproductive cycle. This information would provide a better understanding of the hormonal regulation of reproductive processes in this species.

In salmonids, FSH (previously named GTH1, Quérat, 1994) is released during the period of gonadal growth and stimulates the follicular production of E_2 and T (Prat *et al*., 1996; Nagahama, 1997). When follicles reach the postvitellogenic stage, LH (previously named GTH2, Quérat, 1994) regulates final maturation and ovulation/spermiation and stimulates a shift in steroidogenesis from the synthesis of T and E_2 to that of maturation-inducing steroids (MIS) thus promoting the maturation and ovulation of oocytes (reviewed by Nagahama, 1994, 1997; Nagahama *et al*., 1995; Prat *et al*., 1996). This shift in gonadal steroidogenesis has been observed in several species of the genus *Morone*, closely related to the European sea bass (King *et al*., 1994, 1995; Mylonas *et al*., 1997a).

In the female European sea bass, several studies have described the potential role of progestagens during final oocyte maturation (Prat *et al*., 1990; Scott *et al*., 1990). Both 17,20β-dihydroxy-4-pregnen-3-one (17,20βP) and 17,20β,21-trihydroxy-4-pregnen-3-one (20βS) were shown to induce maturation *in vitro* (Sorbera *et al*., 1999) and Asturiano *et al*. (2000) showed plasma peaks of 17,20βP and 20βS *in vivo* coinciding with the beginning of maturation and with final maturation, respectively, suggesting that both progestagens may be the MIS in this species. However, until now no studies have been done concerning the role of progestagens in the regulation of the male reproductive process in this species.

The aim of the present study was to elucidate the hormonal mechanism which enables maturation to occur in the first clutches of oocytes without inducing maturation in subsequent clutches. Moreover, this study attempted to confirm the involvement of 17,20βP and 20βS in the reproductive cycle of both male and female European sea bass.

MATERIAL AND METHODS

Fish

Three-year-old female (882.4 ± 57 g B.W.; n=15) and five-years-old male (852.16 ± 23 g; n=21) Euro-

pean sea bass were maintained under natural photoperiod and temperature conditions in separate fibre-glass tanks supplied with aerated flow-through sea water (37.8 g l^{-1} salinity). Fish were hand fed three days a week with a natural diet consisting of *Boops boops* at the rate of 2.2% tank biomass per day from June to October and 1.2% the rest of the year. The amount of food given to broodstock was adjusted monthly after sampling.

Sampling protocol

All samplings were performed between 9:30 and 10:30 am. At each sampling, males and females were anesthetized with ethylenglycol-monophenylether (1 ml l^{-1}) and blood was taken from the caudal vein and dispensed into heparinized tubes kept on ice. Plasma was obtained by centrifugation (1600 g, 20 min at 4ºC), aliquoted and stored at -20ºC until analysis.

Males were bleed and checked for the presence of sperm by gently stripping, between 9:30 and 10:30 am, at 7-day intervals from October to April. After cleaning the genital area with fresh water and throughly drying to avoid contamination of samples with feces, urine and sea water, total expressible milt was collected (from January to April) by applying gentle abdominal pressure to anesthetized males and by placing a vacuum glass tube around the genital area to collect sperm in a graduate cilinder. Total expressible milt was noted in ml.

Females were first sampled at 1-month intervals from the beginning of September to mid-January and later at 7-day intervals until April. A sample of oocytes was obtained from each ovary of every female by inserting a polyethylene tubing into the oviduct to the mid-portion of the ovary and sucking orally as the cannula was withdrawn. Oocytes were placed in Ringer's solution modified for the sea bass (SBR; Sorbera *et al.*, 1999) and maintained on ice for measurement of oocyte diameter using an ocular micrometer under light microscopy immediately after obtaining the sample. In each sample, 60-100 of the largest oocytes were measured. A sample of each group of measured oocytes was fixed separately in 4% formaldehyde, 1% glutaraldehyde fixative (McDowell and Trump, 1976). Follicles were dehydrated in a 75-90% ethanol series and embedded in plastic Technovit (Kulzer). The blocks were cut in 2-µm sections and were stained with Cleveland-Wolfe (Herlant, 1960). These preparations were used to determine the developmental stage of largest oocytes in each female at every sampling. The staging criteria used for oocyte development was as described by Asturiano *et al.* (2000).

Plasma vitellogenin and steroid hormone analyses

All plasma VTG and steroid hormone samples were assayed in duplicate. Plasma levels of T were measured by an EIA as described by Rodríguez *et al.* (2001). T was triple extracted with methanol using 100 µl of plasma diluted 1:32 in potassium phosphate buffer (0.1 M, pH 7.4) containing 0.01% sodium azide, 0.4 M NaCl, 0.001 M EDTA and 0.1% BSA. The inter-assay coefficient of variation was 7.54% (n=10).

Plasma levels of 11KT were measured by EIA as described by Cuisset *et al*. (1994) and the inter-assay coefficient of variation was 10.04% (n=3).

Plasma levels of VTG were quantified using a homologous ELISA following the procedure described by Mañanós *et al*. (1994). Prior to analysis, plasma samples were diluted 1:10,000 or 1:100,000-fold dilution depending on the reproductive stage of the females in order to ensure that all measurements were within the confidence range of the standard curve. The inter-assay coefficient of variation was 10.39% (n=5).

E_2 was extracted from 100 µl of plasma using cyclohexane:ethylacetate (1:1, v/v) and E_2 concentrations were determined by RIA according to Prat *et al*. (1990). The intra- and inter-assay coefficients of variation were 8.34% (n=4) and 4.27% (n=12), respectively.

Plasma concentrations of 17,20βP and 20βS were measured by specific ELISAs based on the method described by Nash *et al*. (2000). Cross-reactivities of the antisera to 17,20βP and 20βS are as in Canario *et al.* (1989). We have not carried out a full test of cross-reactivity using ELISA for these steroids, but previous studies (Cuisset *et al*., 1994) and our own more limited trials have shown no significant difference in cross-reactivity between ELISA and RIA.

RESULTS

Females

Individual samples collected from weekly samplings from September to April revealed that in all females the first ovulation took place after the maximum levels of plasma VTG (Figs. 1 and 2).

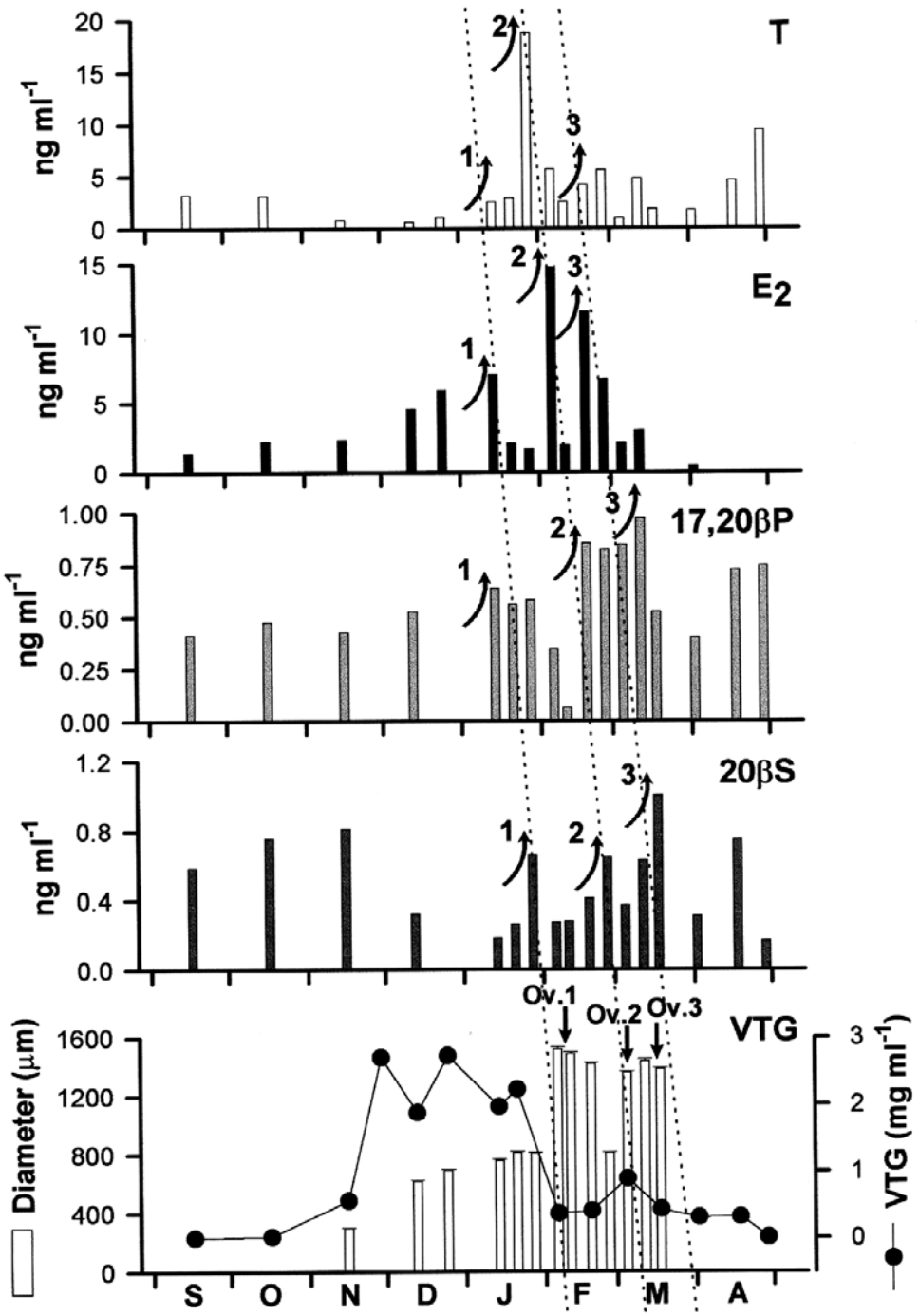

FIG. 1. – The dynamics of oocyte growth and ovulation in one female (September-April) showing three ovulations and the plasma level variations in T, E_2, VTG, 17,20βP and 20βS. Bars on the lower graph show mean diameter (μm) of the most advanced clutches of oocytes. Arrows in the lower graph indicate when ovulations were detected by histological examination of intraovarian samples. Ascendent arrows in the upper graphs indicate the associated peaks of T, E_2, 17,20βP and 20βS, respectively. Vertical long-dotted lines correlate different peaks related with every ovulation.

Both T and E_2 plasma levels showed important variations during the spawning season, with peaks observed prior to ovulation of the successive clutches of oocytes in each female (Figs. 1 and 2). In addition, all the females showed a wave-like profile of plasma 17,20βP and 20βS. Peaks of these progestagens occurred slightly before or coincided with maturation-ovulation of the different clutches of oocytes detected in each female. Figures 1 and 2 illustrate the dynamics of oocyte growth together with the plasma variations of T, E_2, VTG, 17,20βP and 20βS in two different individual females. Histological

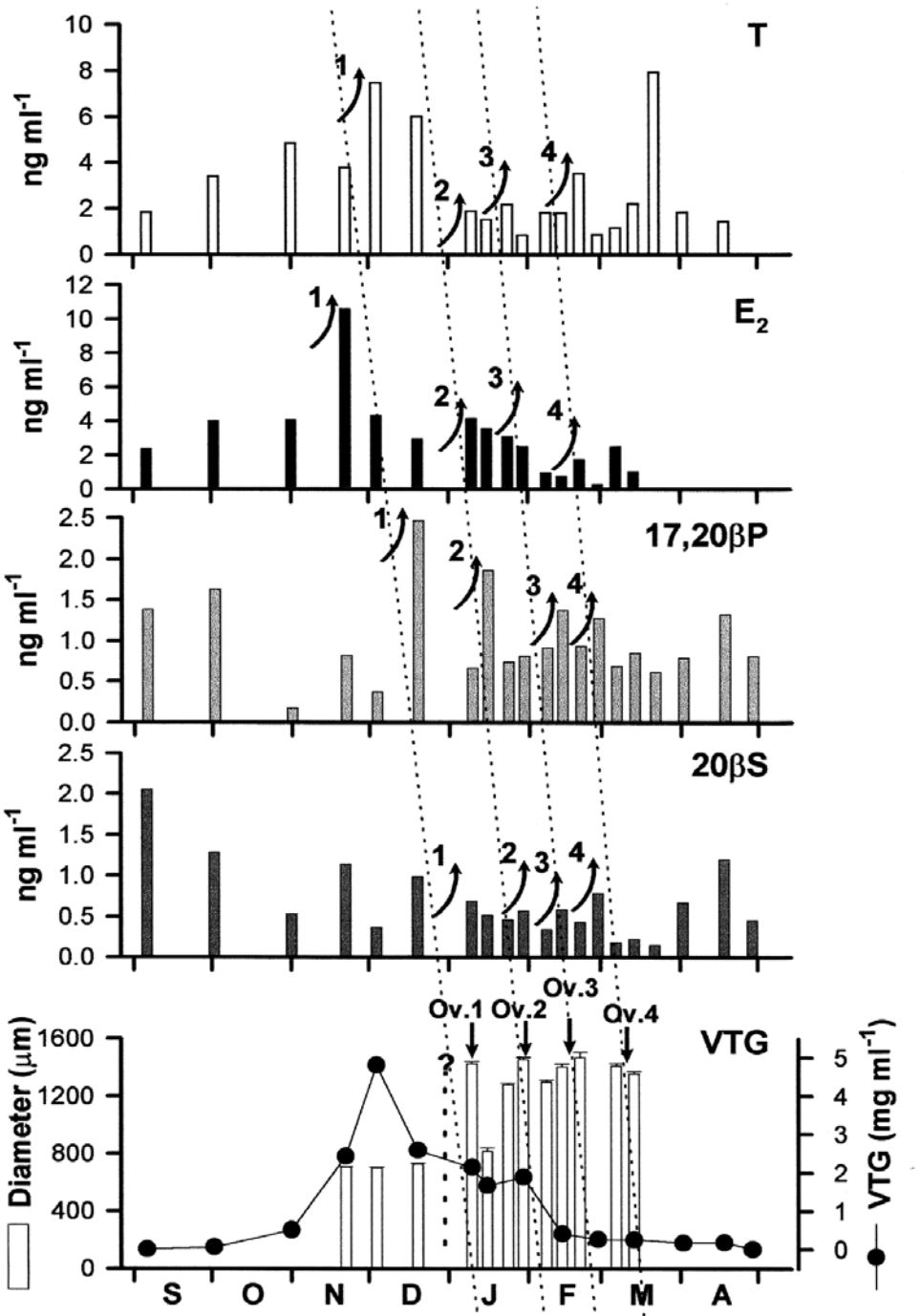

FIG. 2. – The dynamics of oocyte growth and ovulation in one female (September-April) showing four ovulations and the plasma level variations of T, E_2, VTG, 17,20βP and 20βS. Bars on the lower graph show mean diameter (μm) of the most advanced clutches of oocytes. Arrows in the lower graph indicate when ovulations were detected by histological examination of intraovarian samples. Ascendent arrows in the upper graphs indicate the associated peaks of T, E_2, 17,20βP and 20βS, respectively. The short-dotted line shows one interval without samplings when the first ovulation may have started. Vertical long-dotted lines correlate different peaks related with every ovulation.

analysis of ovarian samples collected at each sampling and measurement of the diameters of the largest oocyte led to the conclusion that these two females had three and four ovulations, respectively. For instance, Figure 1 shows the first increases of T and E_2 during the 2nd week of January. Next, a shift in gonadal steroidogenesis was observed resulting in diminishing plasma T and E_2 and increases in 17,20βP (from the 2nd week) and 20βS (between the 3rd-4th weeks of January); the first ovulation was detected in the 1st week of February. Following each MIS wave which resulted in maturation and

ovulation of the most advanced clutch of oocytes, a new shift in gonadal steroidogenesis was observed. This shift was associated with a new elevation of plasma T and E_2 and a reduction in MIS. This pattern was repeated several times depending on the number of ovulations per female. Figure 1 illustrates how new increases of 17,20βP (3rd week of February) and 20βS (4th week of February) resulted in a second ovulation during the 1st week of March and how increases of 17,20βP (1st-2nd weeks of March) and 20βS (3rd week of March) resulted in a third ovulation during the 3rd week of March. This mechanism was observed in all ovulating females (80%); unovulated oocytes from the remaining females appeared to regress and undergo atresia in spite of reaching a postvitellogenic oocyte diameter.

Males

A similar mechanism was also observed in the males. Figures 3 and 4 show the total sperm volume collected from two individual males together with

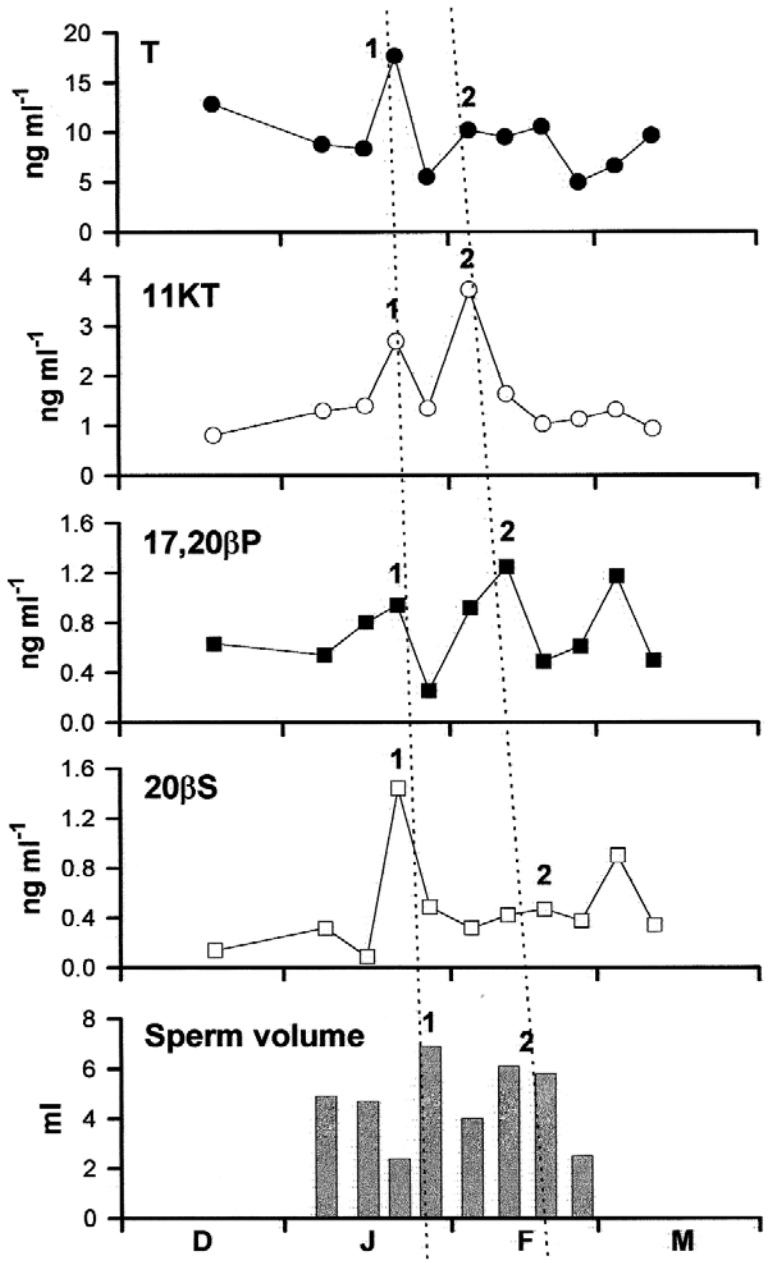

FIG. 3. – The dynamics of sperm production in one male from January to March with plasma level variations of T, 11KT, 17,20βP and 20βS. Numbers indicate the peaks in sperm production during this period and the associated peaks of androgens and progestagens, respectively. Vertical long-dotted lines correlate different peaks with every increase in sperm production.

plasma variations of T, 11KT, 17,20βP and 20βS. In the 21 considered males, total sperm volume was not constant, instead exhibiting successive peaks in production. T and 11KT peaks preceded the MIS elevations responsible for increases in sperm production. For instance, results from a male shown in Fig. 4 displayed the first peaks of T and 11KT during the 2nd week of January that were followed by 17,20βP and 20βS peaks during the 3rd week (coinciding with a reduction in androgens) and by the first increase in sperm production during the 3rd-4th weeks of the same month. A second peak in androgens was observed in the 1st week of February and was followed by progestagen peaks during the 2nd-3rd weeks of this month which coincided with a second increase in sperm production. New increases in androgens and progestagens were detected as well as a third peak of sperm production during the 1st week of March.

Most ovulations were detected from the end of January to the end of February. The milt release period extended from the end of October to the end

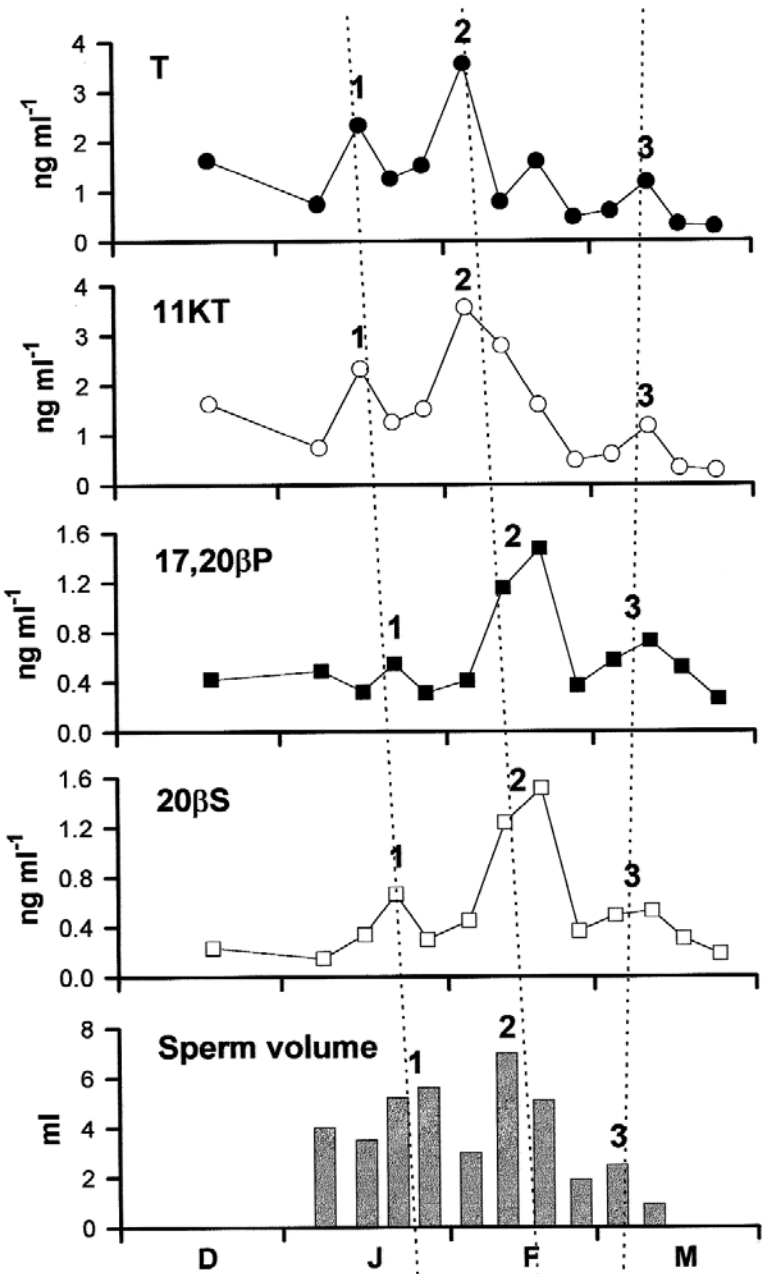

FIG. 4. – The dynamics of sperm production in one male from January to March with plasma level variations of T, 11KT, 17,20βP and 20βS. Numbers indicate the peaks in sperm production during this period and the associated peaks of androgens and progestagens, respectively. Vertical long-dotted lines correlate different peaks with every increase in sperm production.

of March. Although experimental males were maintained separated from females throughout the experiment, the length of the spermiation period was not affected. Thus other males maintained in the same conditions and fed the same diets, but housed with females, exhibited a similar duration of the spermiation period (data not shown).

DISCUSSION

In a species with group-synchronous ovarian development such as the European sea bass, a mechanism is required which enables maturation to occur in the first clutches of oocytes without inducing maturation in subsequent clutches. Results from the present study suggest, for the first time in this species, the existence of this mechanism which is based on shifts in gonadal steroidogenesis. Moreover, results from this study corroborate that both 17,20βP and 20βS have a role as MIS in male and female European sea bass.

All the females that presented ovulations displayed successive short-term elevations of plasma T and E_2 prior to peaks of 17,20βP and 20βS. Each MIS wave resulting from the shift in gonadal steroidogenesis coincided with a reduction in T and E_2 plasma levels and occurred slightly prior to ovulation of the most advanced clutch of oocytes. Following each MIS wave which resulted in the maturation and ovulation of one clutch of oocytes, a new shift in gonadal steroidogenesis was observed. This shift signified a new elevation in T and E_2 plasma levels and a reduction in MIS plasma levels preventing maturation of the rest of clutches.

A similar pattern of hormonal regulation was observed in males. Each wave of plasma progestagens was observed to coincide with increases in the sperm production and was preceded by increases in the synthesis of androgens.

Studies *in vivo* and *in vitro* in salmonids have demonstrated that when follicles reach the postvitellogenic stage, an increase in LH level induces the synthesis of androgens and a decrease in their aromatization to E_2 while the enzymes involved in the synthesis of MIS are activated (Zohar *et al*., 1986; Nagahama, 1994, 1997; Nagahama *et al*., 1995). A similar mechanism was observed by Vizziano *et al*. (1996) in male rainbow trout (*Oncorhynchus mykiss*) in which the synthesis of 17,20βP was inhibited by E_2 until the spermiation period, when the release of E_2 became discontinuous. The same mechanism was previously proposed to explain the changes in the release of 17,20βP during the period of final maturation of oocytes in the ovary of the rainbow trout (Jalabert and Fostier, 1984). Murayama *et al.* (1994) found that the changes in plasma levels of E_2 correlated with the successive spawnings in the Japanese sardine (*Sardinops melanostictus*). This shift in gonadal steroidogenesis from the production of T and E_2 to that of MIS has also been observed in the striped bass (*Morone saxatilis*) and other species of the genus *Morone*, closely related to the European sea bass. King *et al*. (1994) observed *in vitro* that human chorionic gonadotropin (hCG) induced the production of MIS and the reduction of E_2 and T synthesis in ovarian fragments of striped bass (*M. saxatilis*). Similar results were reported both *in vitro* and *in vivo* in *M. americana* and *M. chrysops* (King *et al*., 1995). Recently, Rahman *et al*. (2001) reported a similar steroidogenic shift in hCG-treated ovarian fragments of Japanese yellowtail (*Seriola quinqueradiata*) during final oocyte maturation.

In the white bass (*M. chrysops*) *in vivo* treatment with gonadotropin releasing hormone agonist (GnRHa) induced final oocyte maturation associated with a significant elevation in plasma LH levels and important shifts in gonadal steroidogenesis (Mylonas *et al*., 1997b). In female European sea bass, Navas *et al*. (1998) observed a peak of LH at the middle of the ovulation. Moreover, males exhibited the highest LH plasma levels at the middle of the spermiation period (Navas *et al*., 2001; Rodríguez *et al*., 2001). Although these studies did not examine plasma progestagen variations, female treatment with GnRHa resulted in an important elevation of plasma LH levels that was followed by a peak of 17,20βP and the appearance of ovulated oocytes (Fornies *et al*., 2000). Both 17,20βP and 20βS have been detected in the plasma of female European sea bass (Prat *et al*., 1990; Scott *et al*., 1990; Asturiano *et al*., 2000) and both progestagens have been shown to induce maturation *in vitro* (Sorbera *et al*., 1999). Results from the present study support a role for both 17,20βP and 20βS as MIS both in male and female European sea bass.

The correlation between the beginning of spermiation and increases in plasma levels of progestagens has been observed in several species. Ueda *et al*. (1985) reported that injections of 17,20βP causes an increase in sperm volume in the goldfish (*Carassius auratus*) and suggested that this steroid could play a role as a testicular mediator in the pro-

duction of sperm induced by LH. A similar suggestion has been made for salmonids (reviewed by Nagahama, 1994). In the striped bass (*M. saxatilis*) GnRHa treatment resulted in a significant increase of LH, 11KT, and 17,20βP plasma levels as well as a significant elevation in total expressible milt (Mylonas *et al*., 1997c). In accordance with our results, Baynes and Scott (1985) and Barry *et al*. (1990) proposed that LH induces the production of progestagens which causes an inhibition in androgen synthesis thus regulating final maturation of sperm in fish.

In the plaice (*Pleuronectes platessa*), which also exhibits group-synchronous ovarian development, Scott *et al*. (1998) found that 17,20βP plasma levels during oocyte maturation were not higher than basal levels, although its metabolites were considerably higher in females that ovulated. The present results support the idea suggested by Scott and Canario (1987) that 17,20βP has a paracrine function, influencing the follicles where it is synthesized and that enzymes could inactivate it before it enters the circulatory system. If it entered the circulatory system, 17,20βP would have an endocrine role and induce the maturation of all follicles. This mechanism is necessary in species like the European sea bass, that have group-synchronous ovarian development resulting in several spawnings during a reproductive season, and could explain the slight variations in the plasma levels of 17,20βP and 20βS detected in this study in comparison with the levels reached in other species, especially salmonids, that display synchronous oocyte development.

Furthermore, studies have also revealed rapid changes in MIS levels in some teleosts and a weekly sampling strategy, as used in this study, or monthly sampling would give a limited view of the real amplitude of the progestagens' variations. For instance, in the Japanese sardine (*S. melanostictus*), final oocyte maturation occurs daily between 18:00 and 23:00 hours (Matsuyama *et al*., 1994; Murayama *et al*., 1994), and only during this period plasma concentrations of 17,20βP are detected. Moreover, it is known that the gonad transforms free steroids to glucuronides and sulfates (Kime, 1993) which are undetectable by the assays used in this study. In addition to these changes there may also be short term diurnal changes or a pulsatile release of steroids throughout the day as described by Nash *et al.* (1999, 2000) for rainbow trout. Thus, all these factors could be affecting our measurements of progestagen plasma level variations.

This study has identified a possible mechanism based on shifts in gonadal steroidogenesis which may be responsible for regulation of group-synchronous ovarian development, ovulation and spermiation in the European sea bass. However, *in vitro* studies are required to confirm this mechanism and to explain the involvement of GTHs, gonadal steroids, enzymes, and follicular factors in the regulatory system of reproduction in this species.

ACKNOWLEDGEMENTS

This study was supported by EEC (AIR-CT93-1005) and CICYT (MAR96-1859) and a Spanish-British Concerted Action (HB1997-0072) to M.C. and D.E.K. J.F.A. was supported by the Generalitat Valenciana. The authors wish to thank Dr. A.P. Scott (Lowestoft) for providing the antibodies to 17,20βP and 20βS.

REFERENCES

Alvariño, J.M.R., M. Carrillo, S. Zanuy, F. Prat and E. Mañanós. – 1992. Pattern of sea bass oocyte development after ovarian stimulation by LHRHa. *J. Fish Biol.,* 41: 965-970.

Asturiano, J.F., L.A. Sorbera, J. Ramos, D.E. Kime, M. Carrillo and S. Zanuy. – 2000. Hormonal regulation of the European sea bass (*Dicentrarchus labrax*, L.) reproductive cycle: an individualized female approach. *J. Fish Biol.,* 56: 1155-1172.

Barry, T.P., K. Aida, T. Okumura and I. Hanyu. – 1990. The shift from C-19 to C-21 steroid synthesis in spawning male common carp, *Cyprinus carpio*, is regulated by the inhibition of androgen production by progestogens produced by spermatozoa. *Biol. Reprod.,*43: 105-112.

Baynes, S.M. and A.P. Scott. – 1985. Seasonal variations in parameters of milt production and in plasma concentration of sex steroids of male rainbow trout (*Salmo gairdneri*). *Gen. Comp. Endocrinol.,* 57: 150-160.

Canario, A.V.M., A.P. Scott and A.P.F. Flint. – 1989. Radioimmunoassay investigations of 20β-hydroxylated steroids in maturing/ovulating female rainbow trout (*Salmo gairdneri*). *Gen. Comp. Endocrinol.,* 74: 77-84.

Carrillo, M., N. Bromage, S. Zanuy, R. Serrano and F. Prat. – 1989. The effect of modifications in photoperiod on spawning time, ovarian development and egg quality in the sea bass *Dicentrarchus labrax* L. *Aquaculture,* 81: 351-365.

Cerdá, J., S. Zanuy and M. Carrillo. – 1997. Evidence for dietary effects on plasma levels of sexual steroids during spermatogenesis in the sea bass. *Aquaculture International*, 5: 473-477.

Cuisset, B., P. Pradelles, D.E. Kime, E.R. Kühn, P. Babin, S. Davail and F. Le Menn. – 1994. Enzyme immunoassay for 11-ketotestosterone using acetylcholinesterase as label: application to the measurement of 11-ketotestosterone in plasma of Siberian sturgeon. *Comp. Biochem. Physiol.,*180C: 229-241.

Fornies, M.A., M.J. Bayarri, E. Mañanós, M. Carrillo, C.C. Mylonas, Y. Zohar and S. Zanuy. – 2000. Endocrine effects of different GnRH-delivery systems in female sea bass. In Programme and Abstracts of the 20th Conference of European Comparative Endocrinologists. Pp: 103. Centre of Marine Sciences, University of Algarve, Faro, Portugal.

Herlant, M. – 1960. Etude critique de deux techniques nouvelles destinées à metre en évidence les différents catégories cellulaires présente dans la glande pituitaire. *Bulletin de microscopie appliquée,*10: 37-44.

Jalabert, B. and A. Fostier. – 1984. The modulatory effect *in vitro* of oestradiol-17β, testosterone and cortisol on the output of 17©-hydroxy- 20β-dihydroprogesterone by rainbow trout (*Salmo gairdneri*) ovarian follicles stimulated by the maturational gonadotropin s-GtH. *Reprod. Nutr. Dev.,* 24: 127-136.

Kime, D.E. – 1993. "Classical" and "non-classical" reproductive steroids in fish. *Rev. Fish Biol. Fish.,* 3: 160-180.

King, W.V., P. Thomas and C.V. Sullivan. – 1994. Hormonal regulation of final maturation of striped bass oocytes *in vitro*. *Gen.Comp. Endocrinol.,* 96: 223-233.

King, W.V., D.L. Berlinsky and C.V. Sullivan. – 1995. Involvement of gonadal steroids in final oocyte maturation of white perch (*Morone americana*) and white bass (*M. chrysops*): *in vivo* and *in vitro* studies. *Fish Physiol. Biochem.,*14: 489-500.

Mañanós, E., J. Núñez, S. Zanuy, M. Carrillo and F. Le Menn. – 1994. Sea bass (*Dicentrarchus labrax* L.) Vitellogenin. II. Validation of an enzyme-linked immunosorbent assay (ELISA). *Comp. Biochem. Physiol.,* 107B: 217-223.

Mañanós, E.L., S. Zanuy and M. Carrillo. – 1997a. Photoperiodic manipulations of the reproductive cycle of sea bass (*Dicentrarchus labrax*) and their effects on gonadal development, and plasma 17β-estradiol and vitellogenin levels. *Fish Physiol. Biochem.,*16: 211-222.

Mañanós, E.L., S. Zanuy and M. Carrillo. – 1997b. Identification of vitellogenin receptors in the ovary of a teleost fish, the Mediterranean sea bass (*Dicentrarchus labrax*). *Reprod. Nutr. Dev.,* 37: 51-61.

Matsuyama, M., T. Fukuda, S. Ikeura, Y. Nagahama and S. Matsuura. – 1994. Spawning characteristics and steroid hormone profiles in the wild female Japanese sardine *Sardinops melanostictus*. *Fisheries Science,* 60: 703-706.

Mayer, I., S.E. Shackley and P.R. Witthames. – 1990. Aspects of the reproductive biology of the bass, *Dicentrarchus labrax* L. II. Fecundity and pattern of oocyte development. *J. Fish Biol.,* 36: 141-148.

McDowell, E.M. and B.F. Trump. – 1976. Histologic fixatives suitable for diagnostic light and electron microscopy. *Arch. Pathol. Lab. Med.*, 100: 405-414.

Murayama, T., M. Shiraishi and I. Aoki. – 1994. Changes in ovarian development and plasma levels of sex steroid hormones in the wild female Japanese sardine (*Sardinops melanostictus*) during the spawning period. *J. Fish Biol.,* 45: 235-245.

Mylonas, C.C., A.P. Scott, and Y. Zohar, – 1997a. Plasma gonadotropin II, sex steroids and thyroid hormones in wild striped bass (*Morone saxatilis*) during spermiation and final oocyte maturation. *Gen. Comp. Endocrinol*., 108: 223-236.

Mylonas, C.C., Y. Magnus, Y. Klebanov, A. Gissis and Y. Zohar. – 1997b. Reproductive biology and endocrine regulation of final oocyte maturation of captive white bass. *J. Fish Biol*., 51: 234-250.

Mylonas, C.C., A.P. Scott, E.L.M. Vermeirssen and Y. Zohar. – 1997c. Changes in plasma gonadotropin II and sex steroid hormones, and sperm production of stripped bass after treatment with controlled-release gonadotropin-releasing hormone agonist-delivery systems. *Biol. of Reprod*., 57: 669-665.

Nagahama, Y. – 1994. Endocrine regulation of gametogenesis in fish. *Int. J. Dev. Biol.,* 38: 217-229.

Nagahama, Y. – 1997. 17",20β-dyhidroxy-4-pregnen-3-one, a maturation-inducing hormone in fish oocytes: mechanisms of synthesis and action. *Steroids,* 62: 190-196.

Nagahama, Y., M. Yoshikuni, M. Yamashita, T. Tokumoto and Y. Katsu. – 1995. Regulation of oocyte growth and maturation in fish. *Curr. Top. Dev. Biol.*, 30: 103-145.

Nash, J.P., W.H. Holtz and D.E. Kime. – 1999. Pulsatile steroid hormone profiles, diel metabolic rhythms and reproductive seasonality in the female rainbow trout, *Oncorhynchus mykiss*. In: B. Norberg, O.S. Kjesbu, G.L. Taranger, E. Andersson and S.O. Stefansson (eds.), *Proceedings 6th International Symposium on Reproductive Physiology of Fish*. Bergen (Norway). Pp. 331.

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

Navas, J.M., E. Mañanós, M. Thrush, J. Ramos, S. Zanuy, M. Carrillo, Y. Zohar and N. Bromage. – 1998. Effect of dietary lipid composition on vitellogenin, 17β-estradiol and gonadotropin plasma levels and spawning performance in captive sea bass (*Dicentrarchus labrax* L.). *Aquaculture,*165: 65-79.

Navas, J.M., E. Mañanós, J. Ramos, Y. Zohar, S. Zanuy and M. Carrillo. – 2001. Los niveles plasmáticos de hormona luteinizante en machos de lubina (*Dicentrarchus labrax* L.) se ven influidos por los niveles de lípidos de la dieta. In Proceedings of the VIII Congreso Nacional de Acuicultura: Acuicultura y desarrollo sostenible; pp: 182-184. Dirección General de Pesca y Alimentación. Gobierno de Cantabria, Spain.

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

Prat, F., J.P. Sumpter and C.R. Tyler. – 1996. Validation of radioimmunoassays for two salmon gonadotropins (GtHI and GtHII) and their plasma concentrations through the reproductive cycle in male and female rainbow trout (*Oncorhynchus mykiss*). *Biol. Reprod*., 54: 1375-1382.

Prat, F., S. Zanuy, N. Bromage and M. Carrillo. – 1999. Effects of constant short and long photoperiod regimes on the spawning performance and sex steroid levels of female and male sea bass. *J. Fish Biol.,* 54: 125-137.

Quérat, B. – 1994. Molecular evolution of the glycoprotein hormones in vertebrates. In: Davey, K.G., Peter, R.E. and Tobe, S.S. (eds.), *Perspectives in Comparative Endocrinology*, pp. 27-35. National Research Council of Canada, Ottawa.

Rahman, M.A., K. Ohta, H. Chuda, S. Nakano, K. Maruyama and M. Matsuyama. – 2001. Gonadotropin-induced steroidogenic shift towards maturation-inducing hormone in Japanese yellowtail during final oocyte maturation. *J. Fish Biol*., 58: 462-474.

Rodríguez, L., I. Begashi, S. Zanuy, and M. Carrillo – 2001. Development and validation of an enzyme immunoassay for testosterone: Effects of photoperiod on plasma testosterone levels and gonadal development in male sea bass (*Dicentrarchus labrax* L.) at puberty. *Fish Physiol. Biochem*., 23(2): 141-150.

Scott, A.P. and A.V.M. Canario. – 1987. Status of oocyte maturation-inducing steroids in teleosts. In: D.R. Idler, L.W. Crim and J.M. Walsh (eds.), *Proceedings of the Third International Symposium on Reproductive Physiology of Fish*, pp.: 224-234, St. John´s, Newfoundland. St. Johns: Marine Sciences Research Laboratory.

Scott, A.P., A.V.M. Canario and F. Prat. – 1990. Radioimmunoassay of ovarian steroids in plasmas of ovulating female sea bass (*Dicentrarchus labrax*). *Gen.Comp. Endocrinol.*, 78: 299-302.

Scott, A.P., P.R. Witthames, R.J. Turner and A.V.M. Canario. – 1998. Plasma concentrations of ovarian steroids in relation to oocyte final maturation and ovulation in female plaice sampled at sea. *J. Fish Biol.,* 52: 128-145.

Sorbera, L.A., J.F. Asturiano, M. Carrillo, J. Cerdà, D.E. Kime, and S. Zanuy. – 1999. *In vitro* oocyte maturation in the sea bass: effects of hCG, pituitary extract and steroids. *J. Fish Biol.,* 55: 9-25.

Ueda, H., A. Kambegawa, and Y. Nagahama. – 1985. Involvement of gonadotrophin and steroid hormones in spermiation in the amago salmon, *Oncorhynchus rhodurus*, and goldfish, *Carassius auratus*. *Gen.Comp. Endocrinol.,* 59: 24-30.

Vizziano, D., F. Le Gac and A. Fostier. – 1996. Effect of 17β-estradiol, testosterone, and 11-ketotestosterone on 17,20β-dihydroxy-4-pregnen-3-one production in the rainbow trout testis. *Gen.Comp. Endocrinol.,* 104: 179-188.

Zanuy, S., M. Carrillo and F. Ruiz. – 1986. Delayed gametogenesis and spawning of sea bass (*Dicentrarchus labrax* L.) kept under different photoperiod and temperature regimes. *Fish Physiol. Biochem.,* 2: 53-63.

Zanuy, S., F. Prat, M. Carrillo and N. Bromage. – 1995. Effects of constant photoperiod on spawning and plasma 17β-oestradiol levels of sea bass (*Dicentrarchus labrax* L.). *Aquat. Living Resourc.,* 8: 147-152.

Zohar, Y., B. Breton, and A. Fostier. – 1986. Short-term profiles of plasma 17"-hydroxy, 20β-dihydroprogesterone levels in the female rainbow trout at the periovulatory period. *Gen.Comp. Endocrinol.,* 64: 189-198.

Scient. ed.: C. Pedrós-Alió