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Evidence of Temperature-Dependent Sex Determination in the European Sea Bass (*Dicentrarchus labrax* L.)

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ABSTRACT To test the hypothesis that sex determination in the European sea bass (*Dicentrarchus labrax* L.) can be affected by the incubating temperature during the very early developmental stages, eggs from the same batch of spontaneously spawned broodstock were divided at the stage of half-epiboly into three groups according to rearing temperature: G13 = 13°C, G15 = 15°C, and G20 = 20°C. Temperature treatment lasted until the middle of metamorphosis (17–18 mm total length, [TL]), and, with the exclusion of water temperature, all biotic and abiotic conditions were identical for the three experimental groups. The on-growing phase was performed under ambient photoperiod and temperature conditions for all groups. Sex proportions were determined by histological examination of the gonads of fish at 308, 467, and 568 days posthatch (DPH). At 308 DPH (TL: 135–201 mm), 100% of the specimens had differentiated into males and females. A significantly higher ($P < 0.01$) proportion of females was found in groups G13 (72–74%) and G15 (67–73%) than in group G20 (24–28%). At the final sampling there was no statistically significant difference in body weight between the experimental groups. However, in all groups, female fish were larger than males ($P < 0.001$). Results provide for the first time clear evidence that temperature during the very early developmental stages is the crucial factor affecting the process of sex differentiation of the sea bass, with low rearing temperatures (13 or 15°C) resulting in sex proportions consistently skewed in favor of females. *J. Exp. Zool. 287:225–232, 2000.* © 2000 Wiley-Liss, Inc.

Sex differentiation in fish is controlled ultimately by specific sex-determining genes, but in contrast with other taxa, sexual development in teleosts is protracted and plastic. Genotypic and phenotypic sex do not necessarily coincide, and, in several species, interactions between the genome and variable environmental and internal factors may determine sex (Shapiro, '88; Redding and Patiño, '93). In addition, the diversity of reproductive strategies shown by teleosts does not help to obtain a clear picture, and typical genetic mechanisms of sex differentiation are inadequate to explain sexual phenotype.

Among the environmental factors implicated in sex differentiation, temperature is the most studied. The rearing temperature has been shown to influence sex differentiation in several vertebrates such as amphibians, reptiles, and fish (Ewert et al., '94; Mrosovsky, '94; Lang and Andrews, '94; Viets et al., '94; Chardard et al., '95; Strüssmann and Patiño, '95; Blázquez et al., '98a; Baroiller et al., '99). It is possible that temperature exerts its action on the metabolic pathways for steroid biosynthesis or on the brain that has been proposed to be the initial site of sex differentiation in fish (Reinboth, '88; Francis, '92; Shapiro and Rasotto, '93; Elofsoon et al., '97).

The European sea bass, *Dicentranchus labrax*, is a gonochoristic species with the gonad remains sexually undifferentiated until the end of the first year of life (7–12 months of life) (Roblin and Buslé, '83; Blázquez et al., '95). However, in fish maintained under intensive culture conditions the sex ratio is consistently skewed in favor of males (reaching in some instances values over 90%), and a significant proportion of precocious males (males

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that mature soon after sexual differentiation) is always present (Blázquez et al., '95, '98a; Carrillo et al., '95). This is undesirable for the fish farmers, because male fish exhibit reduced somatic growth, resulting in an 18–40% smaller body weight at 2 years of age (Carrillo et al., '95). Therefore, during the last 10 years, for both scientific and economical reasons, research has been focused on the genetic and endocrine basis of sexual differentiation in sea bass. Sea bass lack heteromorphic sex chromosomes, and ploidy manipulation failed in modifying sex ratio (Cataudella et al., '73; Sola et al., '93). The labile period (period of responsiveness to the action of exogenous sex steroids) has been located around 100 days of age (Blázquez, '96). During this period administration of adrogens or estrogens result in all-male or increased female populations, respectively (Blázquez et al., '95; Blázquez, '96). However, due to the increasing concern of consumers to the use of chemical agents to food products, research has been shifted to the investigation of possible environmental factors implicated in sex control. In a recent study, Blázquez et al. ('98a) suggested that rearing temperature at the alevin stage (57–137 days post fertilization) could, although slightly, modify sex differentiation and growth of sea bass. However, no study on the effect of rearing temperature at the first stages of ontogenesis exists.

The present study was undertaken to investigate the hypothesis that the bias toward males in cultured sea bass is due to environmental factors and to test whether sex determination can be affected by the incubating temperature during the very early developmental stages of eggs and larvae.

MATERIALS AND METHODS

Experimental groups

Sea bass eggs were obtained from a broodstock of the same genetic origin (wild-caught specimens, kept in captivity for 6 years) maintained at the Aquaculture Research Station of IMBC. Fish were fed special pellets (BioMar S.A., France) supplemented twice a week with raw fish. Maturation and spawning occurred spontaneously under local photoperiod and temperature conditions (35°N, Fig. 1). Released eggs passed through a water overflow pipe into a collector (500-litre volume) equipped with a cylidro-conical planktonic mesh.

At the stage of half epiboly (30 hr after fertilization, water temperature 15.8°C) and following an acclimatization period of 2–3 hr, eggs from the same batch were collected and distributed into 6 × 500-litre larval rearing tanks at an initial stocking density of 100 per litre. Three experimental groups were formed (duplicate tanks per group), according to inflow water temperature; G13, 13°C; G15, 15°C; and G20, 20°C (Table 1).

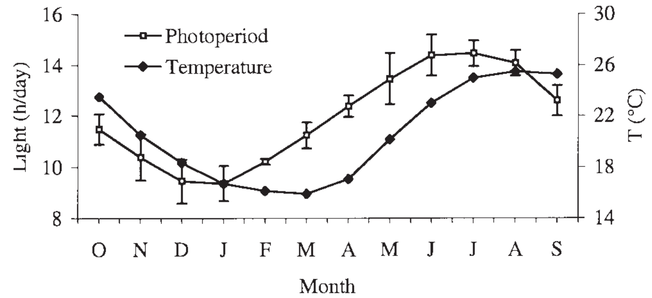

Fig. 1. Ambient photoperiod and temperature (mean ± SD) conditions during the on-growing phase (Crete, 35°N).

At the middle of metamorphosis stage (17–18 mm total length [TL]) juveniles of the different experimental groups were transferred into 3 × 500-litre on-growing tanks, under the same photoperiod and temperature conditions. Following a 2-week acclimatization period, 1,000 fish per experimental group were randomly shared into 2 × 500-litre tanks (duplicate tanks per group) (Table 1).

Experimental conditions

Incubation and larval rearing

Tanks for the egg incubation and larval rearing were connected to a closed recirculation system, equipped with biological filter and a thermo-regulating system (heater/cooler), to maintain constant the desired temperature for the three experimental groups up to the middle of the metamorphosis stage. Egg incubation and yolk-sac larval stage were carried out under total darkness, with gentle homogenization provided by aeration (150 ml/sec, 150–200 μm bubble diameter) and water inlet from the tank bottom (25% of the tank volume per hr). The subsequent larval rearing was performed under 14L:10D artificial illumination (10–15 lux), water exchange rate 10–50% of the tank volume per hr, 39–40 ppt salinity, and oxygen saturation over 85%. Water surface was maintained free from any lipid film with means of an air blower skimmer.

Larvae rearing followed the classic method (Divanach et al., '97). Larvae were fed enriched *Brachionus plicatilis* (Selco, INVE S.A., Belgium), from the complete consumption of the lipid globule to 6.3 mm TL, and a mixed diet consisted of rotifers and instar I *Artemia nauplii* (Platinum

TABLE 1. Abiotic and biotic parameters (range or mean ± SD) during the different rearing phases[1]

	G13	G15	G20	G13	G15	G20
Phase		Eggs–larval			On-growing	
Duration (days)	92	73	64	476	495	504
Initial total length (TL, mm)		Eggs		18.0 ± 1.1	17.8 ± 1.4	17.6 ± 1.1
Final TL (mm)	18.0 ± 1.1	17.8 ± 1.4	17.6 ± 1.1	254 ± 21	254 ± 22	255 ± 14
Temperature (°C)	13.3 ± 0.2	15.2 ± 0.1	20.0 ± 0.2		Ambient	
Initial fish density (ind/litre)		100			1	
Tank volume (litres)		500			500	
O_2 saturation (%)		>85%			>85%	
Salinity (ppt)		39–40			33–38	
Photoperiod (hr)		14L:10D			Natural	
Water exchange (%/hr)		10–50			50–300	
Feeding		Live zooplankton			Dry pellets	

[1]Thermal treatment (G13 = 13°C, G15 = 15°C, G20 = 20°C) lasted from the stage of half-epiboly until the middle of metamorphosis. During the on-growing phase, fish in all groups were exposed to ambient photoperiod and temperature conditions (Fig. 1). The final sampling was performed on September 1, 1999 (568 days posthatch).

Grade, Argent, Seattle, WA), from 5.5 to 9.0 mm. After 7.0 mm TL, enriched instar II *A. nauplii* (EG, INVE S.A., Belgium) were also given. Prey concentration was adjusted 2–5 times a day at a concentration of 2–5 and 1–4 individuals per ml for rotifers and *Artemia*, respectively. During larval rearing, the water surface was maintained free from any lipid film (a prerequisite for swimbladder inflation) thanks to an air blower skimmer.

Up to the middle of metamorphosis (17–18 mm TL), with the exclusion of the water temperature, all biotic and abiotic conditions were the same for the experimental groups (Table 1). At the end of the larval rearing phase, fish were counted and subjected to salinity floating test (Chatain and Corrao, '92), and individuals without an inflated swimbladder were removed.

On-growing phase

The on-growing was performed under identical biotic and abiotic conditions for all groups. Fish were kept under ambient photoperiod and temperature conditions, water exchange rate 50–300% of the tank volume per hr, 33–38 ppt salinity, and oxygen saturation over 85% (Table 1). During the 2-week adaptation period to the new rearing conditions, juveniles were progressively weaned on an inert diet (Lansy, INVE S.A., Belgium), which was distributed by automatic feeders. Fish were then fed ad libitum by means of self-feeders. An extruded commercial feed for sea bass was administered, with feed size changing according to feed manufacturers' recommendations (BioMar S.A., France).

Sampling and histological procedure

During the on-growing phase fish were sampled at the age of 308, 467, and 568 days posthatch (40–60 specimens/group/sampling). Fish were anaesthetized (ethylene glycol–monophenyl ether, Merck, Germany, 0.5 ppm), measured (±1.00 mm TL), and weighed (±0.01 g), gonads were dissected and weighed (±0.01 g), and the gonadosomatic (GSI = 100 × gonad weight/body weight) index calculated.

Gonad differentiation in sea bass starts in the posterior part of the gonads (Roblin and Bruslé, 1983). Therefore, the posterior parts of each of the two gonads (left and right) were fixed in 10% neutral-buffered formalin, dehydrated, and embedded in paraffin, and sliced sections of 4–6 microns were stained with Mayer's hematoxylin and eosin (Clark, '81).

Statistical analysis

Data were analyzed for normality (Kolmogorov–Smirnov test) and homoscedacity of variance (Bartlett's test), and when necessary, they were log-transformed before being treated statistically. The "*t*-test" performed comparison of body weight, TL, and GSI between male and female fish within each experimental group. One-way analysis of variance (ANOVA) was applied to check for significant changes between different groups within a single parameter. If significant ($P < 0.05$), Tukey's significant means test was applied to identify groups that were significantly different. The replicated goodness of fit test (*G*-statistic; Sokal and Rohlf, '81) was applied to check differences in survival and sex proportion between groups.

RESULTS

Temperature treatment affected the duration of the embryonic development: hatching occurred at 105, 85, and 55 hr post-fertilization for Groups G13,

G15, and G20, respectively. At hatching, the yolk-sac larvae were 3.3 ± 0.1 mm in total length (TL). Similarly, due to the incubation temperature larvae reached the middle of metamorphosis (17–18 mm TL) at 92, 73, and 64 days posthatch for Groups G13, G15, and G20, respectively (Table 1).

At the egg and larval stages, a higher survival rate was observed in G13 (36.1%, $P < 0.05$) with respect to G15 (23.0%) and G20 (23.7%). This difference was due to mechanical reasons related to the different spatial distribution of larvae in the rearing tanks. Larvae of groups G15 and G20 tend to float on the water surface and, subsequently, were trapped by the skimmer. At the subsequent developmental stages and until the end of the experiment, there was a high survival rate in all groups (94–98%).

At the first sampling (December, 308 DPH), all gonads from the specimens examined had differentiated into males (presence of lobules) and females (presence of primary oocytes). At that time, TL ranges were 135–194, 149–200, and 147–201 mm for groups G13, G15, and G20, respectively. There was a significantly higher ($P < 0.01$) proportion of females in G13 (74%) and G15 (67%) than in G20 (24%) (Fig. 2). At the subsequent sampling (May, 467 DPH) a similar pattern was observed, with higher proportions of females in groups G13 (72%) and G15 (73%) than in G20 (27%). This pattern was verified at the final sampling (September, 568 DPH), where sex proportions in groups G13 and G15 were consistently skewed in favor of females (73% and 67%, respectively). Conversely, a low percentage of female fish was observed in G20 (28%). At that time, females reached the primary oocyte stage,

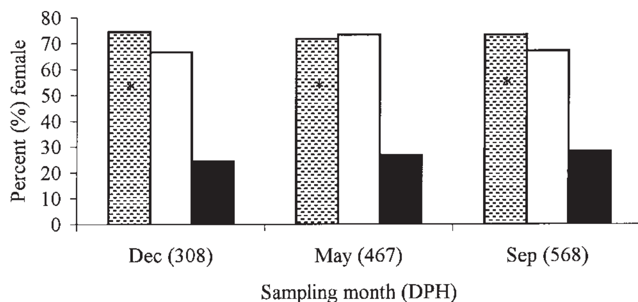

Fig. 2. Female percentages in sea bass exposed to different thermal treatment from the stage of half-epiboly until the middle of metamorphosis (G13 = 13°C, G15 = 15°C, G20 = 20°C). Shaded bars, G13; open bars, G15; black bars, G20 (DPH, days posthatch; N = 40–60 fish per group per sampling point). Asterisks indicate significant differences between G20 and the other groups ($P < 0.01$).

i.e., ovaries contained oocytes (diameter, 77–134 μm) with a homogenous cytoplasm, circular nucleus, and numerous nucleoli and males were at the spermatogenesis stage (Fig. 3). There were no significant differences in mean GSI values between the experimental groups (Table 2). However, in all groups, female fish had higher GSI than males (Table 2).

At the first sampling G15 fish were larger (body weight: 176.2 ± 2.4 g, $P < 0.001$) than fish in groups G13 (162.1 ± 2.5 g) and G20 (170.0 ± 2.5 g). However, during the subsequent samplings there were no differences in body weight between the experimental groups. In all groups, female fish were larger than males, a difference that was statistically significant ($P < 0.001$) during the final sampling (Table 2).

DISCUSSION

The present study aimed to investigate the effect of incubation temperature at the early stages of development on the sex ratio of cultured sea bass. To exclude any possible side effect of other factors on sex differentiation, eggs of the same spawning batch (genetic origin) were used, and all experimental populations were reared, with the exception of water temperature, under identical abiotic and biotic conditions and stocking densities.

As pointed out by Baroiller et al. ('99), the kinetics of gonadal development have to be determined prior to each experiment aiming in an accurate correlation between morphological and physiological events. This is of particular importance in the case of sea bass where size, rather than age (expressed as days post-fertilization, DPF), has been shown to be the critical factor for the timing of sex differentiation (Roblin and Buslé, '83). Therefore, for the correct interpretation of results, the experimental design followed was that temperature treatment started and ended at unique developmental event, i.e., from embryogenesis to the middle of metamorphosis (17–18 mm TL, in all groups). During this developmental period, fish undergo extreme changes with respect to morphology, anatomy, physiology, and behavior, being transformed from an embryo to a larva and then to a juvenile (Kendall et al., '84; Blaxter, '88; Youson, '88). Thus, duration of the thermal treatment was related to size rather than age (fish reached the middle of metamorphosis at different chronological time-ages, due to the effect of temperature on the developmental and growth rates, Table 1).

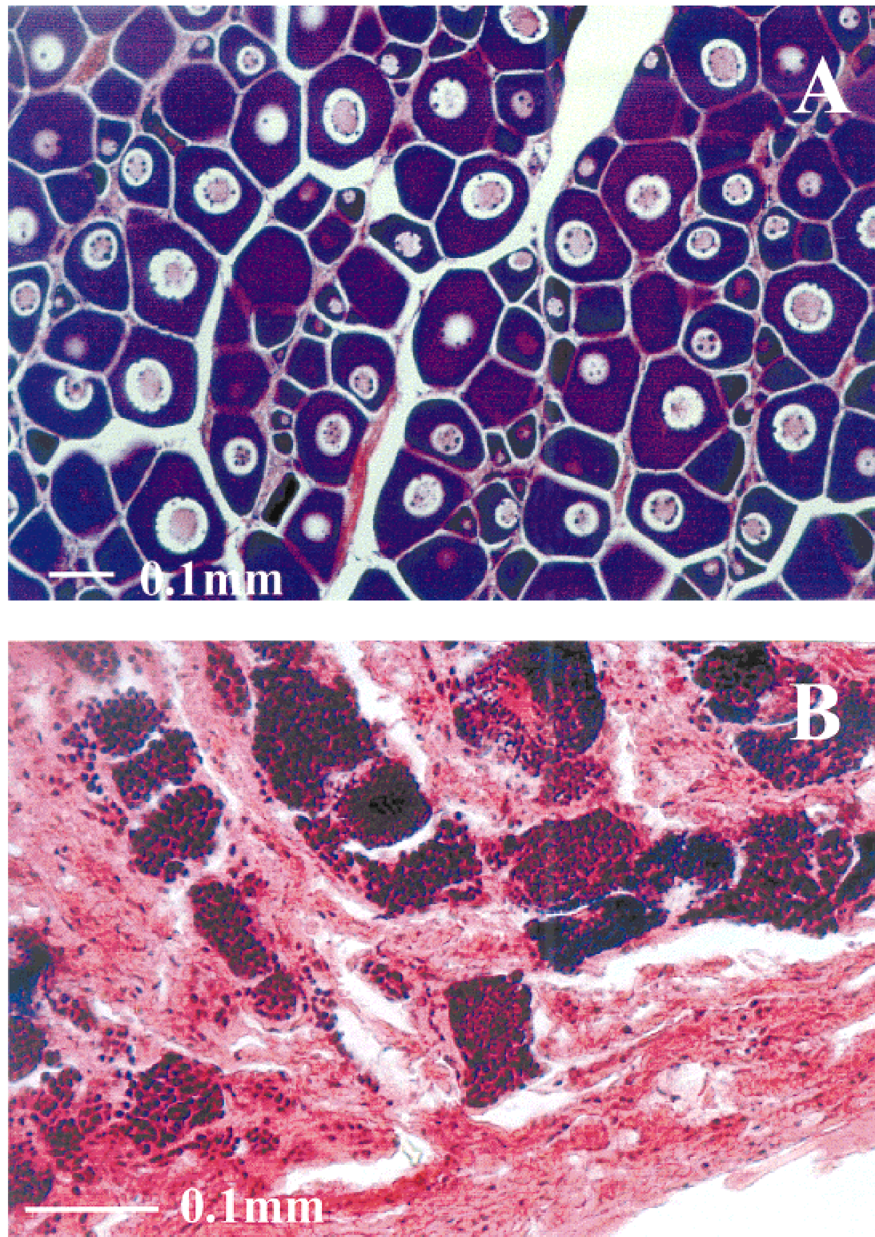

Fig. 3. Microphotographs of gonadal sections of sea bass. **A:** Ovary at the primary oocyte stage. **B:** Testis at spermatogenesis.

Results provided strong evidence of temperature-dependent sex determination in *D. labrax*. Exposure of fish at low temperatures (13 or 15°C) affected consistently and permanently the sex ratio in favor of females, whereas the high-temperature (20°C) treatment resulted in a male-biased sex ratio. Thus, sea bass is a thermosensitive species with a temperature response type similar to that described in most of the teleosts identified as thermosensitive; the proportion of males increases with temperature, and/or female-biased populations are produced at low temperatures (Conover and Kynard, '81; Schultz, '93; Hostache et al., '95; Römer and Beisenherz, '96; Strüssmann et al., '96, '97; Baroiller et al., '99).

In the tilapias, some atherinids, and in reptiles the thermosensitive period is similar to the labile period, i.e., the critical period during which the gonads are still undifferentiated but exhibit maximum sensitivity to the action of exogenous hormonal treatment (Baroiller et al., '99). According to Blázquez et al. ('98b) this period is located from

TABLE 2. Growth and somatic indices in the experimental sea bass groups at the different sampling dates[1]

Sampling date	Group	Age (DPH)	Sex	TL ± SD (mm)	BW ± SD (g)	GSI ± SD (%)	*N*
	G13		F	162 ± 9	45 ± 8	0.18 ± 0.06[a]	29
	G13		M	162 ± 10	48 ± 8	0.08 ± 0.07[b]	10
15 Dec 98	G15	308	F	179 ± 18	64 ± 24	0.19 ± 0.08[a]	27
	G15		M	173 ± 10	57 ± 11	0.10 ± 0.08[b]	13
	G20		F	168 ± 16	52 ± 17	0.20 ± 0.03[a]	10
	G20		M	172 ± 13	56 ± 14	0.06 ± 0.05[b]	31
	G13		F	210 ± 18	97 ± 29	0.25 ± 0.06[a]	43
	G13		M	204 ± 17	90 ± 24	0.07 ± 0.10[b]	17
23 May 99	G15	467	F	218 ± 18	110 ± 34	0.28 ± 0.10[a]	44
	G15		M	209 ± 10	98 ± 16	0.08 ± 0.11[b]	16
	G20		F	212 ± 23	103 ± 37	0.31 ± 0.08[a]	16
	G20		M	209 ± 14	96 ± 22	0.06 ± 0.05[b]	44
	G13		F	260 ± 22[a]	214 ± 53[a]	0.32 ± 0.08[a]	44
	G13		M	244 ± 16[b]	179 ± 37[b]	0.04 ± 0.02[b]	16
1 Sep 99	G15	568	F	257 ± 22	201 ± 58	0.34 ± 0.08[a]	40
	G15		M	246 ± 21	176 ± 55	0.04 ± 0.01[b]	20
	G20		F	264 ± 10[a]	221 ± 22	0.32 ± 0.10[a]	17
	G20		M	252 ± 14[b]	200 ± 34	0.03 ± 0.01[b]	43
	All		F	259 ± 21[a]	209 ± 53[a]	0.33 ± 0.08[a]	101
			M	249 ± 16[b]	190 ± 41[b]	0.04 ± 0.01[b]	79

[1]Thermal treatment (G13 = 13°C, G15 = 15°C, G20 = 20°C) lasted from the stage of half-epiboly until the middle of metamorphosis, and then fish were exposed to ambient photothermal conditions (DPH, days posthatch; F, female; M, male; TL, total length; BW, body weight; GSI, gonadosomatic index; and *N*, number of fish). Different superscript letters indicate statistical differences between groups ($P < 0.01$).

57 to 137 days post-fertilization in sea bass. In the atherinid, *Menidia menidia*, the thermosensitive period occurs just before metamorphosis at approximately 50 days posthatch (Conover and Fleisher, '86). Our data indicate that the thermonsensitive period in sea bass is also located before the completion of metamorphosis (<64 days posthatch at 20°C). This may also explain the differences between our results and those of a recent study (Blázquez et al., '98a) where low temperatures (15°C) produced monosex male populations of sea bass, whereas variable but low proportions of females (<27%) were produced at 24°C. In the later study, sea bass eggs were incubated at a temperature range of 20–24°C from days 0 to 43 post-fertilization (DPF), and, after acclimation, fish were then subjected to different thermal regimes (15 or 25°C) from 57 to 137 DPF. Thus, it seems likely that thermal treatment started after completion of the metamorphosis, and the subsequent temperature treatment could not induce increased female production.

The mechanisms underlying temperature-dependent sex determination in sea bass, and fish in general, are unknown. There is a general perception that sexual development is largely controlled by mechanisms of feedback control in the brain–pituitary–gonadal axis (Francis, '92). However, there is insufficient evidence to determine whether brain or gonads are the initial site of sex differentiation. Studies, mainly in salmonids, have demonstrated that the hypothalamic–pituitary axis is potentially active around the time of sex differentiation and that steroids can have a feedback effect on this axis (Saga et al., '93; Feist and Schreck, '96; Baroiller et al., '99). Growth hormone (GH) immunoreactivity in the pituitary gland of rainbow trout was detected slightly before the appearance of primordial germ cells (PGCs), at an even earlier stage than gonadotropin (GTH I) (Saga et al., '93). In similar manner, during the first day after hatching, cells immunoreactive for GH, thyroid-stimulating hormone (TSH), and adrenocorticotropic hormone (ACTH) are present in the pituitary of sea bass larvae, while no positive reaction can be obtained with anti-LH during the first 26 days after hatching (Cambré et al., '90). Therefore, it might be possible that GH is involved in the sex differentiation process, as it is known that GH can act on regulation of steroidogenesis in adult salmonid gonads (Baroiller et al., '99). Besides, temperature may exert its action in the process of sex differentiation through the brain expression of aromatase enzyme, as in reptiles (Jeyasuria and Place, '98).

Regarding the driving role of sex steroids and aromatase in reptiles, two theoretical models have been proposed to explain the basis of temperature-dependent sex determination. In the first model, temperature can act on the androgen-to-

estrogen ratio and thus 5α-reductase-to-aromatase activity, whereas in the second temperature can act on the transcription of the aromatase gene and thus the presence or absence of aromatase enzyme (Pieau, '96; Bogart, '97; Jeyasuria and Place, '98; Baroiller et al., '99). Recently a parallel hypothesis has been suggested for fish. Instead of the androgen-to-estrogen ratio adopted for reptiles, the 11-oxygenated androgen-to-estrogen ratio in fish would direct either male (excess of 11-oxygenated androgens) or female (excess of estrogens) differentiation (Baroiller et al., '99).

In the present study, incubation temperatures applied in groups G13 and G15 (13 and 15°C, respectively) were in the range normally occurring in nature and led to a high proportion of females (average of all samplings: 71%). This is in accordance with data from field studies where females dominate wild sea bass populations (Barnabé, '73; Arias, '80). Treatment with an incubation temperature typical of intensive larvae rearing (18–20°C) led to a male-biased population (average G20: 74%), i.e., within the range reported in cultured sea bass (70–99%) (Carrillo et al., '95; Blázquez et al., '98a,b). Thus, male-biased populations observed in culture conditions are the result of high temperatures during the intensive rearing.

In this study, female fish grow faster than males, a difference that was statistically significant at approximately 19 months of age. As at that time female fish had higher GSI than males, this difference seems unlikely to be due to differences in metabolic expenditure on gonadal growth. However, the inability to identify sex during the early developmental stages does not allow us to conclude whether within a certain population individuals become males by virtue of their small relative size, and not small by virtue of their maleness.

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