Aquaculture Research, 2007, **38**, 1161–1174 doi: 10.1111/j.1365-2109.2007.01782.x

Reproductive performance and seasonal plasma sex steroid and metabolite levels in a captive wild broodstock of brill *Scophthalmus rhombus* L.

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Abstract

This study reports egg production by captive wild brill *Scophthalmus rhombus*, a potential new flatfish species for Southern Europe-Mediterranean mariculture, as well as seasonal plasma levels of 17β–estradiol, testosterone, 11–ketotestosterone, proteins, triglycerides, glucose and lactate. A mean egg production of 102 800 eggs kg body weight^{-1} was achieved during the 2005 spawning period (January–March), although a continuous egg supply could only be obtained from some females, which had a higher relative fecundity (261 019 ± 10 393 eggs kg^{-1}) with 12–17 eggs batches released at a mean interval of 3.4 days. Most eggs were obtained with water temperatures ranging from 12 to 14 °C, and under increasing temperatures (up to 2.9 °C). Potential egg viability (70.1 ± 2.9%), fertilization (72.2 ± 3.4%) and hatching rates (31.9 ± 3.9%) showed high variability, with potential viability tending to decrease as the water temperature increased (mainly between 16 and 17 °C) and 0% hatching above 16.6 °C. The endocrine changes that brill underwent during late gametogenesis, spawning and post-spawning periods were similar to those reported in other Pleuronectiformes. This study establishes an important basis for further research on the biology and physiology of brill reproduction, directed towards the optimization of the breeding techniques used currently.

Keywords: turbots, artificial breeding, reproductive physiology, sex hormones, metabolic parameters

Introduction

The great expansion of the marine fish aquaculture industry in Europe during the last decades can be attributed to the culture of a few species, whose markets already show signs of saturation. Therefore, introduction of new species (diversification of aquaculture products), as well as solving other biological, technical and environmental problems, seems to be crucial to keep the industry economically viable. Among these new target species, flatfishes occupy a top position, because they normally have very good market prices and present high growth rates (Person-Le Ruyet, Baudin-Laurencin, Devauchelle, Métailler, Nicolas, Robin & Guillaume 1991; Shields, Gara & Gillespie 1999; Arneri, Collella & Gianetti 2001; Imsland, Foss, Conceição, Dinis, Delbare, Schram, Kamstra, Rema & White 2003; Hachero 2006). One of these potential candidate flatfish species for Southern Europe–Mediterranean marine

aquaculture diversification is brill *Scophthalmus rhombus* L., because: (1) it is well adapted to warm climates (it inhabits coastal waters from 5 to 50 m depth along the Eastern Atlantic and the Mediterranean Sea; Bauchot 1987); (2) its landings are scarce; (3) its growth in the wild is rapid during the first 2 years of life (Robert & Vianet 1988; Arneri *et al.* 2001), period in which the market size (500–1000 g) can be reached in culture (I. Hachero, M. Herrera & J. I. Navas, unpubl. data); and (4) mainly due to its high market value [between 20 and 30 € kg^{-1}; Isla Cristina (Huelva, Spain) fish market]. In addition, its similarity to turbot *Psetta maxima* (L.), species which currently dominates the flatfish farming sector (Brown 2002), enables the application of currently available breeding techniques for this species. In fact, the studies examining the biology of both species and their suitability for large-scale culture began together in the early 1970s in the United Kingdom (Jones 1972). The results obtained with brill in those preliminary studies were worse compared with turbot, and therefore, it was determined that the second species had more potential for commercial cultivation (Jones 1972). After these preliminary studies and to our knowledge, no more information has been published on the culture of brill until the present day.

Control of gametogenesis and reproductive events is mediated by the brain–pituitary–gonad (BPG) axis (Weltzien, Andersson, Andersen, Shalchian-Tabrizi & Norberg 2004). It seems to be evident that other endocrine factors linked to growth, nutritional status and metabolism influence the BPG axis functions–and vice versa – (Luquet & Watanabe 1986; Le Gac, Blaise, Fostier, Le Bail, Loir, Mourot & Weil 1993; Holloway & Leatherland 1998; Weltzien *et al.* 2004). One of the most widely used and practical tools to obtain information on the BPG axis and on the reproductive status of the fish is the assessment of plasma levels of sex steroids during the gonadal cycle (Merson, Casey, Martinez, Soffientino, Chandlee & Specker 2000). Moreover, the evolution of nutritional-status-related metabolite plasma levels along the reproductive cycle can be useful to evaluate the health (Guijarro, Lopez-Patiño, Pinillos, Isorna, De Pedro, Alonso-Gómez, Alonso-Bedate & Delgado 2003) and physiological status of the fish, because gonadal maturation and spawning can drastically affect the internal milieu of the organism (see references in Svoboda, Kouřil, Hamáèková, Kalab, Savina, Svobodová & Vykusobá 2001).

From 2002, the flatfish culture group at IFAPA Centro *Agua del Pino* (Cartaya, Huelva, Spain), in collaboration with other Institutions, has been focusing on the study of brill reproductive biology in captivity, among others aspects related to the rearing technique, as a necessary prerequisite for the development of a sustainable culture for this species. Thus, the aims of the present work, as a starting point to gain a comprehensive knowledge on the reproductive biology and physiology of brill in mariculture, are to characterise the annual egg production obtained from a captive wild broodstock of this species and to provide reference plasma levels of several sex steroids [17β–estradiol (E_2), testosterone (T) and 11–ketotestosterone (11–KT)] and metabolites (proteins, triglycerides, glucose and lactate) during the reproductive cycle in captivity.

Material and methods

Collection and management of wild breeders

Brill breeders were caught by trawling or trammel netting in the Gulf of Cádiz (SW Spain) between 2002 and 2004 and thereafter transported to the IFAPA Centro *Agua del Pino* facilities (Cartaya, Huelva, Spain) where they were acclimated to captivity and kept during the experimental period (November 2004 to June 2005). At arrival, animals with serious sores in the skin and/or fins were treated with oxytetracycline through consecutive baths during 7–10 days (dose: 50 ppm) or injected with Terramicine (20% tetracycline; Pfizer Laboratories, Alcobendas, Madrid, Spain) at a dose of 0.25 mL kg^{-1} (one single injection). Preventive 100 ppm formalin baths were applied to those fish without evident wounds after capture. A diet of fresh or frozen European pilchard *Sardina pilchardus* (Walbaum) was used to adapt breeders to feeding in captivity. Fish successfully adapted to captivity ($n = 24$) were sexed by abdominal massage in order to express oocytes or sperm, tagged with an intramuscular passive integrated transponder tag (Trovan ID 100A; EID Ibérica, Madrid, Spain) and stocked in four groups (Table 1). Fish were kept under natural conditions of photoperiod (37°17′N, 7°9′W; Fig. 1) in 4.2 m^2 (5 m^3; 1.2 m water depth) rectangular fibreglass tanks located indoors. No sand substrate was used. The tanks were supplied with running sea water (SW; 200% renovation per day) with 37–39 g L^{-1} salinity and 5–9 ppm oxygen. Water temperature fluctuated naturally (Fig. 1), except during summer when it was maintained below 23 °C (titanium plates heat exchanger; Alfa Laval Iberia, Alcobendas, Madrid, Spain). During the ex-

perimental period, fish were fed (at 14:00 hours) with frozen squid *Loligo* spp. and blue whiting *Micromesistius poutassou* (Risso) twice a week, and frozen mussels *Mytilus* spp., pilchard and dry pellets specific for breeders (Skretting, Cojobar, Burgas, Spain) once a week at a maximum daily ration of 2% of body mass.

Gamete collection and artificial fertilization

Because natural spawnings of brill under the current rearing conditions had very poor potential viability rates during 2004 (6.7 ± 1.6%), from 2005 onwards, gametes were hand-stripped from mature fish and artificially fertilized. Artificial fertilization protocol used in the present study was based on that used currently for turbot at the production scale (Omnes, Normant, Suquet & Fauvel 1991; Person-Le Ruyet *et al.* 1991), and thus it can be extrapolated to industrial hatcheries. During the spawning season (mid January to late March), all females were periodically checked for expression of hyaline eggs. In order to collect freshly ovulated eggs and thus avoid over ripening inside the ovary lumen (McEvoy 1984), the stripping frequency was selected individually by checking the females daily during the beginning of the spawning season to establish their approximated ovulatory rhythms according to their first ovulation date. In the majority of them, a 3-day stripping frequency was found to be effective. However, the females that could not be successfully stripped on the third day were checked daily during the subsequent days until the eggs were released. Tanks were also provided with egg collectors and when eggs were detected on an unexpected ovulation day, the females in the tank were stripped for eggs. Males maintained spermiating over the whole reproductive season, and so they were alternatively chosen for the extraction of sperm. Before handling, fish were anaesthetized in 2–phenoxyethanol (500 ppm, maximum time of exposure: 5 min). Thereafter, eggs were hand –stripped, being collected in a dry 2 L plastic beaker and kept at room temperature (10–15 °C) until fertilization (ca. 20 min). The total number of eggs was calculated volumetrically under dry conditions, counting the eggs contained in one 500 μL aliquot and extrapolating it to the total volume of eggs collected, which was measured in a calibrated cylinder. The same aliquot was used to calculate the potential viability rate and to measure, after adding SW, the diameter of 10 randomly chosen potentially viable eggs. Eggs were estimated to be potentially-viable when they showed a perfect spherical and translucent aspect (McEvoy 1984; Fauvel, Omnes, Suquet & Normant 1992). Each egg batch was fertilized at 18–20 °C with freshly stripped sperm from one male, using a volume ratio eggs:sperm:sea water of

Table 1 Composition (number of fish), mean (± SEM) weights and stocking density of the four groups of brill breeders used in the present study

Group	Composition (females:males)	Weight (g)		Stocking density (kg m^{-2})
		Females	Males	
A	3:3	2664 ± 525	1770 ± 378	3.2
B	4:2	2872 ± 207	2046 ± 444	3.7
C	3:3	2782 ± 513	1949 ± 226	3.4
D	4:2	2823 ± 294	1378 ± 513	3.3

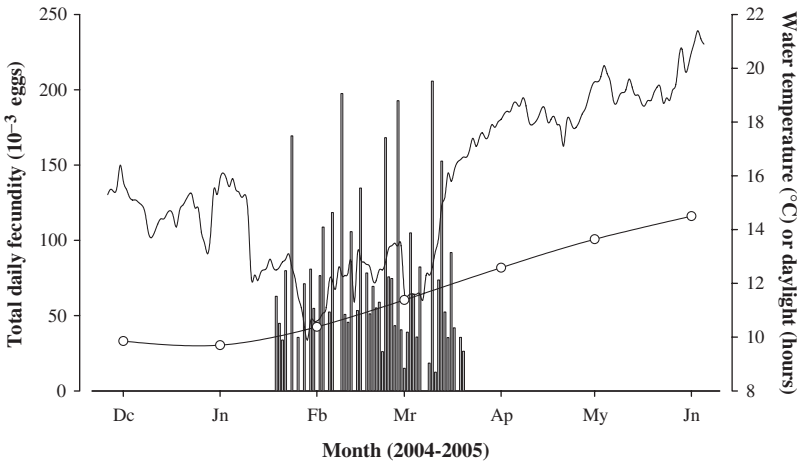

Figure 1 Total daily fecundity during the year 2005 (vertical bars) of female brill held under a naturally fluctuating water temperature (line plot) and photoperiod (line and scatter plot). Data from the four groups of breeders (Tables 1 and 2) have been combined to show a single cycle.

100:0.2:100. Sperm density was not measured, but all sperm used for fertilization were viscous and had high motility. After gentle mixing, the fertilization mixture was left for 3 min (Suquet, Billard, Cosson, Normant & Fauvel 1995) before adding more SW till a total volume of 1.5 L. Thirty minutes later, the floating eggs were collected and one sample of 50 eggs was incubated in 12-well culture plates filled with 3 mL of filtered SW (two eggs per well) at temperatures ranging from 13.2 to 19.8 °C (mean temperature: 15.7 °C) in order to estimate the fertilization and hatching rates. Such a static system was used in order to minimize the mechanical shocks, which can alter the normal egg development, especially at the morula stage and just before hatching (Person-Le Ruyet *et al.* 1991). Two hours after setting up incubation, the number of eggs with cellular division (two to four cell stage) was counted and the fertilization rate was calculated. Five days after fertilization, the number of larvae in the plate was counted and the hatching rate was calculated. All the operations were performed during the morning.

Blood sampling and analytical techniques

From four to 14 females and from four to 10 males stocked in the different groups were randomly blood sampled each month from November 2004 to May 2005. After being anaesthetized as above, a sample of blood was collected from the caudal vessels using cold heparinized syringes. The plasma, obtained by centrifugation (1200 *g*, 10 min, 4 °C), was stored at − 80 °C until further analyses. To reduce stress by successive handling during the spawning season, blood extraction coincided with gamete stripping manipulation. In order to obtain a reliable measurement of plasma metabolite levels, which can vary with time after feeding, blood extraction was always performed between 10:00 and 12:00 hours (feeding at 14:00 hours).

Plasma levels of E_2 (females), 11-KT (males) and *T* were quantified by an enzyme-linked immunosorbent assay (ELISA) according to Rodriguez, Begtashi, Zanuy and Carrillo (2000). The assay was validated for use with brill plasma (data not shown) by confirming parallel displacement of serially diluted pooled plasma samples to the standard curves as well as no significant displacement of steroid-stripped plasma pools (prepared according to Barry, Lapp, Kayest & Malison 1993). Steroids were extracted from 5 μL plasma in 1.5 mL methanol. Reagents and materials were the same as described in García-López, Anguis, Couto, Canario, Cañavate, Sarasquete and Martínez-Rodríguez (2006). The percentages of recovery for E_2, 11–KT and T in steroid-spiked plasma pools were 80%, 85% and 90% ($n = 4$) respectively. The inter-assay coefficients of variation at 50% of binding were 0.2% for E_2 ($n = 2$), 7.0% for 11-KT ($n = 2$), and 8.0% for T ($n = 4$). The intra-assay coefficients of variation (calculated from the sample duplicates) were 3.8 ± 0.6% for E_2, 9.7 ± 1.1% for 11-KT and 7.1 ± 0.8% for T. The lower limit of detection for all assays was 35 pg mL^{-1} plasma. Main cross-reactivity (> 1%; given by the supplier; Cayman Chemical Company, Ann Arbor, MI USA) was detected with estradiol–3–glucoronide (17%) and oestrone (4%) for the E_2 antibody and with 5α–dihydrotestosterone (27.4%), 5β-dihydrotestosterone (18.9%), androstenedione (3.7%) and 11–KT (2.2%) for the T antibody. Cross-reactivity with other steroids was lower than 1% for the 11-KT antibody.

Plasma glucose, lactate, and triglycerides were measured using commercial microplate kits from Spinreact (Girona, Spain). Plasma proteins concentration was measured using the bicinchoninic acid method with a BCA protein kit (Pierce, Rockford, IL, USA) for microplates, with bovine serum albumin as standard. These assays were run on a Bio Kinetics EL-340i Automated Microplate Reader (Bio-Tek Instruments, Winoosky, VT, USA) using DELTASOFT 3 software for Macintosh (BioMetallics, Princeton, NJ, USA).

Statistics

Data, presented as mean ± standard error of mean (SEM), were analysed for statistical differences among groups by one-way ANOVA or by Kruskal–Wallis one–way ANOVA on ranks if data complied or not with normality and homogeneity of variance respectively. In the first case, ANOVA was followed by Student–Newman–Keuls (SNK) while in the second by Dunn's multiple-comparison procedures, both with a significance level (*P*) of 0.05. The interaction between potentially viable eggs diameter and total body mass, water temperature, date or fecundity was studied by linear regression analysis.

Results

Egg production and quality evaluation

Captive brill breeders showed a spawning period during year 2005 lasting, taking into account the whole population, from January 19th to March 21st (10.1–

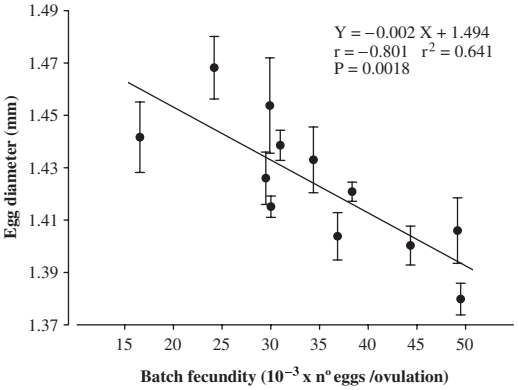

Figure 2 Diameter of potentially viable eggs in relation to batch fecundity per female brill during the year 2005. Data (mean ± SEM) from the four groups of breeders (Tables 1 and 2) have been combined.

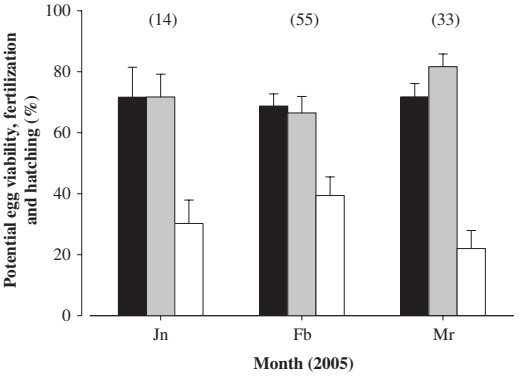

Figure 3 Potential egg viability (black bars), fertilization (grey bars) and hatching rates (white bars) achieved by brill breeders during the year 2005. Data (mean ± SEM) from the four groups of breeders (Tables 1 and 2) have been combined. Means of each parameter are not significantly different among months (Kruskal–Wallis one-way ANOVA on ranks; $P > 0.05$). The numbers in parenthesis indicate the number of egg batches collected each month.

12.0 h light per day) (Fig. 1). A total of 102 egg batches were collected during 48 days out of 61, with a total harvest of 3 654 250 eggs. Daily collection ranged from 12 432 to 205 760 eggs, with a mean of 74 994 eggs. Although eggs were obtained from 13 females out of 14, a continuous supply was attained only from four females, accounting for 67% of the total egg harvest (data not shown). Such females were successfully stripped 12–17 times at a mean interval of 3.4 days. The egg batches obtained from these four females represented 50% of the total egg batches collected. Although the mean relative fecundity was significantly higher in 'good' females ($n = 4$) compared with the remaining ($n = 9$) (261 019 ± 10 393 vs. 52 006 ± 7414 eggs kg^{-1} respectively; $P < 0.001$), batch fecundity was similar (40 541 ± 4185 vs. 31 437 ± 3361 eggs ovulation^{-1}; $P = 0.136$).

The diameter of potentially viable eggs ranged from 1.28 to 1.65 mm, with a mean (± SEM) of 1.42 ± 0.002 mm. No significant correlation was found between egg diameter and total body mass, water temperature at egg collection or during the previous day of stripping, or date along the spawning season (data not shown). However, egg diameter was negatively correlated with total, batch and relative fecundity, with the strongest correlation being with batch fecundity (Fig. 2). Accordingly, mean diameter of potentially viable eggs obtained from the females with higher fecundity (see previous paragraph) was significantly lower ($P < 0.001$) than that of eggs collected from the remaining females (1.41 ± 0.003 vs. 1.44 ± 0.005 mm respectively).

The mean potential egg viability, fertilization and hatching rates, taking into account monthly grouped data from the four groups of breeders, did not show any significant change along the spawning season, ranging from 69% to 72%, from 66% to 82%, and from 22% to 39% respectively (Fig. 3). Females with a higher fecundity also showed significantly higher potential egg viability and fertilization rates compared with the remaining females (76 ± 3% and 69 ± 4% vs. 61 ± 5% and 46 ± 7% respectively). Hatching rate was relatively higher in the former than in the latter (36 ± 5% vs. 23 ± 6%).

Table 2 summarizes the reproductive performance characteristics obtained from the four groups of brill breeders used in the present study.

Relationship between water temperature and egg production and quality

Although egg batches were collected with water temperature ranging from 10.0 to 16.7 °C in the breeding tanks (Fig. 1), between 61% and 69% of the total harvest (64–71 batches out of 102) was obtained when the water temperature ranged from 12 to 14 °C the previous or the same day of collection (Fig. 4). The temperature of the day before egg collection was accounted because stripping was always performed during the morning and therefore, it was probable that eggs were ovulated during the day before collection. Within this temperature range, 67–69% of the

Table 2 Reproductive performance characteristics obtained during year 2005 from the four groups of brill breeders used in the present study

	Group				
	A	**B**	**C**	**D**	**Total**
No. of females (ovulating)	3 (2)	4 (4)	3 (3)	4 (4)	14 (13)
Biomass ovulating females (kg)	4.4	11.5	8.3	11.3	35.5
First ovulation date	15 February	19 January	19 January	21 January	19 January
Last ovulation date	17 March	16 March	21 March	16 March	21 March
Total no. of ovulations	7	28	34	33	102
No. of ovulations female^{-1}	2.3 ± 1.9 (0–6)	7.0 ± 2.7 (4–15)	11.3 ± 3.5 (5–17)	8.3 ± 3.2 (3–17)	7.3 ± 1.5 (0–17)
Ovulation frequency (days)	6.7 ± 3.7 (3–14)	4.2 ± 0.5 (1–13)	3.2 ± 0.1 (2–6)	3.6 ± 0.3 (1–8)	3.8 ± 0.2 (1–14)
Total fecundity (10^{-3} × no. of eggs)	227.6	1,110.7	1,380.5	935.5	3,654.2
Batch fecundity (10^{-3} × no. of eggs ovulation^{-1})	32.5 ± 3.2 ab	39.7 ± 4.0 a	40.6 ± 3.6 a	28.3 ± 2.6 b	35.8 ± 1.9
Relative fecundity (10^{-3} × no of eggs kg^{-1})	51.5	96.7	165.4	82.8	102.8
Potential viability (%)	29.7 ± 11.8 c	76.5 ± 4.3 ab	60.1 ± 5.6 bc	83.1 ± 3.0 a	70.1 ± 2.9
Fertilization (%)	79.0 ± 15.0 a	73.3 ± 6.6 a	66.7 ± 5.4 a	75.8 ± 6.2 a	72.2 ± 3.4
Hatching (%)	0 a	38.9 ± 8.1 a	24.4 ± 5.5 a	36.1 ± 7.1 a	31.9 ± 3.9

Data are expressed as mean ± SEM. Numbers in parentheses indicate the data range. Ovulation frequency was calculated from ovulations with potentially viable eggs. Same notation denotes homogeneous groups ($P > 0.05$).

Figure 4 Proportion of total fecundity achieved by female brill breeders in relation to water temperature during the previous (black bars) and the same day (grey bars) of egg collection. Data from the four groups of breeders (Tables 1 and 2) have been combined. Numbers above bars indicate the number of egg batches.

Figure 5 Proportion of total fecundity achieved by female brill breeders in relation to water temperature changes during the four (black bars), three (grey bars) and two (white bars) days before egg collection. Data from the four groups of breeders (Tables 1 and 2) have been combined. The numbers above bars indicate the number of egg batches.

eggs were collected in February (48–50 eggs batches out of 64–71; data not shown).

The relationship between changes in water temperature 4, 3 and 2 days before egg collection, and the total fecundity is shown in Fig. 5. Between 59% and 62% of the total egg production was collected when the water temperature in the breeding tanks increased from 0.1 to 2.9 °C during the previous days of stripping (59–63 batches out of 102). Despite this gen-

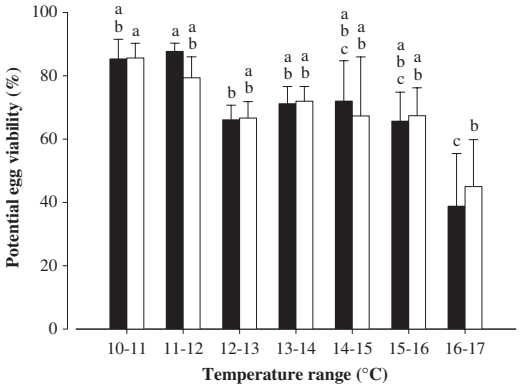

Figure 6 Potential egg viability rate achieved by brill breeders in relation to water temperature during the previous (black bars) and the same day (white bars) of egg collection. Data (mean ± SEM) from the four groups of breeders (Tables 1 and 2) have been combined. Means with different notations are significantly different among temperature ranges in the same parameter and day (Kruskal–Wallis one-way ANOVA on ranks; Dunn's method; $P < 0.05$). See Fig. 4 for sample sizes.

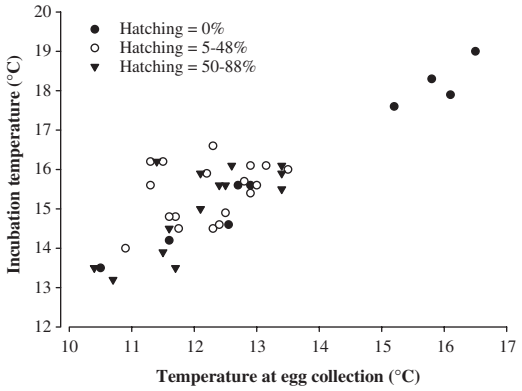

Figure 7 Temperature at egg collection and incubation temperature in relation to hatching rate obtained from artificial fertilization of brill gametes. Values correspond to egg batches with potential viability and fertilization rates equal to or higher than 70% ($n = 41$). Data from the four groups of breeders (Tables 1 and 2) have been combined.

eral trend, the egg batches collected in January were mainly ovulated under steady or decreasing temperatures (9–12-egg batches out of 14, which accounted for 12–17% of the total egg harvest, data not shown).

The potential egg viability rate showed a decreasing tendency among increasing water temperature intervals, considering the temperature in the breeding tanks both at the same day and at the day before egg collection (Fig. 6). No time effect was observed (see Fig. 3).

Egg batches with potential viability and fertilization rates equal to or higher than 70% ($n = 41$) but with low to null hatching rates were obtained within a wide range of collection and incubation temperatures (from 10 to 17 and from 13 to 19 °C respectively, Fig. 7). However, all of them collected and incubated at temperatures above 13.5 and 16.6 °C (respectively) had 0% hatching (four out of 41), while those egg lots presenting hatching rates equal to or higher than 50% were always obtained at collection and incubation temperatures below 13.5 and 16.3 °C respectively (14 out of 41; Fig. 7).

Plasma sex steroid and metabolite profiles

Females

Plasma E_2 levels remained high during the first half of the spawning period (January 17–February 9th 2005; Fig. 8a), although with elevated individual variability as denoted by the high values of SEM Thereafter, they declined progressively until baseline values in late April. The presence of T in the blood stream was low both before and after the spawning period, while it was detected at its highest levels in the sampling performed in the middle of the reproductive season (Fig. 8b). In the case of plasma total protein levels (Fig. 8c), they increased markedly from December 1 2004 to January 17 2005, remained high till March 17th and subsequently decreased on April 21st. Both plasma triglycerides and glucose levels (Figs. 8d and e respectively) increased gradually till peaking in February 2005 and thereafter, they decreased progressively until reaching in late April lower or similar values than those found in December. Plasma lactate levels remained steady at relative high values from December to March, decreasing significantly in April (Fig. 8f). Available data did not allow us to correlate endocrine profiles with individual spawning performance, as well as to compare steroid and metabolite levels between high-and low-fecundity females.

Males

Both plasma 11-KT and T levels (Figs. 9a and b respectively) increased gradually till peaking in February 2005 (some males showed maximum androgen con-

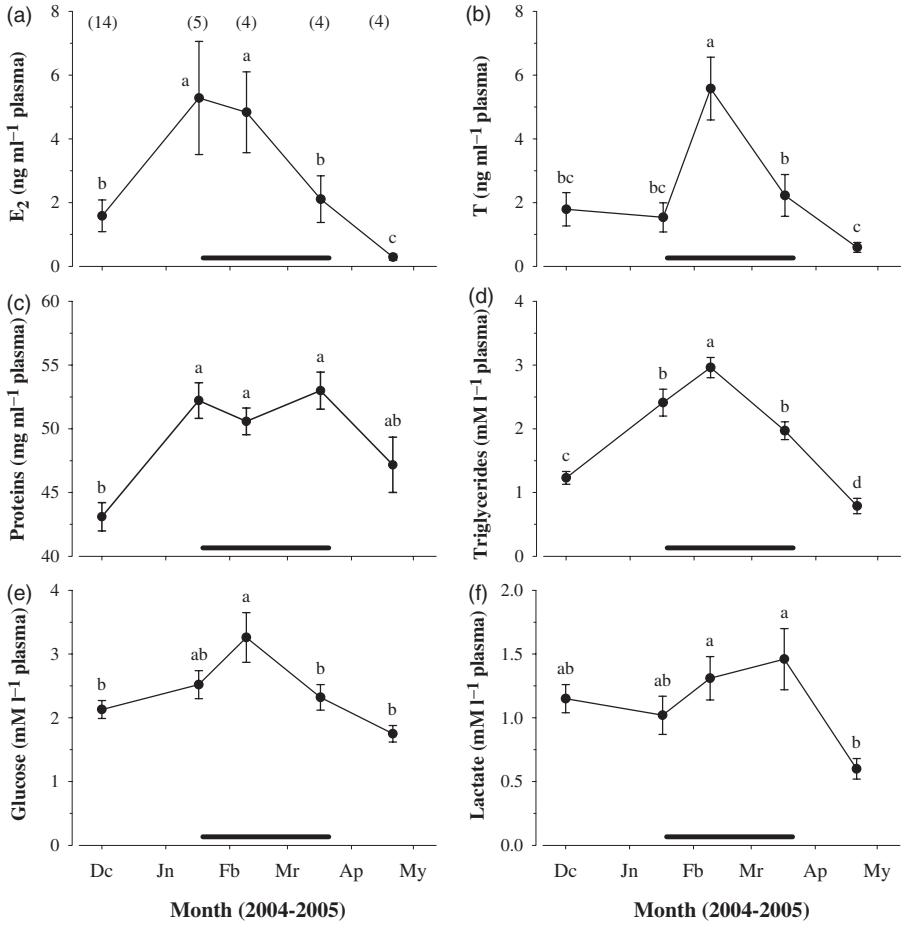

Figure 8 Monthly changes in plasma levels of (a) E_2, (b) *T*, (c) proteins, (d) triglycerides, (e) glucose and (f) lactate in female brill. The numbers in parenthesis indicate the sample sizes for all the plasma parameters. Horizontal bars at the lower side of each panel represent the spawning period. Means (± SEM) with different notations are significantly different (one-way ANOVA; Student–Newman–Keuls test; $P < 0.05$).

centrations in January 2005, data not shown) and thereafter, they decreased progressively until reaching in late April significantly lower values than those found in December. Male plasma levels of total proteins (Fig. 9c), triglycerides (Fig. 9d), glucose (Fig. 9e) and lactate (Fig. 9f) showed slight changes during the experimental period, remaining at relatively or significantly lower values than those measured in females.

Discussion

Egg production and quality

Wild captive brill showed in the current study at Southwest Iberian Peninsula a spawning period lasting from mid-January to mid-March. In the Mediterranean and Black Seas, spawning also occurs in late winter (Bauchot 1987), although in the Adriatic Sea, the spawning season seems to be prolonged until July (Caputo, Candi, Colella & Arneri 2001). In the North Sea, conversely, brill spawns (as turbot) from May to August (Jones 1972), as a consequence of the expected lower temperature.

Both the number of egg batches per female (up to 17; mean: 7.3) and ovulation frequency (3.8 days) obtained for brill were similar to those reported for turbot, which is able to spawn up to 12–16 times per season (Howell & Scott 1989; Person-Le Ruyet *et al.* 1991) with a ovulatory period between 80 and 90 h (3.3–3.7 days; McEvoy 1984). However, the relative fecundity was lower in the case of brill (102 800 vs. 430 000 eggs kg^{-1} in turbot; Person-Le Ruyet *et al.*

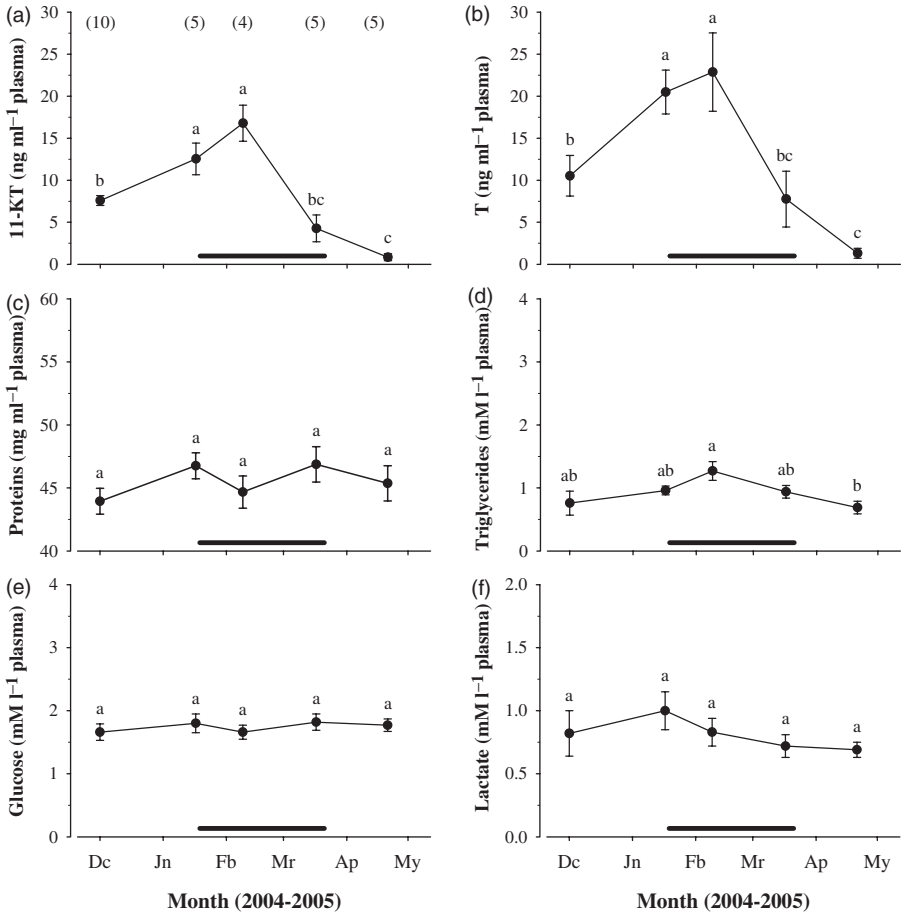

Figure 9 Monthly changes in the plasma levels of (a) 11-KT, (b) T, (c) proteins, (d) triglycerides, (e) glucose and (f) lactate in male brill. Numbers in parenthesis indicate the sample sizes for all the plasma parameters. Horizontal bars at the lower side of each panel represent the spawning period. Means (± SEM) with different notations are significantly different (one-way ANOVA; Student–Newman–Keuls test; $P < 0.05$).

1991), a difference possibly related to the egg size differences between both species (1.28–1.65 mm for brill vs. 0.9–1.2 mm for turbot, this study, Jones 1972). Interestingly, this possibility matches the lower egg diameter reported for female brill showing the higher fecundities. Another (not mutually exclusive) possibility would be the lower weight of our breeders in comparison with turbot breeders used by Person-Le Ruyet *et al.* (1991) (1.2–3.9 vs. 3.5–8 kg). Similar to American plaice *Hippoglossoides platessoides* (Fabricius) (Nagler, Adams & Cyr 1999), but in contrast to Atlantic cod *Gadus morhua* L. (Kjesbu, Kryvi & Norberg 1996), Senegalese sole *Solea senegalensis* Kaup (Dinis, Ribeiro, Soares & Sarasquete 1999), turbot (Howell & Scott 1989; Mugnier, Guennoc, Lebegue, Fostier & Breton 2000) and Atlantic halibut *Hippoglossus hippoglossus* (L.) (Brown, Shields & Bromage 2006) egg diameter in brill did not decrease during the course of the spawning season. The small number of eggs per batch measured ($n = 10$) and/or the fact that only potentially viable eggs were taken into account in the present study cannot be discarded as the origin of such differences. However, a circumstance supporting our data is that the number of eggs (both potentially viable and non-viable) contained in the 500 μL aliquot taken from each batch to calculate the total egg number, despite fluctuating along the spawning season for each female, was similar between the beginning and the end of this period.

The present study obtained relatively high potential egg viability rates, similar to those reported by Fauvel *et al.* (1992) and higher than those by Devau-

chelle, Alexandre, Le Corre and Letty (1988), both for turbot. However, we also found a high variability among individuals and egg batches, which indicates that the stripping strategy used was efficient to avoid egg overripening inside the ovary lumen only in some females, i.e. stripping of eggs was not well synchronized with ovulatory patterns in all the females.

The fertilization rates obtained in the present study were also relatively elevated, although with high variability. This variability could also be associated with the mismatch between the ovulatory rhythms and the stripping frequency in some females leading to egg overripening. Another possibility to explain this variability would be the use of inadequate insemination conditions as pointed out by Suquet *et al.* (1995). Therefore, we cannot rule out the 'male effect' on such variability (as well as in that found in the hatching rate), because we used sperm from only one male instead of a pool of sperms to fertilize each egg batch and sperm density was not measured (although all sperm used for fertilization was viscous and had high motility). The fertilization values obtained here were similar to those reported for turbot by Omnes *et al.* (1991) at the pilot scale and by Chereguini, Garcia de la Banda, Rasines and Fernández (1999) at the experimental scale. This was true even though the quantity of sperm used in the present work was lower than that used by Chereguini *et al.* (1999) (0.2 vs. 0.5 mL per 100 mL of eggs).

Regarding hatching rates, the values obtained in the present work with brill were similar to those reported by Fauvel *et al.* (1992), although significantly lower than those found by McEvoy (1984), both for turbot. In the latter study, such high hatching rates (up to 97%) were obtained only for 'freshly stripped' eggs (collected within 10 h of ovulation), because after being retained in the ovary lumen for 1 day, the hatching rate declined to 0%. Therefore, it can be suggested that, similar to turbot (McEvoy 1984) and Atlantic halibut (Norberg, Valkner, Huse, Karlsen & Leröy Grung 1991), brill eggs overripen quickly when retained in the ovary lumen and this is probably a major cause of poor or variable egg quality in captive broodstocks.

Water temperature ranges for egg collection in the present work were very similar to those reported for spawning of turbot; from 9.5–11 to 16–17 °C (Bromley, Sykes & Howell 1986; Person-Le Ruyet *et al.* 1991), with the maximal production between 13 and 15 °C (Devauchelle *et al.* 1988; Person-Le Ruyet *et al.* 1991). Our data suggest that an increase in temperature may induce ovulation-spawning in brill, as demonstrated previously in southern flounder *Paralichthys lethostigma* Jordan & Gilbert (from 14.0–15.5 to 17 °C; Smith, McVey, Jenkins, Denson, Heyward, Sullivan & Berlinsky 1999) or Senegalese sole (up to 2.5 °C; Anguis & Cañavate, 2005). It has been reported that water temperature is able to act on the synchronization of final phases of gamete maturation, ovulation, and spawning (Bromage, Porter & Randal 2001). Therefore, it is probable that the thermal increase stimulates the final maturation and ovulation of a batch of oocytes in all the species mentioned above.

The potential egg viability rate in brill tended to decrease as the water temperature in the breeding tanks increased, especially in the interval 16–17 °C. This temperature threshold coincides with that reported for turbot, whose eggs always showed a lower potential viability over 16–17 °C (Devauchelle *et al.* 1988). This circumstance may be related to the quicker ageing and overripening of ovulated eggs inside the ovary lumen (Billard 1985; Howell & Scott 1989) and/or to disturbances of different endocrine-dependent developmental processes before ovulation under inadequately high temperatures (van der Kraak & Pankhurst 1997; Davies & Bromage, 2002). Impaired egg viability with increasing temperatures has also been reported, among others, in European seabass *Dicentrarchus labrax* (L.) (> 16 °C; Carrillo, Zanuy, Prat, Serrano & Bromage 1993), Atlantic halibut (> 8 °C; Brown *et al.* 1993) and Atlantic cod (> 9.6 °C; van der Meeren & Ivannikov 2006).

Available data suggest the existence of a thermal threshold affecting hatching, because all the egg batches incubated at temperatures above 16.6 °C had 0% hatching. A similar upper limit for adequate incubation temperature has been reported for turbot (17 °C in the North Sea, Devauchelle *et al.* 1988; 18 °C in the Baltic Sea, Nissling, Johansson & Jacobsson 2006). Moreover, temperatures higher than 17 °C have been reported to induce additional deformities in turbot embryos (Devauchelle *et al.* 1988).

The results obtained show that an acceptable brill eggs supply can be achieved using artificial fertilization techniques similar to those currently available for turbot (Omnes *et al.* 1991; Person-Le Ruyet *et al.* 1991). However, it is necessary to study precisely the ovulatory rhythms to improve the gamete collection method, the implication of water temperature variations on the final stages of maturation/ovulation, the characteristics of brill sperm to optimize the artificial fertilization protocol and the embryonic development under the current incubation system to improve the hatching success. Studies of these condi-

tions that would make spontaneous natural spawning possible and controllable may also be of high interest to brill broodstock management. In addition, the use of hormonal stimulation to overcome spawning problems in some females, to synchronize ovulation within the broodstock and to maximize egg production should be evaluated in future research.

Plasma sex steroid and metabolite profiles

In general, the evolution of plasma sex steroid levels in brill reported here agrees with that described previously for other flatfish species, like turbot (Howell & Scott 1989) Atlantic halibut (Methven, Crim, Norberg, Brown, Goff & Huse 1992; Weltzien, Taranger, Karlsen & Norberg 2002), English sole *Parophrys vetulus* Girard (Sol, Olson, Lomax & Johnson 1998), summer flounder *Paralichthys dentatus* (L.) (Merson *et al.* 2000), spotted halibut *Verasper variegatus* (Temminck & Schlegel) (Koya, Watanabe, Soyano, Ohta, Aritaki & Matsubara 2003) and Senegalese sole (García-López *et al.* 2006).

Plasma sex hormone concentrations were highly variable among brill individuals during the spawning season, which may reflect the asynchrony in the final phases of gamete maturation and spermiation/ovulation, as reported in turbot (Howell & Scott 1989). In group-synchronous multi-spawning fish, such as brill (Caputo *et al.* 2001; this study), circulating levels of steroids fluctuated in relation to the cycles of oocyte batch recruitment, maturation, and release throughout the spawning season (Howell & Scott 1989; Methven *et al.* 1992; Asturiano, Sorbera, Ramos, Kime, Carrillo & Zanuy 2002). A similar pattern has been observed in males, because each increase in the sperm production used to be preceded by increases in the synthesis of androgens (Asturiano *et al.* 2002).

Regarding steroid profiles in females, it is interesting to note the delayed peak of T concentration in relation to E_2 surge and decline. The fact that T levels increased markedly or remained high whereas E_2 levels started to decrease is characteristic of the final oocyte maturation of most teleost (Scott, Witthames, Turner & Canario 1998; Sun & Pankhurst 2004). At this stage, T seems to be involved in the maintenance of oocytes once vitellogenesis has been completed (Kime 1993) or in the enhancement of gonadotropin-releasing hormone sensitivity of the pituitary (Trudeau, Murthy, Habibi, Sloley & Peter 1993) in preparation for the preovulatory surge in plasma luteinizing hormone (Mylonas, Scott & Zohar 1997). In turbot, Howell and Scott (1989) reported a close relation between short-term peaks of T plasma levels and maximum in egg production over the spawning season.

It is worth noting the relatively high levels of T compared with those of 11-KT found in male brill. In the majority of flatfish studied to date (i.e. Harmin, Crim & Wiegand 1995; Weltzien *et al.* 2002; García-López *et al.* 2006), the concentrations of plasma 11-KT during gametogenesis and spawning are 5–10-fold higher than those of T, as indicative of the greater relevance of 11-KT in the control of spermatogenesis and reproductive behaviour in teleosts (Borg 1994). However, the high levels of T found in our study suggest a more relevant role for T in brill spermatogenesis. To our knowledge, no information regarding the ratio between the levels of these steroids is currently available for turbot to compare our results.

The evolution of plasma levels of metabolites in brill showed a clear sex-specific pattern, which suggests the considerable differences in the metabolic and energy requirements for female and male reproductive processes, with oogenesis requiring a higher energy demand than spermatogenesis. Similar differences have been reported in the plasma levels of triglycerides and proteins of tench *Tinca tinca* (L.) at prespawning (Svoboda *et al.* 2001; Guijarro *et al.* 2003), although no differences in serum glucose and lipid levels were found between male and female European plaice *Pleuronectes platessa* L. at any time of the reproductive cycle (White & Fletcher 1985; White, Fletcher & Pope 1986).

The high plasma proteins levels (in conjunction with elevated E_2 concentrations) during the brill spawning season seem to be mainly associated with increased synthesis of vitellogenin (see references in Johnson, Casillas, Myers, Rhodes & Olson 1991), which would be elevated during the whole reproductive season, as reported in other multiple spawners (i.e. Methven *et al.* 1992; Sun & Pankhurst 2004). Plasma triglyceride levels also peaked in female brill during spawning, indicating the mobilization of lipids from the liver and other body tissues, both to be incorporated by growing oocytes (Johnson *et al.* 1991) and to cover the great energy investment required for vitellogenesis (Bon, Corraze, Kaushik & Le-Menn 1997). Some fish species show improved tolerance and better dietary glucose use during gonadal maturation (Luquet & Watanabe 1986), which may indicate the importance of carbohydrates as energy source at this stage of the life cycle (Washburn, Bruss,

Avery & Freedland 1992). Increased hepatic glycolytic activity (Soengas, Barciela & Aldegunde 1995) and exportation of generated glucose to the ovary (Mommsen & Walsh 1988) may also have contributed to hyperglycaemia found in female brill during prespawning and spawning, as reported in flounder *Platichtys flesus* (L.) (Petersen & Emmersen 1977) and tench (Svoboda *et al.* 2001). Conversely, plasma glucose levels declined at spawning in English sole (Johnson *et al.* 1991), while they were unaffected by the reproductive cycle in European plaice (White & Fletcher 1985). The changes in plasma lactate levels in female brill were also influenced by the considerable energy demand required for gonadal development. Lactate-derived energy seems to be more important towards the end of the spawning season, when plasma levels of triglycerides and glucose started to decrease.

The present work provides reference plasma levels of sex steroids and metabolites for future studies on the physiology of the reproductive events of brill. Such physiological studies will complement those related to spawning performance in our goal to establish a reliable breeding technique for this species.

Acknowledgments

This research has been funded by the projects DIVERAQUA (Interreg IIIA: SP5.E36). The authors thank J. F. Ferrer, J. M. Márquez and M. Rosano at Desarrollo Agrario y Pesquero S.A. (Junta de Andalucía) and A. Rodiles at IFAPA Centro *Agua del Pino* for their essential help with fish husbandry. Thanks are also due to R. Osta at ICMAN for her skilful assistance in steroid measurements.

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