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Sperm production and quality in brill *Scophthalmus rhombus* L.: relation to circulating sex steroid levels

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Abstract The aims of the present study were to characterize sperm quality and to quantify seasonal changes in sexual hormone (testosterone [T], 11-ketotestosterone [11-KT] and 17,20β-dihydroxypregn-4-en-3-one [17,20β-P]) levels in male brill (*Scophthalmus rhombus*) plasma, as well as to test a more intensive sampling strategy to establish relationships between sex steroid levels and sperm production parameters. Sperm concentration ranged from 0.5 to 3.1 $\times$ 10^9 spermatozoa mL^{-1}, and changes in sperm quality parameters depending on sampling date were observed. Plasma sexual steroid levels remained high and changed in parallel during the spawning season and afterwards decreased to very low levels in summer. The analysis of annual changes of 11-KT and T ratios suggests that 11-KT can be the main circulating androgen for stimulating spermatogenesis in *S. rhombus* and that T could be involved in the beginning of spermatogenesis through the positive feedback on brain-pituitary-gonad axis. Finally, daily 11-KT and T levels showed similar patterns of variation in males sampled, whereas 17,20β-P amounts showed somewhat opposite trends. These differences could be related with the different role of androgens and progestin during the spermatogenesis.

Keywords Motility · Sperm concentration · *Scophthalmus rhombus* · Sex steroids

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Introduction

Brill (*Scophthalmus rhombus* L.) is a promising flatfish species for marine aquaculture diversification in Southern Europe, due to its good market prize, high growth rate and good adaptation to captivity in warm climates.

A recent study has provided substantial information on egg production from wild broodstocks adapted to captivity, as well as reference plasma sex steroid levels during part of the reproductive cycle (Hachero-Cruzado et al. 2007). However, no information is available on the sperm density and motility changes associated with the spawning season for this species. Also, Hachero-Cruzado et al. (2007) performed

monthly samplings from November to May, giving a limited view of the real changes in steroid levels during annual cycle, and hence, rapid changes, especially during the spawning season, may be missed.

Consequently, the aims of the present study were to characterize *S. rhombus* sperm quality during the spermiation period, to quantify seasonal changes in plasma sex steroids (testosterone [T], 11-keto testosterone [11-KT] and 17,20β-dihydroxypregn-4-en-3-one [17,20β-P]) and to test a more intensive sampling strategy to establish relationships between levels of sex steroids and sperm production parameters.

Materials and methods

Breeders were caught and maintained in captivity as described in Hachero-Cruzado et al. (2007). During an annual cycle, six males were monthly checked to study sperm as well as plasma sex steroids variations. Throughout the spawning season (from January to April 2007), males were sampled every seven and 15 days to collect sperm and blood samples, respectively. Furthermore, three males were sampled daily for one week in February 2008 in order to study daily changes in plasma sex steroids and sperm quality parameters. For each sampling, breeders were individually captured and anaesthetized, and blood and plasma samples were extracted as described in Hachero-Cruzado et al. (2007). Sperm collection was made according to the method described by Cabrita et al. (2006).

Sperm samples were pre-diluted 1:5 in Ringer's immobilizing solution of 200 mosmol kg^{-1} (Chereguini et al. 1997) and stored on ice until further analysis (<20 min). Sperm density was determined using a Neubauer chamber, afterwards sperm was again diluted in Ringer's until a final dilution of 1:400. This procedure was repeated four times, with four different sub-samples. Over 100 sperm cells were counted in sub-samples. Spermatozoa motility was measured after activating 1 µL of sperm pre-diluted 1:5 in Ringer's immobilizing solution with 19 µL of seawater. Motility was scored immediately under light microscopy according to Sánchez–Rodríguez (1975). This procedure evaluates (scale from 0 to 5) the percentage of cells moving continuously after activation: 0, no movement; 1, 0–20 %; 2, 20–40 %; 3, 40–60 % 4, 60–80 %; and 5, 80–100 % cells moving. In order to minimize the measurement subjectivity, two people scored every sample independently and the final scores were the mean values.

Plasma levels of 11-KT, T and 17,20β-P were quantified by enzyme-linked immunosorbent assay (ELISA) according to a similar procedure described in Rodríguez et al. (2000). 11-KT and T assays were previously validated for *S. rhombus* (Hachero–Cruzado et al. 2007). 17,20β-P assays were validated for use with *S. rhombus* 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 et al. 1993). Reagents and materials were the same as described in García–López et al. (2006) for T and 11-KT. Antisera, AChE tracers and standards for 17,20β-P assays were provided by Cayman Chemical (Michigan, USA).

Data, presented as mean ± standard error of mean (SEM), were analysed for statistical differences among groups by one-way ANOVA or Kruskal–Wallis tests if data complied or not with normality, respectively. Following, Student–Newman–Keuls (homogeneous variances) or Dunnett T3 (non-homogeneous variance) tests were performed after the ANOVA test in order to detect homogenous subgroups. For non-normal data, Kruskal–Wallis test was followed by Dunn's multiple-comparison procedures. The significance level was 0.05.

Results

All males produced sperm between January and April 2007 (Fig. 1). Sperm density ranged from 0.5×10^9 (January 26th) to 3.1×10^9 (March 9th) spermatozoa mL^{-1} ($p = 0.02$) (Fig. 1a). Sperm motility tended to increase from January 22nd to March 9th, decreasing thereafter towards the end of the spawning season, although differences were not significant in both cases (Fig. 1b).

Patterns of variation in plasma T and 11-KT levels were similar (Fig. 1c, d). However, T values were highly variable, and only the values measured in January 18th, February 2nd and March 2nd were significantly higher than those detected at the end of the spawning period (April 25th). Plasma 11-KT values had less variability, and they were always

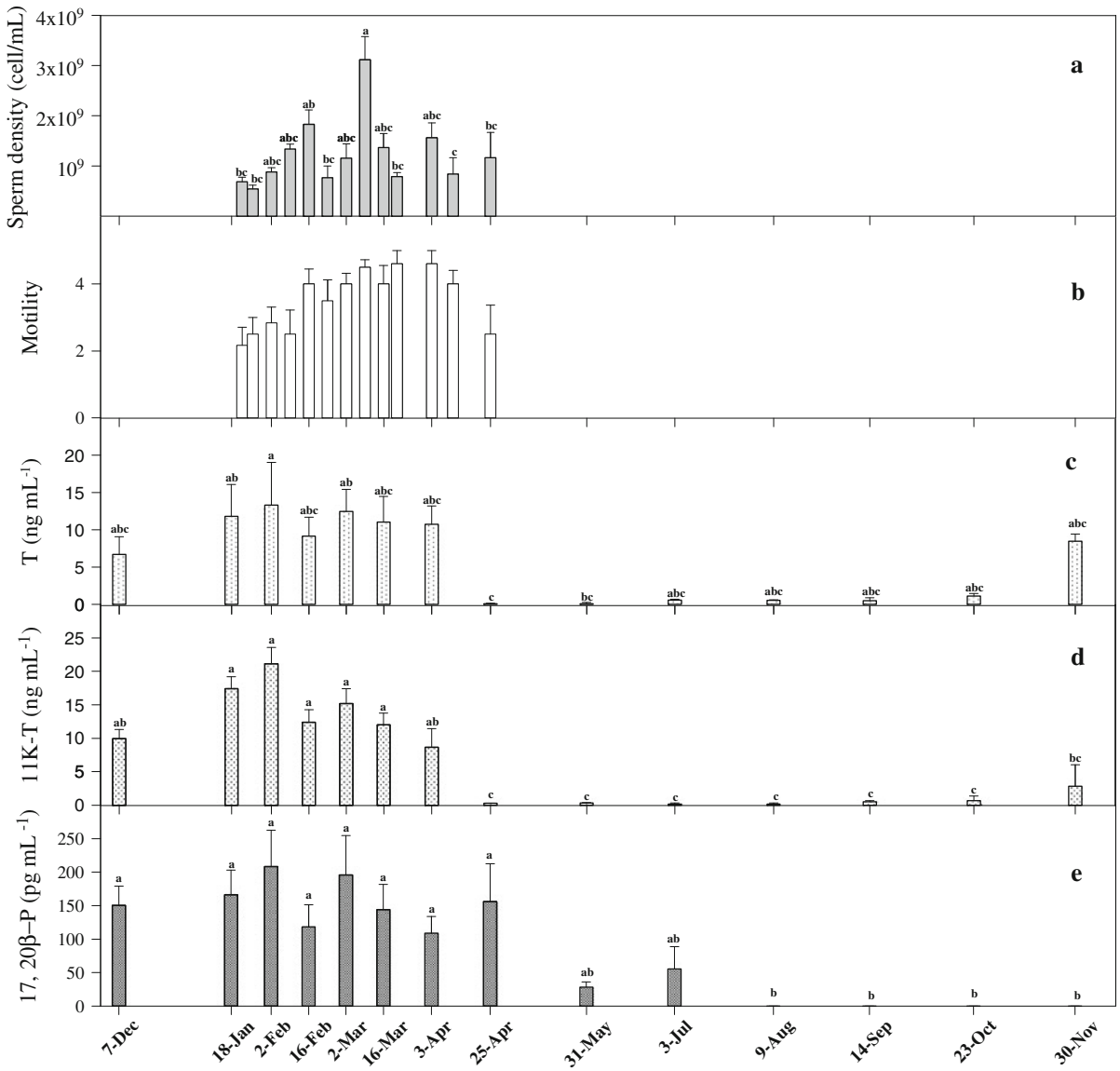

Fig. 1 Sperm density (**a**) motility (**b**) plasma T (**c**) 11-KT (**d**) and 17,20β-P (**e**) levels during an annual cycle. Data (mean $\pm$ SEM) from the six males have been combined. Means with different notations are significantly different ($p < 0.05$)

significantly higher for the spawning period than that for the period between late April and October. 17,20β-P plasma concentrations remained at high values (>100 pg mL^{-1}) from December 2006 to April 2007. Afterwards, they decreased to undetectable levels until December 2007 (Fig. 1e).

Daily analyses of sperm characteristics (motility, density and volume) as well as plasma sexual hormone levels (11-KT, T and 17,20β-P) during one week are shown in Fig. 2. In general, 11-KT and T levels presented similar patterns, decreasing along the week, whereas 17,20β-P amounts showed somewhat opposing trends. Overall, there was a clear relationship neither among the different sperm characteristics, nor between sperm characteristics and plasma sexual hormone levels. Nevertheless, the sperm density and motility increases (significant for motility values) could be directly related with the 17,20β-P variation.

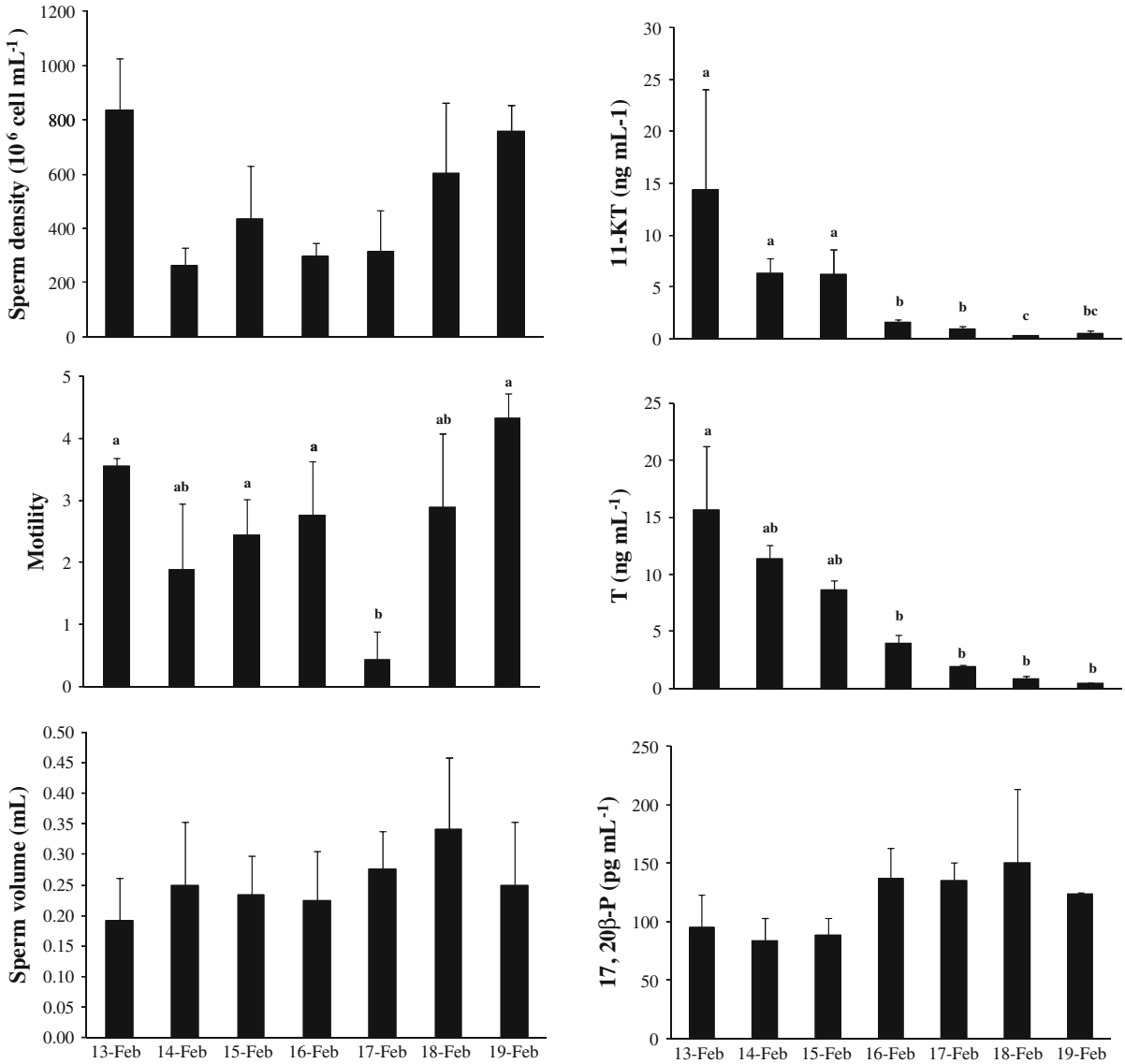

Fig. 2 Daily changes in sperm motility, density and volume, as well as in plasma levels of 11-KT, T and 17,20β-P from three *S. rhombus* males sampled at 1-day interval throughout one week during February 2008. Data (mean ± SEM) from the three males have been combined. Means with different notations are significantly different ($p < 0.05$)

Discussion

This is the first study reporting parameters related to sperm production in *S. rhombus*, specifically sperm volume, density and motility. Sperm concentration ranged from 0.5 to 3.1 $\times$ 10^9 spermatozoa mL^{-1} and was lower than the most of the values reported for other marine fishes (see the review by Suquet et al. 1994; Babiak et al. 2006). Changes in *S. rhombus* sperm quality in relation to the sampling date were observed, as sperm density and motility showed the maximum values at the half of the spermiation period. In fact, it has been described that seasonal variations in sperm density and motility are physiological phenomenon during the spermiation period and vary depending on fish species (Suquet et al. 1998; Fauvel et al. 1999; Mylonas et al. 2003; Rouxel et al. 2008).

In the present study, plasma androgen levels (11-KT and T) remained high during the spawning season and afterwards decreased to very low levels in summer. This androgen profile has also been reported in other flatfish species *Pleuronectes americanus* (Harmin et al. 1995),

Pleuronectes vetulus (Sol et al. 1998) and *Solea senegalensis* (García–López et al. 2006). The highest ratios between 11-KT and T (data not shown) coincided with the spermiation period, suggesting that 11-KT can be the main circulating androgen for stimulating spermatogenesis in *S. rhombus*. However, this ratio decreased to values close to zero during summer and autumn 2007 (data not shown). These results also suggest that T could be involved in the beginning of the spermatogenesis through the positive feedback on brain-pituitary-gonad axis. Sex steroids feedback effects on this axis have been reported in *Oncorhynchus mykiss* W. (Davies et al. 1999), *Carassius auratus* L. (Kobayashi et al. 1997), *Micropogonias undulates* L. (Khan et al. 1999) and *Dicentrarchus labrax* (Rodriguez et al. 2001), which supports our hypothesis. Moreover, in vivo and in vitro studies on several teleost species showed that 11-KT is the most effective as direct stimulator for spermatogenesis, while T is more effective than 11 androgens in feedback mechanisms on the brain-pituitary-gonad axis (Miura et al. 1991; Borg 1994; Rodríguez et al. 2001).

The analysis of the annual evolution of plasma steroid levels showed 11-KT, T and 17,20β-P levels changed in parallel during the spawning season. However, the analysis of the daily data on plasma steroids values revealed different patterns for androgens (11-KT and T) and progestin 17,20β-P. These differences could be related with the different role of androgens and progestin during the spermatogenesis. 11-KT is related to spermatogonial renewal and proliferation, while 17,20β-P induced spermatogonia to the meiotic prophase and final maturation of male gametes (Schulz et al. 2010). Therefore, sperm density and motility increase observed during the daily analyses of sperm characteristics (statistically significant change for the latter) could be due to a 17,20β-P concentration rising.

The present report provides the first information on sperm production and quality in *S. rhombus* related to circulating sex steroid levels both in monthly and daily bases. Nevertheless, our data does not allow us to establish significant correlations between the parameters analysed. Further experiments using a higher number of fish and a longer daily sampling period would be required to that aim.

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