Seasonal cycle of gonadal development and serum levels of vitellogenin of the red porgy, *Pagrus pagrus* (Teleostei: Sparidae)

Received: 16 March 2000 / Accepted: 10 April 2001 / Published online: 9 June 2001 © Springer-Verlag 2001

**Abstract** Histological examination of gonads of female and male red porgy, *Pagrus pagrus*, reared in captivity, was carried out in order to describe the main gonadal changes related to gametogenesis and the seasonal changes of environmental factors. The gonadosomatic index (GSI) and the concentration of serum vitellogenin (VTG) were also determined. The frequency distribution of gonad development stages and the GSI and vitellogenin concentration during the annual cycle indicated the separation of the female and male reproductive cycles into three main periods. The autumn period when gametogenesis begins (October–November), the period of exogenous vitellogenesis (December–March) or spermatogenesis (December–March), and the spawning season (March–May). The spawning period coincided with an increase in temperature (15–19°C) and daylength. Serum levels of vitellogenin rose significantly in January, reached a peak at the beginning of the spawning period (March, 405.5 µg ml⁻¹) and remained high until the end of the spawning period (May).

**Introduction**

The red porgy, *Pagrus pagrus*, is a sparid fish, widely distributed throughout the Atlantic Ocean and Mediterranean Sea, and living at depths from 18 to 280 m (Manooch and Hassler 1978; Vassilopoulou and Papakonstantinou 1992). It is a highly prized fish, consumed by humans along the Mediterranean and Atlantic coasts, where it contributes significantly to commercial fisheries, as well as to the recreational harvest of reef fishes (Manooch 1976; Harris and McGovern 1997). Because of these activities, red porgy stocks have experienced serious landing declines in some areas of their distribution (Vaughan et al. 1992; Harris and McGovern 1997).

*P. pagrus* grows rapidly and also shows adaptability to heavy stocking, criteria which should be satisfied by new candidate species for fish farming (Divanach et al. 1993; Kentouri et al. 1994). Additionally, owing to its wide distribution, there is a large market in Europe and in the USA. Thus, it is considered a new species which could be included in the diversification efforts of marine fish production in the Mediterranean.

In order to effectively rear red porgy, control of its reproduction is needed. Basic knowledge on its reproductive biology is of prime importance for control measures. The present study is part of a broader investigation programme on the study of red porgy reproductive biology and growth under rearing conditions. The study examined the reproductive cycle by monitoring the histological changes in female and male gonads together with serum vitellogenin in females, in relation to season.

**Materials and methods**

Fish population and sampling

Red porgies, *Pagrus pagrus*, used in this study belonged to 3- to 5-year-old populations, captured in the wild and acclimatised to rearing conditions (Kokokiris et al. 1999). Experimental fish were maintained at the Institute of Marine Biology of Crete (eastern
Mediterranean) in outdoor tanks of 10 m³ each, under the natural photoperiod and water temperature. Fish were fed commercial pellets (sea bream pellets, Biomar) provided by self-feeders, accessible throughout the day (ad libitum feeding). Experimental fish were followed for 1 year, from September 1994 to September 1995; sampling was carried out on a monthly basis (Kokkiris et al. 1999). Each month 41–100 fish were sampled, for a total of 670 individuals over the year.

During sampling, blood was collected from the caudal vessel using a sterile 1 ml syringe and was placed in Eppendorf tubes kept on ice for clotting. After centrifugation (2,500 g, 15 min) serum was frozen and stored at −20°C for further analysis.

Whole fish were weighed (± 1.0 g) and dissected. Viscera were weighed (± 1.0 g) and gonads dissected and weighed (± 0.01 g). Gonadosomatic index (GSI) was defined as follows:

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GSI = \frac{GW}{(BW - GW - VW)} \times 100
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where BW is body weight, GW is gonad weight and VW is visceral weight.

Gonad histology

Tissue samples from the middle of the right gonad were fixed in Bouin’s solution and prepared using routine histological procedures (Gabe 1968), sectioned into 4–6 μm sections, stained with Harris’ haematoxylin and eosin and examined under light microscopy (Kokkiris et al. 1999). Mean oocyte diameter (MOD) was determined as the mean diameter of the 30 largest oocytes within one histological cross-section of the ovary. Measurements were obtained using an ocular micrometer (mm) and were corrected by a factor of 0.15 for shrinkage due to the histological preparation protocol.

Vitellogenin purification

Blood serum vitellogenin (VTG) levels were measured by a homologous radioimmunoassay in all fish sampled. Vitellogenin was obtained by in vivo estrogenisation of male red porgies. Estradiol benzoate (E2b, Sigma) was dissolved first in 5% benzylic alcohol and then in saline for a priming injection (300 μg NaCl ml⁻¹, 0.2 M) or in cocoa butter for implantation (5 mg cocoa butter ml⁻¹). Individually tagged males, kept in outdoor tanks (2 m³; salinity: 40–41‰; O₂ concentration: 4.7–6.4 mg l⁻¹) received 300 μg of E2b ml⁻¹ kg⁻¹ body weight of priming injection, and 24 h later, 5 mg ml⁻¹ kg⁻¹ body weight for implantation. Blood sampling was performed from the caudal vessels, before priming injection and 30 days after implantation, in the presence of phenylmethylsulfonyl fluoride (PMSF, Sigma) in order to inhibit proteolysis. After centrifugation (4,000 g, 10 min) serum was collected and stored at −20°C for further analysis.

To determine the efficiency of induction of VTG synthesis, for each male, aliquots of serum were subjected to SDS-PAGE (sodium dodecyl sulphate polyacrylamide gel electrophoresis), and the males that responded better were selected for the purification of VTG. Vitellogenin was purified using a gel filtration, followed by ion-exchange, low-pressure chromatography, performed at 4°C. Serum samples were loaded on a gel filtration Sepharose 6B column (80×2.8 cm, Pharmacia), equilibrated and eluted at a flow rate of 60 ml h⁻¹ with a 100 mM Trizma base buffer (2 mM CaCl₂, pH 7.8). Fractions were collected (5.5 ml) and analysed by SDS-PAGE. Fractions containing VTG were pooled and concentrated using an Amicon cell (cutting level: 10,000 Da), and were loaded on an ion-exchange DEAE Trisacryl M column (32×1.8 cm, IBF) equilibrated with the same buffer. The elution was performed by using a gradient from 100 to 500 mM of Trizma base buffer (2 mM CaCl₂, pH 7.8) at a flow rate of 36 ml h⁻¹, followed by a chase with 3 ml of Tris M + 2 mM CaCl₂. The purity of eluted VTG was checked by SDS-PAGE. The fractions containing purified VTG were aliquoted and stored at −80°C.

Specific antibodies against VTG were raised in rabbits using electrophoretic VTG excised from Coomassie-blue-stained SDS-PAGE, according to the procedure described by Harrington (1990). For each injection, 500 μl of saline containing 100 μg of electrophoretic VTG was emulsified in 500 μl of Freund’s adjuvant (v:v) and introduced subcutaneously at various points. The schedule of immunisation and the collection of immunoserum was according to Bon et al. (1997).

Vitellogenin assay procedure

1. Antigen coating: The coating was performed in 96-well microtiter plates (Nunc) with 200 μl of 0.05 M carbonate buffer pH 9.6, containing 125 ng VTG ml⁻¹. The blank values were obtained by coating eight wells with 125 ng bovine serum albumin (BSA) (bovine serum albumin) ml⁻¹. The plates were then covered and incubated for 3 h at 37°C. The content of the wells was discarded by inverting the plates, and three successive washes of 30 s each were done using 0.01 M phosphate buffer pH 7.4, 0.15 M NaCl and 0.05% Tween 20 (PBS-T). The saturation of non-specific binding sites was achieved by incubating the plates with 2% normal pig serum in PBS-T (PBS-T-NPS). The plates were maintained for 30 min in an oven at 37°C, followed by a three-wash cycle with PBS-T.

2. Primary antibody incubation: In separate tubes, specific antibodies diluted 1:100,000 in PBS-T-NPS were incubated with (a) serial dilutions of samples (diluted at least 1:50) or (b) of the VTG standards (VTG from 2,000 to 0.98 ng ml⁻¹) for 16 h at 37°C. The content of these tubes was then distributed in duplicates in the coated microtiter plates (200 μl well⁻¹), and the plates were incubated for 2 h at 37°C, followed by a three-wash cycle with PBS-T.

3. Secondary antibody incubation: Each well received 200 μl of swine IgG (immunoglobulin of type G) anti-rabbit IgG (DAKO) diluted 1:5,000 in PBS-T-NPS. The plate was incubated for 2 h at 37°C and rinsed as before.

4. Peroxidase anti peroxidase (PAP) complex incubation: As in step 3, the PAP complexes (DAKO) obtained in rabbits (diluted 1:5,000 in PBS-T-NPS) were distributed in the wells (150 μl well⁻¹), and the plate incubated for 1 h at 37°C and then washed.

5. Colour development: Each well received 200 μl of the following solution prepared immediately before use – 20 ml of citrate-phosphate buffer 0.1 M, pH 5, 10 μg of o-phenylene diamine (OPD, Sigma) and 10 μl of 30% H₂O₂. Colour development reached its maximum after 15 min in the dark at room temperature, and the reaction was stopped by adding 50 μl well⁻¹ of 4 M H₂SO₄.

6. Absorbance measurement: The absorbance of each well was measured at 490 nm using an EL311 Biotech autoreader. The results of the absorbance measurement were expressed by a linear or non-linear model.

In order to test the specificity of the ELISA assay, serum from P. pagrus containing VTG was diluted from 10⁻² to 10⁻⁷, and the binding curve was compared to the standard curve. Besides, sera from Spondylus canthus, S. aurata, Diplois annularis, Lithognathus morrhueus, Dentex dentex and Puntazzo puntazzo were diluted from 10⁻² to 10⁻⁷, and their binding curves were compared to the standard curve.

Statistical analysis

Statistical errors are expressed as the standard error of the mean (SEM). One-way analysis of variance (ANOVA) and a Scheffé’s multiple range test were applied to compare mean levels. Bartlett’s test was used to verify the homogeneity of variances. When necessary (non-homogeneity of variance), data were log-transformed before subjecting them to ANOVA.