

Observations on oocyte maturation and hydration *in vitro* in the black sea bass, *Centropristis striata* (Serranidae)

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Abstract

A protocol for the incubation of *Centropristis striata* ovarian follicles *in vitro* from females that spawn repeatedly under laboratory conditions was developed. Isolated follicles underwent maturation after 15-24 h of incubation at 25°C in 75% Leibovitz L-15 medium, containing relatively low doses of human chorionic gonadotropin (hCG) or homologous pituitary extract (50% response with approximately 2 IU/ml and 0.02 pituitary equivalents/ml, respectively). During maturation *in vitro*, follicles underwent a remarkable volume increase (about 340%) that was correlated with proteolysis of primarily the largest yolk protein in the oocyte. The maturation response of follicles to hCG was size-dependent within a range from 400 to 600 µm in diameter, with a maximum response displayed by 530-600 µm follicles. The response of these follicles to hCG was not inhibited within a range of incubation temperatures from 15 to 28°C, but the length of time required for the follicles to achieve 50% maturation was negatively correlated with increasing temperatures ($r^2 = 0.97$; $p \leq 0.01$). However, the largest follicles (530-600 µm) did not undergo maturation in response to high doses (1 µg/ml) of the major maturation-inducing steroids (MISs) known in teleosts, 17,20-dihydroxy-4-pregnen-3-one (17,20β) and 17,20β,21-trihydroxy-4-pregnen-3-one (20βS), even after a previous incubation period of follicles with 10 IU/ml hCG for 1, 3 or 6 h. These observations indicate that *C. striata* follicles are very sensitive to increasing doses of gonadotropin, and that their responsiveness to this hormone is highly size-dependent within a very narrow range. The preliminary results also suggest that 17,20β and 20βS are not the MIS in this species.

Keywords: *Centropristis striata*, gonadotropin, steroids, oogenesis, ovaries, yolk protein.

Observations de la maturation et de l'hydratation des ovocytes in vitro chez Centropristis striata.

Résumé

Un protocole a été développé pour l'incubation des follicules ovariens de *Centropristis striata* provenant de femelles dont la ponte a eu lieu en conditions de laboratoire. Les follicules isolés ont subi la maturation après 15-24 h d'incubation à 25°C et dans un milieu Leibovitz L-15 à 75% contenant relativement de faibles doses d'hormone chorionique humaine gonadotrope (hCG) et d'extrait pituitaire homologue (50% de réponse avec approximativement 2 IU/ml et 0,02 d'équivalent pituitaire par ml respectivement). Durant la maturation *in vitro*, les follicules ont subi une augmentation remarquable de volume (environ 340%), ceci est lié à la protéolyse des plus grosses protéines du vitellus de l'ovocyte. La maturation des follicules dépend de leur taille entre 400 à 600 µm de diamètre, avec une réaction maximum entre 530 et 600 µm. La réponse à l'hormone hCG n'est pas inhibé à des températures d'incubation comprises entre 15 et 28°C mais le délai nécessaire aux follicules pour atteindre 50% de maturation est corrélé négativement avec

des hausses de températures ($r^2 = 0,97$; $p \leq 0,01$). Cependant les plus gros follicules (530-600 μm) ne subissent pas de maturation avec des doses fortes (1 $\mu\text{g/ml}$) de stéroïdes connus pour induire la maturation chez les téléostéens (MISs) 17,20b-dihydroxy-4-pregnen-3-(17,20 β) et 17,20 β ,21-trihydroxy-4-pregnen-3-(20 β S) même après une première incubation des follicules avec 10 IU/ml hCG durant 1, 3 ou 6 h. Ces observations montrent que les follicules de *C. striata* sont très sensibles à l'augmentation des doses de gonadotropine et la sensibilité à cette hormone est hautement dépendante de leur taille dans une gamme de tailles réduites. Ces résultats préliminaires suggèrent également que 17,20 β et 20 β S ne sont pas des stéroïdes majeurs (MIS) induisant la maturation pour cette espèce.

Mots-clés : *Centropristis striata*, gonadotropine, stéroïde, ovogénèse, ovaire, vitellus, protéine.

INTRODUCTION

Many species of serranids and sparids are highly valued for food or they are highly rated as ornamentals (New, 1991). In consequence, an increasing number of studies on the culture of new species of these families have been reported in recent years (Divanach and Kentouri, 1983; Garrat *et al.*, 1989; Fernández-Palacios *et al.*, 1994; Tucker *et al.*, 1994). *Centropristis striata* (Linnaeus), or black sea bass, is a protogynous serranid that occurs along the U.S. Atlantic coast, from northeast Gulf of Mexico to the Gulf of Maine (Lindall *et al.*, 1973). It is represented by two subspecies, *C. striata striata* and *C. s. melana*, corresponding to the Gulf of Mexico and northwest Atlantic populations, respectively. The females mature between years 1 and 2 (Wenner *et al.*, 1986), and they present two spawning periods during the year: a major spawning from January to April and a minor spawning during September to October (Wenner *et al.*, 1986; Hood *et al.*, 1994).

Despite that the life history of *C. striata* has been extensively reported due to its importance in commercial and recreational fisheries in the U.S., the reproductive physiology of this species is poorly understood. Reinboth *et al.* (1966) examined ovarian steroid synthesis *in vitro* and identified pregnane-3,20-dione and 5 β -reduced androgens in the incubation medium when labelled progesterone or testosterone, respectively, were added as steroid precursors. More recently, Cochran and Grier (1991) found seasonal fluctuations of plasma 11-ketotestosterone in males and females, whereas the estradiol-17 β levels were high and cycling only in females. In captivity, females with a maximum initial oocyte diameter greater than 400 μm can be induced to ovulate during the natural spawning season after injections of human chorionic gonadotropin (hCG; 275-1600 IU/kg) on two consecutive days (Tucker, 1984). However, natural spawnings under captive conditions or artificial manipulation of the spawning time by photoperiodic or temperature treatments have not been achieved (Tucker, 1994).

Members of the family Serranidae are typically protogynous hermaphrodites that spawn more than once during a season, and some even very

frequently (Tucker, 1994). Therefore, these species offer excellent experimental models to study the hormonal regulation of fractional spawning in marine teleosts. In addition, serranids produce pelagic eggs, probably as a consequence of a remarkable hydration during oocyte maturation (Wallace and Selman, 1981; Thorsen *et al.*, 1993). It is clear, however, that the experimental approaches to explore the cellular mechanisms underlying oocyte maturation and hydration in these species may require the availability of adequate protocols to incubate ovarian follicles *in vitro*, preserving their responsiveness to hormonal stimulation. Consequently, in the present work our objective was to define the best conditions for the incubation of *C. striata* ovarian follicles *in vitro*, as well as to explore the hormonal control of final oocyte maturation in this species. The extent of oocyte volume increase that occurs during maturation, and the pattern of proteolysis of yolk proteins associated, have also been documented.

MATERIALS AND METHODS

Fish and egg collections

Approximately 60-80 black sea bass were collected 25-30 km offshore from St. Augustine (Florida, USA) at 25-30 m depths several times during January and February (1994, 1995 and 1996) by hook and line or trap fishing. Fish were transported to the laboratory where their swimbladders were deflated with a hypodermic needle, and both sexes (total=60-80) were placed together in a 950-l tank supplied with running and aerated sea water, under natural temperature conditions (15-22°C) and constant photoperiod (12 L:12 D). Fish were maintained in reproductive condition until the end of the natural spawning season (late April at St. Augustine) by feeding a diet consisting of trash fish, squid and shrimp *ad libitum* three times a week. Twice during the maintenance period, eggs were collected at 2-h intervals over a 48-h period by placing a fine collecting net in the effluent stream from the holding tank. The number of eggs gathered was estimated

volumetrically, based on a value of 753 ± 14 eggs/ml (six determinations per triplicate).

Chemicals and hormones

The reagents, culture medium and hormones were purchased from Sigma (St. Louis, MO), unless indicated otherwise. hCG (10 000 IU) was dissolved in 3.3 ml culture medium (*see* below) and the solution was stored at 4°C for up to one week. The stock solutions of 17,20 β -dihydroxy-4-pregnen-3-one (17,20 β P) and 17,20 β ,21-trihydroxy-4-pregnen-3-one (20S) were prepared in 95% ethanol and added to the culture media in 5 μ l ethanol vehicle. The pituitary gland from selected females showing mature ovaries (*i.e.* containing large follicles and/or hydrated oocytes) was used to prepare the black sea bass pituitary extract (sbPE). This extract was prepared by homogenizing 3 pituitaries in 1 ml of culture medium at 0°C. The homogenate was centrifuged at 14 000 rpm for 15 min and the supernatant stored in 300- μ l aliquots at -20°C. The same homogenate was used for all the experiments.

Follicle size-frequency profiles

Experimental females were collected from the holding tanks using a bated hook and line always at the same time of day (between 10:00 and 12:00 h). After anesthetization with tricaine (MS-222), they were weighed and measured, and the ovaries and pituitaries removed and also weighed. The body and gonad weights were used to calculate the gonadosomatic index [GSI = $100 \times (\text{gonad weight}/\text{total body weight})$].

The ovaries of 18 females (mean weight = 298.8 \pm 31.5 g and mean length = 22.7 \pm 0.8 cm) were studied in 1994, 1995 and 1996; none had obvious male tissue in ovarian lamellae from the posterior region of the ovary (*i.e.* were transitional females). Consequently, an arbitrarily selected fragment of the ovary was transferred to 35 \times 10-mm polyurethane petri dishes (Falcon No. 3001, NJ, USA) containing about 3 ml media (preliminary results also indicated that different parts of the ovary were equivalent). Approximately 100 individual follicles were randomly separated using watchmaker's forceps, measured to the nearest 40 μ m at a magnification of 25 \times using a Wild stereomicroscope equipped with an ocular micrometer, and scored for normal or abnormal appearance (*see* Results).

In vitro follicle incubations

The competence of oocytes to undergo final maturation was determined using an incubation protocol modified from Greeley *et al.* (1986b). From a different piece of the same ovary used for an oocyte size-frequency profile, individual follicles that did not appear abnormal (atretic?) were transferred either individually or in groups (25-30) to 24-cell

culture trays (Falcon No. 3047, NJ, USA) containing 1 ml culture medium per well. The medium consisted of 75% Leibovitz L-15 medium (with L-glutamine), diluted with distilled water and containing 100 μ g gentamicin per ml, and adjusted to pH 7.5 with HCl. Preliminary experiments did not detect differences in the percentage of maturation or abnormal follicles incubated in L-15 at 50, 75 or 100%, therefore a 75% dilution was chosen for subsequent experimentation (Greeley *et al.*, 1986b; Wallace *et al.*, 1993). Follicles were incubated in a humidified controlled temperature incubator for up to 48 or 72 h and scored for maturation (*see* Results). Photomicrographs of living follicles undergoing maturation *in vitro* were made with a Wild M5 stereomicroscope equipped with substage illumination and a Wild MPS 50 automatic camera.

Electrophoretic analysis (SDS-PAGE)

Samples of follicles (13-33) at different stages of hCG-induced maturation were transferred to 1.5-ml Eppendorf tubes and briefly centrifugated at 14 000 rpm at 4°C in order to remove the remaining culture media. The pelleted follicles were then stored at -20°C until electrophoretic analysis. Follicles were homogenized in sample buffer (40 mM dithiothreitol, 1.0 mM ethylenediamine tetracetic acid, 0.0025% bromophenol blue, 1.25% SDS, 62.5 mM Tris-Cl, pH 6.8, and 12.5% glycerol), heated at 100°C for 5 min, and then centrifuged prior to being run on linear gradient gels (6.93-20.44% acrylamide) (Wallace and Selman, 1985). Samples were electrophoresed at room temperature with a constant current (7 mA/gel) until the dye front reached the bottom of the gel (approximately 15 h). Gels were fixed and then stained with Coomassie blue R-250 as previously described (Wallace and Selman, 1985). Molecular weights were calculated as indicated by Lambin (1978), using molecular weight standards purchased from Bio-Rad.

Statistical analysis

The data are presented as mean SEM. Statistical comparisons of means were carried out by one-way analysis of variance followed by Duncan's multiple range test. Regression analyses were performed using the Statgraphics (version 5.0) package for statistical analysis (Graphic Software Systems, Inc.). Statistical differences were considered significant at $p \leq 0.05$.

RESULTS

Spawning activity of captive fish

Within 1-2 days after being collected from the field and brought to a holding tank at the Whitney Laboratory, animals began to feed eagerly when offered shrimp and cut fish and soon developed a

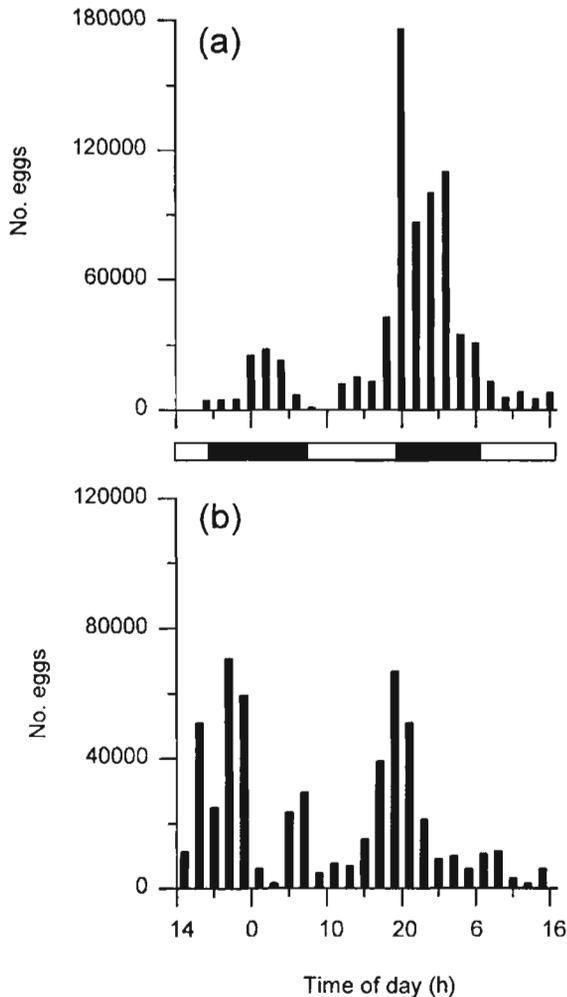


Figure 1. – Number of spawned eggs collected from 30-40 females (together with an equal number of males) at 2-h intervals over 48 h on (a) March 8-10, 1994 and (b) March 22-24, 1994. Numbers were estimated volumetrically with confidence limits of $\pm 2\%$. The horizontal bar shows light (open) and dark (black) periods.

positive visual response to caretakers; their behavior thus provided little indication that they had suffered a stressful ordeal. After 2-3 weeks in captivity, animals showed signs of spawning activity (females darting to the surface pursued by males), so the number of eggs appearing in the effluent from the tank was monitored over a 48-h period (Fig. 1a). Spawning was again monitored two weeks later (Fig. 1b). The results indicated that our population of captive fish was indeed spawning on a daily basis and that maximum egg production occurred during the early evening hours. Experimental females were collected from this population during the morning (10:00 to 12:00) when spawning activity tended to be minimal.

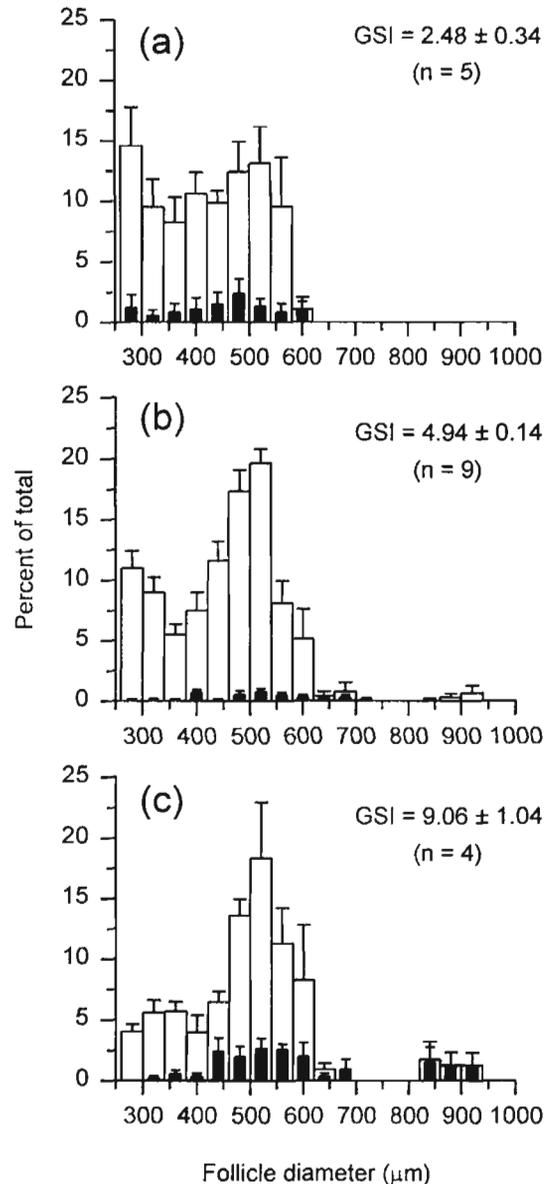


Figure 2. – Size-frequency profiles (mean \pm SEM) for follicles ≥ 280 μm diameter in ovaries of females (a,b,c) with increasing gonadosomatic index (GSI). Approximately 100 follicles were measured to the nearest 40 μm and the results for each size category are expressed as the percent of the total number measured. The percentage of normal and abnormal follicles are indicated by open and black bars, respectively. The GSI and the number of females are indicated for each panel.

Ovarian profiles

Under the stereomicroscope, ovarian follicles with a diameter from 300 to 600 μm appeared spherical and opaque, with the yolk surrounded by a thin translucent layer, and the germinal vesicle (nucleus) was not readily apparent. However, the largest follicles occasionally found in the ovary (840-920 μm diameter) were translucent and usually contained a large oil droplet

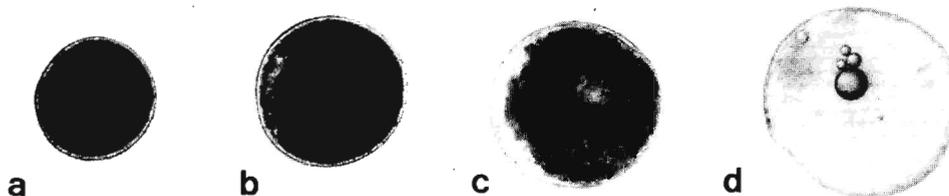


Figure 3. – Follicles undergoing maturation *in vitro*. A 0.56-mm diameter pre-maturational follicle (A) was incubated in 100 IU hCG/ml and photographed 8 h (B), 12 h (C) and 24 h (D) later. Note that the germinal vesicle is never apparent in these follicles and that a large oil droplet is visible in the postmaturational follicle (D), $\times 28.6$.

in the center (see below); sometimes a few smaller droplets were also present. Oocytes that had ovulated out of the surrounding follicle (=eggs) were also observed in the lumen of some ovaries and were approximately within the same size range as the largest ovarian follicles. Some of the follicles (from 0 to 20%, depending of the female) appeared less opaque than similar size follicles within the same ovary and/or had a partially or totally retracted yolk mass; these were judged to be abnormal.

The size-frequency distribution of ovarian follicles $\geq 280 \mu\text{m}$ diameter was determined for all experimental females (Fig. 2a,b,c). Different ovarian profiles were found in females as the GSI increased from 2.48 ± 0.34 to 9.06 ± 1.04 : whereas the number of follicles with a diameter $\geq 480 \mu\text{m}$ increased coordinately with GSI, the proportion of follicles 280–400 μm in diameter decreased. Mature and/or ovulated oocytes ($\geq 840 \mu\text{m}$) were absent when the GSI was 2.48, but their frequency was at a maximum at a GSI of 9.06. Accordingly, the mean follicle volume calculated for each female showed a significant positive correlation ($r^2 = 0.65$; $p \leq 0.001$) with the GSI (data not shown).

Induction of oocyte maturation *in vitro*

Preliminary experiments were done to define the process of oocyte maturation *in vitro*. The largest immature ovarian follicles (500–600 μm diameter) present in the ovary were manually isolated, placed in 75% L-15 culture medium, and exposed to ± 100 IU hCG/ml at 25°C. Initially, such follicles appeared spherical and opaque (Fig. 3a). Follicles not exposed to hCG (control follicles) did not undergo any further changes in size or in external appearance over the entire culture time (up to 48 h). However, after several hours of incubation in hCG, follicles began to enlarge and acquire a stippled or granular appearance in one region (Fig. 3b); by approximately 15 h the changed appearance became quite extensive (Fig. 3c). The opaque granulation eventually dispersed and by 24 h the follicle became translucent and contained an oocyte with a large oil droplet (and sometimes a few additional small ones) and no visible germinal vesicle (Fig. 3d). After 34 h, a small proportion of oocytes (approximately 40%) also ovulated, although after ovulation these ovulated oocytes (=eggs) soon

became abnormal in the culture medium. Thus, at 48 h the proportion of abnormal follicles/eggs (*i.e.* mis-shapen and/or dehydrated) reached approximately 65% (Fig. 4b). Based on these observations and those previously made in other marine species (*e.g.* Wallace *et al.*, 1993; Murayama *et al.*, 1994; Matsubara *et al.*, 1995; Okumura *et al.*, 1995), translucent (“cleared”) follicles containing a large oil droplet were considered to have undergone germinal vesicle breakdown. A time course of this process for *C. striata* follicles at 25°C is provided in Figure 4a.

Subsequent experiments were done to establish which size follicles were competent to undergo maturation *in vitro*. Accordingly, various size follicles (350–600 μm diameter) were isolated from females and incubated in the presence or absence of 100 IU hCG/ml for 48 h. No spontaneous maturation was observed in the control groups not exposed to hormone. In the presence of hCG, follicles underwent an increasing incidence of maturation corresponding to their size: no response was observed among follicles 350–390 μm diameter, whereas a maximum response was displayed by follicles 490–600 μm diameter (Fig. 5). The analysis of the maturation response to increasing doses of hCG and sbPE also indicated a high sensitivity of these larger follicles to GtH, since a 50% maximum response was achieved at a concentration of approximately 2 IU/ml hCG and 0.02 pituitary equivalents (PEq)/ml sbPE (Fig. 6).

The effect of incubation temperature on oocyte maturation over time was also examined for a definition of the permissible temperature range. Follicles with a diameter of 500–600 μm were incubated in the presence of 100 IU hCG/ml at 28, 25 and 15°C and periodically scored for maturation for up to 60 h (Fig. 7a). Over this range of temperatures, we did not observe any inhibitory effect on oocyte maturation or any overinduction of abnormal follicles (data not shown). On the contrary, the length of time required by 500–600 μm follicles to achieve 50% maximum response appeared negatively correlated ($r^2 = 0.97$; $p < 0.01$) with increasing incubation temperatures (Fig. 7b). Thus, follicles underwent 50% maturation in 10.3 ± 0.3 h at 28°C, whereas at 15°C the same process required 37.9 ± 0.1 h.

Based on previous results, follicles larger than 500 μm diameter and an incubation temperature of 25°C were selected for further experiments in which

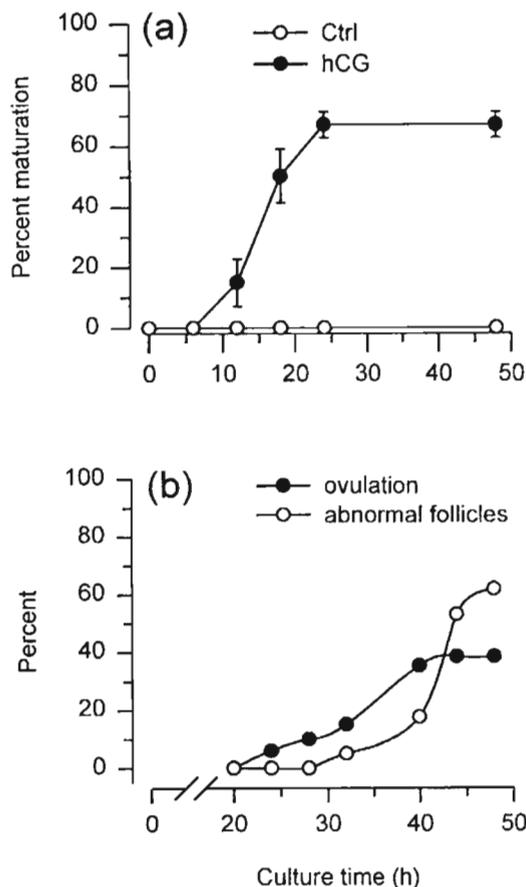


Figure 4. – Maturation response of black sea bass ovarian follicles to human chorionic gonadotropin (hCG) *in vitro*. (a) Twenty-five to 30 immature follicles (500-600 μm in diameter) were incubated in duplicate at 25°C in the presence (hCG) or absence (Ctrl) of hCG (100 IU/ml). Values are means \pm SEM (n=4 females). (b) Ovulation response and abnormality over time of follicles matured *in vitro* and transferred to fresh culture medium from two different females.

the effectiveness of two major maturation-inducing steroids (MISs) identified in teleosts, 17,20 β P and 20 β S (Nagahama *et al.*, 1995), inducing maturation of *C. striata* oocytes was tested. To assess this, two groups of follicles (500-600 μm) were incubated in

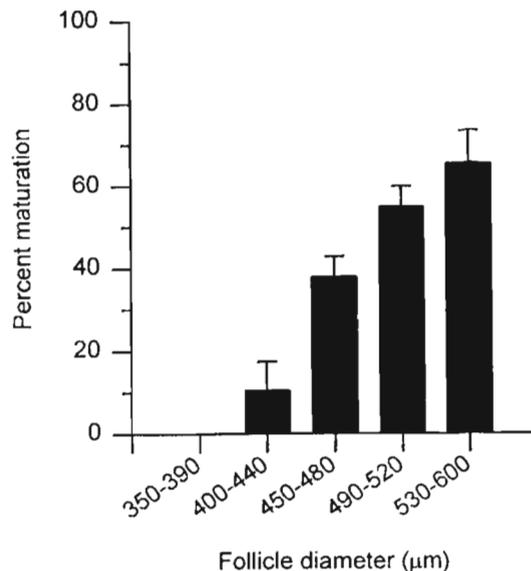


Figure 5. – Maturation response of different size follicles (from 350-390 μm to 530-600 μm diameter) to 100 IU/ml of human chorionic gonadotropin (hCG) after 48 h. Values are means \pm SEM (n=3 females). Follicles not exposed to hCG did not show spontaneous maturation (data not shown).

hormone-free culture media or in media containing 10 IU/ml hCG for 1, 3 or 6 h, washed with fresh medium, and subsequently each group randomly distributed in four treatment groups, 0.3% ethanol (control group), hCG (10 IU/ml), 17,20 β P (1 $\mu\text{g}/\text{ml}$) or 20 β S (1 $\mu\text{g}/\text{ml}$), for an additional 42-45 h. Follicles incubated with hormone-free culture media and transferred to media containing hCG underwent maturation after 42-45 h of culture, whereas the follicle groups transferred to media not containing hormones (control group) or containing 17,20 β P or 20 β S did not mature or showed very low incidence of maturation (Table 1). Similar results were observed on follicles incubated with hCG for either 1, 3 or 6 h and transferred to the different treatments, since the maturation observed in the control and steroid-stimulated follicles was comparable and significantly

Table 1. – Effect of 17,20 β -dihydroxy-4-pregnen-3-one (17,20 β P) and 17,20 β ,21-trihydroxy-4-pregnen-3-one (20 β S) on maturation of black sea bass ovarian follicles *in vitro* previously incubated in the absence or presence of human chorionic gonadotropin (hCG)¹.

Preincubation time (h)	Control				hCG			
	Ctrl	hCG	17,20 β P	20 β S	Ctrl	hCG	17,20 β P	20 β S
1	0	60.7 \pm 8.5	0	0	5.3 \pm 3.1	50.7 \pm 6.5	7.8 \pm 4.3	9.2 \pm 5.1
3	0	65.3 \pm 10.2	2.5 \pm 1.5	4.5 \pm 3.2	8.5 \pm 4.5	48.9 \pm 4.5	15.6 \pm 7.7	14.9 \pm 6.3
6	0	55.9 \pm 5.7	1.9 \pm 1.1	2.2 \pm 1.2	18.8 \pm 7.3	53.5 \pm 6.8	19.3 \pm 5.5	23.6 \pm 2.1

¹ Two groups of follicles (500-600 μm diameter) were incubated in the absence (Control group) or presence (hCG group) of 10 IU/ml hCG for 1, 3 or 6 h. After this period, each group was washed three times in fresh, hormone-free culture media, and groups of 25-30 follicles were subsequently cultured in duplicate in the presence of 0.3% ethanol (Ctrl), 10 IU/ml hCG, 1 $\mu\text{g}/\text{ml}$ 17,20 β P or 1 $\mu\text{g}/\text{ml}$ 20 β S for an additional 42-45 h at 25°C. Data show the percentage of oocyte maturation (mean \pm SEM, n=3 females) at the end of culture (see statistics in Results).

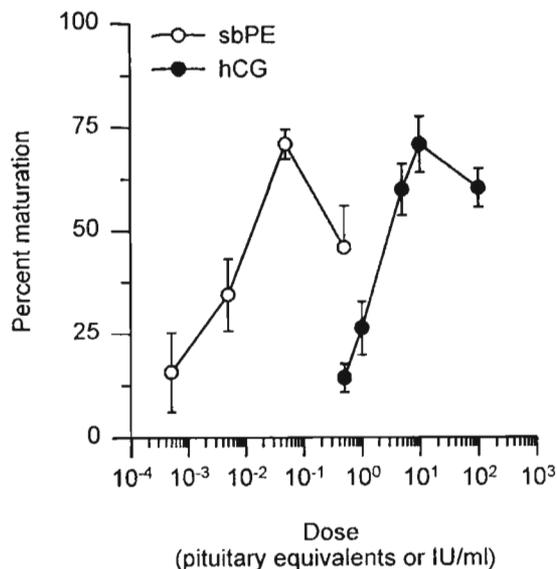


Figure 6. – Maturation response of the largest immature ovarian follicles (500-600 μ m diameter) exposed to logarithmic dilutions of black sea bass pituitary extract (sbPE) and human chorionic gonadotropin (hCG). Groups of 25-30 follicles were manually isolated and incubated at 25°C. Values are means \pm SEM (n=3 females).

($p \leq 0.05$) lower than that displayed by hCG-stimulated follicles (Table 1).

Finally, another set of experiments was designed to reproduce and measure *in vitro* the relative volume increase of large, maturing oocytes compared to immature, non-clearing oocytes. Accordingly, large, immature follicles (500-600 μ m in diameter) were cultured individually in the presence or absence of hCG (100 IU/ml) at 25°C and measured at different times in culture for up to 24 h. Follicle diameters increased rapidly between 6 and 15 h after exposure to hCG, and more slowly thereafter. In contrast, follicles not exposed to hCG did not show any significant increase in diameter. The volume increase of hCG-stimulated follicles after 24 h was 340 20% (Fig. 8).

The protein composition of follicles induced to undergo maturation *in vitro* was examined by SDS-PAGE (Fig. 9). Several major protein bands (presumably oocyte yolk proteins) were apparent in prematuration follicles (lane a), but by 24 h incubation in hCG (lane d) at least two of these major proteins (107 and 17 kDa) became relatively diminished. Although the reduction of these proteins appeared to occur between 18 h (lane c) and 24 h (lane d) incubation, in some experiments the reduction occurred by 18 h (data not shown).

DISCUSSION

Recent reports have shown that hormone-induced ovulation can be achieved with appropriate *C. striata* females collected from the Atlantic coast during

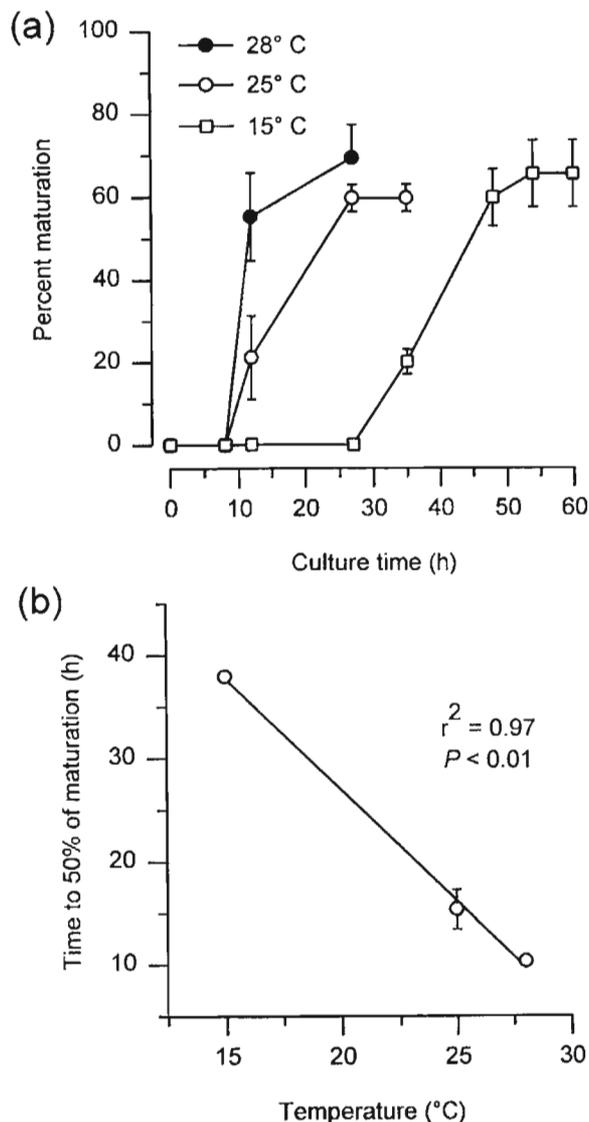


Figure 7. – (a) Maturation of sea bass ovarian follicles (500-600 μ m diameter) induced by 100 IU of human chorionic gonadotropin/ml and incubated for up to 60 h at different temperatures. (b) Relationship between temperature of incubation and time to reach 50% maximum response. Values are means \pm SEM (n=3 females).

the spring (*e.g.* Tucker, 1984), although natural spawning of captive fish has not yet been reported for this species (Tucker, 1994). The present work was performed in February to April, 1994, 1995 and 1996 on females collected from the Atlantic coast population. Specimens brought to the laboratory showed few signs of trauma associated with capture and transport and began daily spawning within a few weeks after which the females were used as a source of ovarian follicles. The reasons for spawning in our laboratory-maintained fish, in contrast to the experience of previous investigators (Tucker 1994), need to be elucidated. For the purpose of this study,

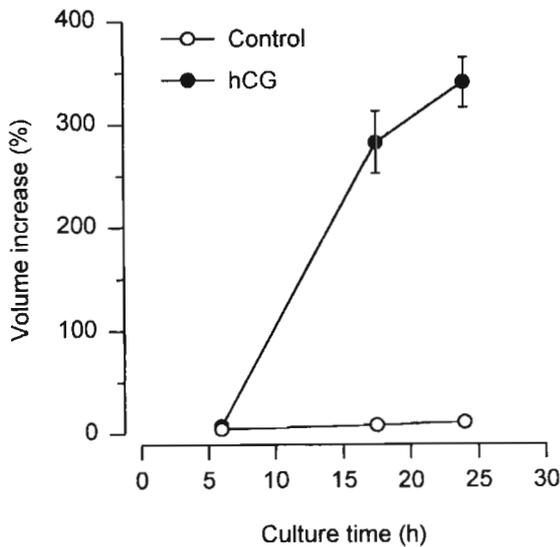


Figure 8. – Volume increase of follicles during human chorionic gonadotropin (hCG)-induced oocyte maturation *in vitro*. Twenty-four follicles (500-600 μm diameter) were incubated individually (1 follicle/ml) in the presence (hCG group) or absence (Control group) of 100 IU hCG/ml at 25°C. Values are mean \pm SEM (n = 4 females).

however, such spawning helped both to establish the reproductive normalcy of our experimental population and to provide a basis for daily sampling times.

The size-frequency distribution of ovarian follicles from females with different GSIs indicated that in the late morning hours all females had a relatively asynchronous population of follicles up to approximately 600 μm diameter, but for those females with a $\text{GSI} \geq 5$, a distinct “clutch” of large ovarian follicles was present. In retrospect, the size of the follicles in this clutch (840-920 μm diameter) indicated that they were in late oocyte maturation and presumably would have ovulated and been spawned that evening. The lack of such a clutch in females with a $\text{GSI} < 5$ also indicates that not all females in our laboratory population spawned at the same time. These observations, together with the range of GSIs encountered in our daily sampling, suggest that the *C. striata* ovary may be classified as being group-synchronous during our sampling period (Wallace and Selman, 1981), but that the reproductive condition of the population was not completely synchronous. However, definitive conclusions concerning the dynamics of follicle recruitment during the natural reproductive season of *C. striata* need to be drawn from follicle size-frequency profiles performed at various times during the day and on different days over the spawning period. Based on our observations, however, it appears likely that individual *C. striata* spawn repeatedly during their reproductive season and can thus be categorized as multiple spawners, which is typical for many serranids (Tucker, 1994).

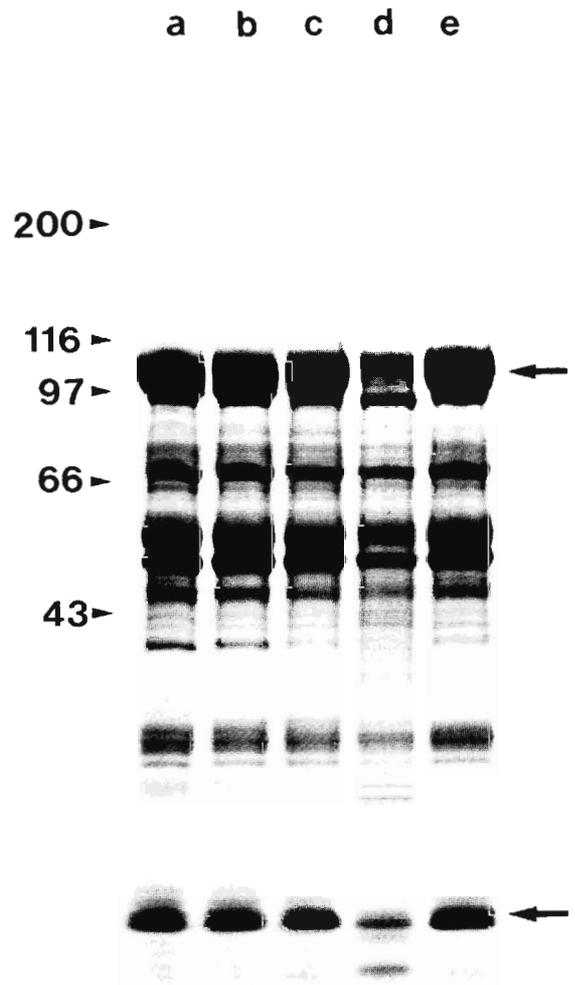


Figure 9. – SDS-PAGE of the major proteins present in extracts of prematurational follicles (a) and follicles sampled 8 h (b), 18 h (c) and 24 h (d) after incubation in 75% L-15 medium containing 100 IU hCG/ml, or control follicles incubated for 24 h without hCG (e). Each lane contain extract from 1.8 follicles. Molecular mass standards are indicated in kDa on the left. Arrows on the right mark the major protein bands that become less prominent during maturation.

In this study, we have depicted some of the morphological changes that occur during the maturation of *C. striata* ovarian follicles, and our efforts were directed towards establishing a well defined protocol for gonadotropin (GtH)-induced oocyte maturation and hydration *in vitro*. For incubation of the ovarian follicles, we routinely used a temperature of 25°C as a matter of convenience, although under natural conditions, *C. striata* appears to spawn at 16-21°C. When a temperature other than the spawning temperature is used for follicle/oocyte

incubations some assurance must be provided that the conditions are not detrimental for follicle survival. Our own observations over a temperature range of 15-28°C indicated that a similar response to hCG was eventually achieved regardless of temperature, and that abnormal follicles were not more frequently encountered at the extreme temperatures. These observations also allowed us to plot the 50% maximum response vs time which, since the points fell on a straight line, has predictive value for future experiments that would require the manipulation of the progress of oocyte maturation. After maturation, however, we noted that about 40% of the oocytes ovulated, and a short time later they became abnormal. Since ovulation may require other compounds in addition to the MIS (Goetz *et al.*, 1991), this observation was not entirely surprising and coincides with those reported for other species (*e.g.* Selman *et al.*, 1994). Nevertheless, it also seems that *C. striata* oocytes may need the presence of additional substances in the surrounding media, since our efforts to improve the survival of ovulated eggs by transferring them to fresh sea water were unsuccessful (data not shown).

Numerous studies for teleosts have established that oocyte maturation is triggered by MIS secreted by the follicle cells under GtH stimulation. From studies performed on salmonids, cyprinids and sciaenids, two major steroids have been conclusively identified as naturally occurring MIS, 17,20 β P and 20 β S, although very little is known about the changes of follicle responsiveness to GtH during follicle development or at different times during the spawning season (Nagahama *et al.*, 1995). In the present work, we have shown that the maturation response of *C. striata* follicles to hCG could be generated *in vitro* within several hours (Fig. 4a) and was highly size-dependent within a very narrow range (from 400 to 600 μ m diameter), with a maximum response being displayed by 530-600- μ m follicles. These results are difficult to compare with other studies since analogous experiments have been carried out for only a few teleost species, although they seem to be similar to those reported for medaka (Hirose, 1980), snook (Wallace *et al.*, 1993) and zebrafish (Selman *et al.*, 1994). Taken together, such observations suggest that small changes in follicle size are accompanied by dramatic changes in the number of GtH and/or MIS receptors, in their ligand binding properties, in the developing status of the follicle steroidogenic pathways, or in some combination of these possibilities (Nagahama *et al.*, 1995). In species with a relatively asynchronous population of prematurational follicles, therefore, special care must be taken to define carefully measured sized classes of limited size range when follicles are pooled to perform experiments *in vitro* in order to minimize such sources of variation.

Nevertheless, *C. striata* oocytes did not undergo maturation *in vitro* after exposure of follicles to high doses of either 17,20 β P or 20 β S for 45 h. The oocytes from other marine teleosts are also unresponsive to these MISs unless they are treated *in vivo* or *in vitro* with GtH, which seems to increase the ability of oocytes to respond to steroid by a mechanism that apparently requires the RNA-dependant synthesis of new protein(s) (Kobayashi *et al.*, 1988; Patiño and Thomas, 1990; Degani and Baker, 1992; Yoshikuni *et al.*, 1992; Zhu *et al.*, 1994; Kagawa *et al.*, 1994). However, the preincubation of *C. striata* follicles for a short time period (1-6 h) with a dose of hCG similar to that used in other species (Patiño and Thomas, 1990; Zhu *et al.*, 1994; Kagawa *et al.*, 1994) did not develop sensitivity to steroids in the treated oocytes. These results, therefore, suggest that 17,20 β P or 20 β S are not possibly the MIS in *C. striata*, which also appears to be the case for other teleost species (*see* Kime, 1993). Since *C. striata* may serve as an excellent model for experimental studies on the hormonal control of fractional spawning in marine teleosts, future experiments should be directed towards the purification and characterization of the naturally occurring MIS in this species.

One of the features of oocyte maturation in *C. striata* includes a dramatic increase in oocyte volume both *in vivo* and *in vitro* together with a decrease of especially the largest polypeptide present in the oocyte. Increases in oocyte volume due to rapid water uptake during maturation have been reported for many teleosts, and have ranged from slight in many freshwater and euryhaline species with demersal eggs to several-fold in marine species with pelagic eggs (Wallace and Selman, 1981, 1985; Craik and Harvey, 1986; Greeley *et al.*, 1986a; Watanabe and Kuo, 1986; Wallace *et al.*, 1993). The osmotic effectors driving the hydration process appear to be accumulating K⁺ and/or amino acids, respectively (Craik and Harvey, 1987; LaFleur and Thomas, 1991; Wallace *et al.*, 1992; Thorsen *et al.*, 1993). Presumably, therefore, amino acids are the primary osmotic effectors responsible for the hydration of *C. striata* eggs, which are buoyant in sea water. Several authors have correlated the appearance of amino acids within the oocyte with the proteolysis of yolk proteins during oocyte maturation (*e.g.* Greeley *et al.*, 1986a; Craik and Harvey, 1987). Specifically, a consistent feature in such oocytes appears to be the loss or diminution of the largest yolk polypeptide during the maturation process (Greeley *et al.*, 1986a; Carnevali *et al.*, 1992, 1993; Matsubara and Sawano, 1995; Matsubara *et al.*, 1995; Okumura *et al.*, 1995), which is similar to our own findings. The mechanism by which this peptide may be presumably selected and utilized as a source of amino acids during oocyte maturation remains to be elucidated.

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