

## Plasma vitellogenin and 17 $\beta$ -estradiol cycles in the skipjack tuna (*Katsuwonus pelamis*) of the Western Indian Ocean

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### Abstract

Vitellogenin (VTG) levels were measured in the plasma of 143 female skipjack tuna, *Katsuwonus pelamis* caught in the Western Indian Ocean. These levels were correlated with the gonadosomatic index (GSI) and with plasma 17 $\beta$ -estradiol (E<sub>2</sub>) levels during the reproductive cycle. VTG and E<sub>2</sub> levels were measured using a competitive enzyme-linked immunosorbent assay (ELISA) and a radioimmunoassay (RIA), respectively. VTG was purified from a pool of plasma obtained from females with high GSI using a double chromatography method (gel filtration and ion exchange). A specific antibody was obtained in rabbits. The VTG immunoenzyme assay developed gave an assay detection limit (90 % binding) of 15 ng.ml<sup>-1</sup>. Mean VTG levels ranged from 1.2 mg.ml<sup>-1</sup> to 4 mg.ml<sup>-1</sup>. Mean monthly VTG values remained relatively elevated during the resting periods (1.2 mg.ml<sup>-1</sup> in April and 1.9 mg.ml<sup>-1</sup> in September), while maximum levels reached only 4 mg.ml<sup>-1</sup> during the reproductive season. The highest GSI values were observed from November to March during the major reproductive season corresponding to the North monsoon and from early June to late August during the minor reproductive season corresponding to the East monsoon. Lowest GSI values were found in April-May and September-October. Mean E<sub>2</sub> levels exhibited considerable variability among all females sampled ranging from 700 pg.ml<sup>-1</sup> to 9 ng.ml<sup>-1</sup>. This study demonstrated that the sexual maturation was correlated with the monsoon seasons and that there was a positive correlation between monthly variations of GSI, VTG and E<sub>2</sub> levels in the skipjack tuna population from the Western Indian Ocean.

**Keywords:** Vitellogenin, estradiol, reproductive cycle, skipjack tuna, western Indian Ocean.

*Cycles annuels de la vitellogénine et de l'oestradiol chez le listao (Katsuwonus pelamis) de l'ouest de l'océan Indien.*

### Résumé

La vitellogénine (VTG) du plasma est mesurée par immuno-enzymodosage (ELISA) chez 143 femelles de listao capturées dans l'ouest de l'océan Indien. Les variations de VTG sont comparées avec celles du rapport gonadosomatique (GSI) et avec les concentrations en oestradiol du plasma déterminées par radio-immunodosage (RIA) au cours du cycle reproducteur. La VTG est purifiée par une technique basée sur une double chromatographie par filtration sur gel puis sur échangeur d'ions. Un anticorps spécifique est obtenu sur des lapins. Le dosage de VTG nous a permis d'atteindre une sensibilité de 15 ng.ml<sup>-1</sup> correspondant à 10 % de déplacement de la liaison. Au cours du cycle reproducteur, les niveaux moyens de VTG varient de 1,2 à 4 mg.ml<sup>-1</sup>. Au cours des périodes de repos sexuel (avril et septembre), les niveaux de VTG restent

cependant relativement élevés (1.2 mg.ml<sup>-1</sup> en avril et 1.9 mg.ml<sup>-1</sup> en septembre), alors qu'ils n'atteignent que 4 mg.ml<sup>-1</sup> au maximum de la période de reproduction. Le rapport gonadosomatique (GSI) est maximum de novembre à mars (saison de reproduction principale) c'est-à-dire au moment de la mousson de nord, mais également durant la mousson de sud (juin à fin août), ce qui correspond à une petite saison de reproduction. L'oestradiol présente une très grande variabilité au sein de la population de femelles échantillonnées, les taux moyens variant de 700 pg.ml<sup>-1</sup> (limite de détection) à 9 ng.ml<sup>-1</sup>. Cette étude montre que l'activité de reproduction est en relation avec les moussons et qu'il existe une corrélation positive entre les variations mensuelles des trois paramètres étudiés (GSI, taux de vitellogénine et d'oestradiol du plasma) chez le listao.

**Mots-clés :** Vitellogénine, oestradiol, cycle reproducteur, listao, thon, océan Indien.

## INTRODUCTION

Vitellogenin (VTG) has been demonstrated in the female plasma of a wide variety of teleosts, as a female-specific glycolipophosphoprotein which is synthesized by the liver in response to an estrogenic stimulation. Released into the blood, transported to the ovary, and specifically taken up into oocytes, VTG is the major plasma precursor of yolk, used by the embryo for its development.

Although seasonal changes in plasma VTG and steroid hormone levels have been well documented in a number of freshwater fish species (Fostier *et al.*, 1983), less information is available for marine species. However, for serum VTG, such changes have been reported in cod, *Gadus morhua* (Plack *et al.*, 1971), *Pseudopleuronectes americanus*, (Campbell and Idler, 1976), *Pleuronectes platessa* (Ng and Idler, 1978), *Solea vulgaris* (Nuñez Rodriguez *et al.*, 1989), *Hippoglossus hippoglossus* (Norberg, 1995), *Oncorhynchus mykiss* (Sumpter, 1985; Heppell *et al.*, 1995), *Salmo trutta* (Norberg and Haux, 1988), *Gobius niger* (Le Menn, 1979), *Dicentrarchus labrax* (Mañanos *et al.*, 1994a) and *Anguilla japonica* (Okumura *et al.*, 1995). In the same way, changes in serum estrogens specially in marine fish during the spawning have been established for bluefish, *Pomatomus saltator* and king mackerel, *Scomberomorus cavalla* (MacGregor *et al.*, 1981), orange roughy, *Hoplostethus atlanticus* (Pankhurst and Conroy, 1988), red sea bream, *Pagrus major* (Matsuyama *et al.*, 1988; Ouchi *et al.*, 1988), striped bass, *Morone saxatilis* (Berlinsky and Specker, 1991) and sea bass, *Dicentrarchus labrax* (Prat *et al.*, 1990).

Up to now, few physiological data were recorded concerning pelagic fishes with very large oceanic migrations. This paper deals with seasonal changes of estradiol 17 $\beta$ -estradiol (E<sub>2</sub>) and VTG with the annual spawning cycle in the skipjack tuna, *Katsuwonus pelamis*, considered as a large transoceanic migratory fish.

## MATERIALS AND METHODS

### Samples

143 female skipjack tuna were captured in the Western part of the Indian Ocean from 45° E to 65° E and from 5° N to 10° S by the French purse seiners based in Seychelles Islands. Each fish was caught alive in the reduced purse seine net with a 1 meter diameter scoopnet, brought on the deck for sampling. Using a 10 ml heparinized syringe containing a protease inhibitor (PMSF, phenylmethylsulfonyl fluoride, 1 mM), the blood was collected in the ventral aorta, just at its connection with the bulbus arteriosus, and immediately centrifuged (10 min at 6 000 g). The plasma obtained was frozen and stored at -30°C in 2.5 ml aliquots. These females were weighed and measured (fork length). The ovaries and livers were removed, frozen at -30°C and weighed later at the laboratory.

### Enzyme immunoassay method

This assay is based on the competition for the anti-VTG antibody between VTG in the sample and VTG absorbed on microtiter plates and is based on that previously described by Nuñez Rodriguez *et al.* (1989).

### Vitellogenin purification

The skipjack tuna VTG was isolated from pooled plasma of vitellogenic females (GSI > 3). VTG was purified using a double chromatography method similar to that described by Mañanos *et al.* (1994b). Briefly, female plasma (2.5 ml) was applied to Sepharose 6B (Pharmacia LKB) gel filtration column, the VTG containing fractions were pooled and applied to a column of DEAE (Diethylaminoethyl) Trisacryl M (IBF Sepracor, anion exchange chromatography). The elution of VTG was performed with a linear NaCl gradient of 0 to 300 mM. After elution, fractions containing purified VTG identified on SDS-PAGE (Sodium dodecyl sulphate-Polyacrylamide gel electrophoresis) were pooled, concentrated on an Amicon cell and stored at -30°C in aliquots.

### *Specific antiserum preparation*

An immunogen composed of equal parts (100 µg) of purified VTG and Freund's adjuvant (SIGMA) was injected subcutaneously into rabbits in 10 or 12 different points of the back according to the following schedule: 4 injections spaced by a week, 15 resting days and 2 boosting injections spaced by 2 weeks. Seven days after last injection, blood was collected and the antiserum was tested by direct Immunoassay using VTG as coating.

### *Antigen coating*

The coating was performed in 96 well microtiter plates (NUNC maxisorp) with 200 µl per well of 0.05 M carbonate buffer (pH 9.6) containing 350 ng.ml<sup>-1</sup> of VTG. The blank values were obtained by coating with male plasma proteins at the same concentrations. The plates were then covered and incubated for 16 hours at 4°C. The content of the wells was discarded by inverting the plates and 3 successive washes of 60 seconds each (wash cycle) were applied 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) for half an hour at 37°C, followed by a wash cycle.

### *Specific antibody incubation*

In separate microtubes, the specific antibody diluted 1/32 000 in PBS-t-NPS were incubated with serial dilutions (factor 2) of VTG standard preparation (from 6 000 ng.ml<sup>-1</sup> to 11 ng.ml<sup>-1</sup>) for 16 hours at 4°C. The content of these microtubes was distributed in duplicates in microtiter plates (200 µl per well) and the plates were incubated during 2 hours at 37°C, followed by a wash cycle.

### *Second antibody incubation*

All the wells received 200 µl of swine IgG anti-rabbit IgG (DAKO) diluted 1/2 000 in PBS-t-NPS and the plates were incubated for 45 min at 37°C, followed by a wash cycle.

### *Peroxidase anti-peroxidase (PAP) complex incubation*

The PAP complex (DAKO) obtained in rabbits (diluted 1/5 000 in PBS-t-NPS) were distributed in the wells and the plate was incubated for half an hour at 37°C, followed by a wash cycle.

### *Revelation of the reaction*

Each well received 200 µl of the following solution (citrate-phosphate buffer 0.1 M pH 5.0; 10 mg of *o*.phenylene diamine (SIGMA); 5 µl of 30% hydrogen peroxide) prepared immediately before use. The color development reached its maximum after 30 min in the dark and at room temperature. The reaction was stopped by adding 50 µl of 4 M sulfuric acid to each well.

The absorbance of each well was measured 30 minutes later, at 492 nm using an ELISA reader, Titertek.

### **Radio immunoassay methodology**

The methodology used is based on that described by Fostier *et al.* (1982). Briefly, sex steroids were extracted from 500 µl of plasma by two successive extractions with 2 ml ethylacetate-cyclohexane mixture (1:1, v/v). After evaporation of the solvent, the dry residue was redissolved in 200 µl ethanol and stored at -20°C until chromatographic separation. The ethanol fractions were transferred to a 8 cm × 0.5 cm Sephadex LH20 column (Pharmacia). Elution was performed using dichloromethane-methanol (95:5, v/v); the separated fractions, evaporated to dryness, were then dissolved in 200 µl ethanol and stored at -20°C until RIA. Efficiency of steroid extraction and separation was evaluated by addition of 1 000 rpm of labelled standard to several plasma samples before all manipulations (recovery efficiency for 17β-estradiol = 60%).

Just before the assay, the plasma samples were dissolved in 500 µl of gelatinized (0.1%) 0.01M phosphate buffer and 0.15M NaCl (pH = 7.25)

Anti-17β-estradiol was purchased from Simon Klinger-Steranti Co (England).

### **Gonadosomatic index**

Gonadosomatic index (GSI) was calculated using the formula  $GSI = \frac{W_g}{W} \times 10^2$ , where  $W_g$ : gonad weight,  $W$ : fish weight.

### **Statistical analysis**

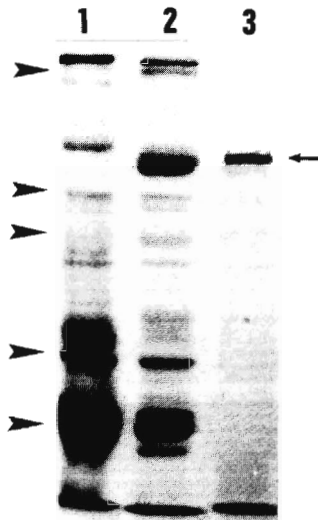
Levels of different parameters measured at each sampling month were averaged and the standard errors (SEM) were calculated. Therefore, the statistical errors are expressed as the standard error of the mean and are written as mean ± SEM. Differences between means were tested by one-way analysis of variance. Significance was accepted for all tests at  $p < 0.05$ .

## **RESULTS**

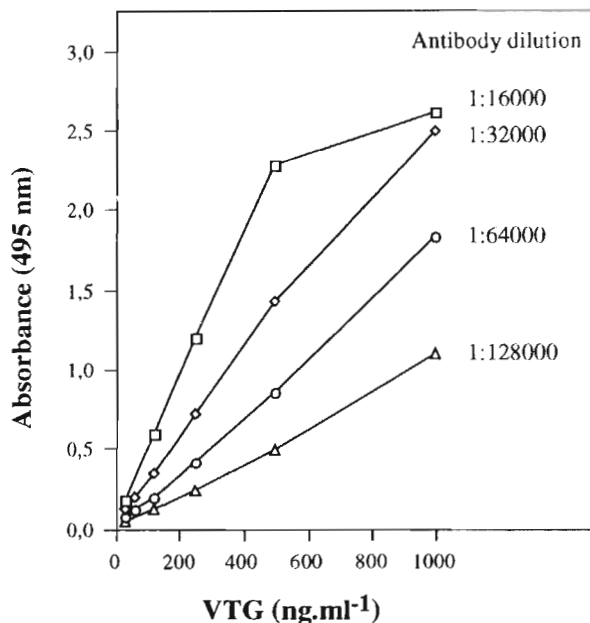
### **Vitellogenin purification, antibody specificity and assay validation**

The integrity of the VTG obtained by a double chromatography technique has been checked by SDS-PAGE electrophoresis (Fig. 1). The VTG migrated as a single band of an apparent molecular mass of 140 kDa (lane 3) corresponding to equivalent VTG band of a maturing female (lane 2). Male plasma has been used as negative control (lane 1).

The results concerning coating and antibody dilution are presented in Figure 2. For the assay, the concentra-



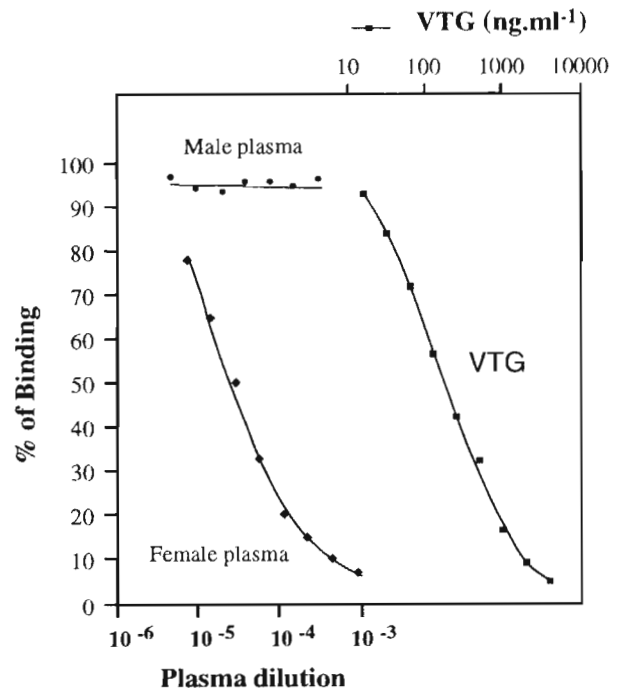
**Figure 1.** – Polyacrilamide gel electrophoresis of 2  $\mu$ l of skipjack tuna male plasma (lane 1), 1  $\mu$ l of skipjack tuna maturing female plasma (lane 2) and 10  $\mu$ g of purified skipjack tuna VTG (lane 3). The migration positions of Biorad's high molecular weight kit (200, 115, 97, 66 and 45 kDa respectively from top to bottom) are indicated on the left ( $\blacktriangleright$ ). Migration position of skipjack vitellogenin (VTG) is indicated on the right ( $\blacktriangleleft$ ).



**Figure 2.** – Coating with serial dilutions (factor 2) of vitellogenin (VTG) incubated with 4 different dilutions of skipjack tuna VTG antiserum.

tion of the VTG coating solution was fixed at 350  $\text{ng.ml}^{-1}$  and an antibody dilution of 1:32 000. At this dilution and coating concentration, maximum absorbance was around 1 ( $B_0$ ) and the blanks coated with male plasma represented 8% of the total binding.

The data concerning competition curves of different antigens are summarized in Figure 3. Parallel displacements were obtained with the standard preparation of VTG and serial dilutions of female skipjack tuna plasma. On the other hand, male plasma gave no significant displacement of the binding.

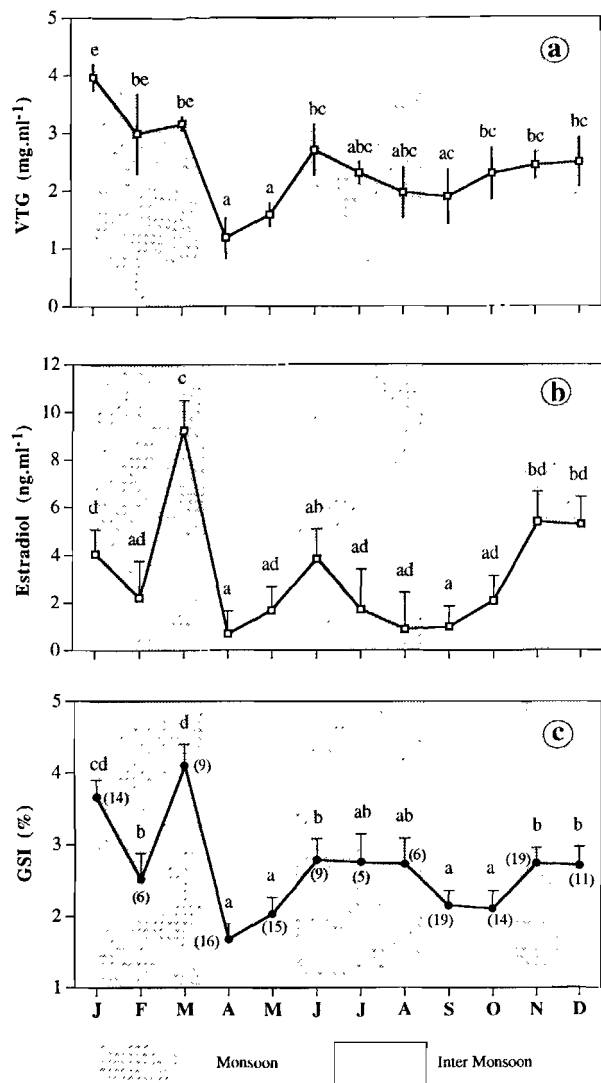


**Figure 3.** – Binding curves [ $\log(\text{dose or dilution}) = f((B_i - N) / (B_0 - N)) \times 100$ ] obtained with serial dilutions (factor 2) of various antigens. ( $B_0$ : absorbance at 0 dose,  $B_i$ : absorbance of samples or standards,  $N$ : background absorbance).

The sensitivity (VTG concentration which gave around 90% of binding) is 15  $\text{ng.ml}^{-1}$  with an intra-assay variation of 2.3% ( $n = 16$ ) and an inter-assay variation of 6.5% ( $n = 16$ ) near 50% of binding. Parallelism of 20 different standard curves was assessed by covariance analysis using the F-test on mean squares (Snedecor and Cochran, 1957; Sokal and Rohlf, 1981). No statistical difference was observed between the different regression lines.

### Vitellogenin annual cycle

Over the year of sampling, the vitellogenin plasma levels revealed some important variations (Fig. 4a). Lowest levels ( $\leq 2 \text{ mg.ml}^{-1}$ ) were recorded first in April and May, and later in August and September. From January to March, highest levels (more than 3  $\text{mg.ml}^{-1}$ ) were registered with a maximum value in January ( $3.97 \pm 0.35 \text{ mg.ml}^{-1}$ ). In June, we also noted another plasma VTG peak, lower than during January-March ( $2.70 \pm 0.44$ ), but nevertheless significantly dif-



**Figure 4.** – Monthly variations, for plasma levels of vitellogenin (VTG) (a), 17β-estradiol (b), and gonado-somatic index (c). Values represent the mean ± S.E.M. Points with different superscripts are significantly different ( $p < 0.05$ ) determined by one-way analysis of variance and Fisher's PLSD test. The number of fish at each sampling time is indicated between brackets (identical for a, b and c).

ferent ( $p < 0.05$ ) from those noted in April ( $1.18 \pm 0.33 \text{ mg.ml}^{-1}$ ) and in September ( $1.89 \pm 0.30 \text{ mg.ml}^{-1}$ ).

**Estradiol profile**

Low levels of plasma estradiol ( $E_2$  less than  $2 \text{ ng.ml}^{-1}$ ) were observed in April and in August and September (Fig. 4b). From September, these levels increased steadily and significantly and peaked in December ( $p < 0.05$ ). After two months of regular decrease (January and February), they increased again sharply during March, to reach their highest values

( $9.24 \pm 1.27 \text{ ng.ml}^{-1}$ ). A strong decrease was then observed in April, which is at the end of most important spawning period. We also observed a new peak in June ( $3.86 \pm 1.27 \text{ ng.ml}^{-1}$ ) corresponding to the beginning of another spawning period (winter period) less important than the previous one (summer period).

**Gonadosomatic index**

The GSI was minimum in April ( $1.67 \pm 0.22$ ) increasing in May to reach a first maximum in June ( $2.78 \pm 0.29$ ) and presented a plateau during July and August (Fig. 4c). For the following months, a gradual decrease in the GSI was observed until October ( $2.10 \pm 0.24$ ). From October to January, the GSI increased and reached one of the highest values in January ( $3.66 \pm 0.24$ ). A decrease was noted in February ( $2.51 \pm 0.36$ ) but immediately followed by the highest mean value of the cycle in March ( $4.10 \pm 0.29$ ).

From these results we conclude that a short spawning period occur from June throughout August, and later, longer spawning period, from November to March appear, possibly with 2 or 3 peaks of more numerous spawnings.

**DISCUSSION**

Among teleost fish having a single spawning period, there is generally a significant correlation between the levels of plasma  $E_2$  and plasma VTG with the vitellogenesis leading to ovary growth (Wingfield and Grimm, 1977; Lambert *et al.*, 1978; Kagawa *et al.*, 1983). It is necessary to note that in most studies done in temperate fish species, there is a time-lag between these correlations. For example, Nuñez Rodriguez *et al.* (1989) stated that the maximum VTG level was observed 1 month before the maximum of the GSI. In the wild brown trout, Norberg *et al.* (1989) noted that the peak of VTG followed the plasma  $E_2$  levels and decreased 1 month prior to ovulation.

In the literature, the levels of plasma  $E_2$  and VTG are variable, with observed maximum values ranging from  $0.5$  to  $50 \text{ ng.ml}^{-1}$  for  $E_2$ , and from  $13$  to  $120 \text{ mg.ml}^{-1}$  for VTG. It seems that these levels are higher for freshwater fish (cultured or wild) than for marine fish. Sometimes, large differences can be observed for the same species.

Concerning salmonids, and specially in the rainbow trout (*Oncorhynchus mykiss*), levels of  $E_2$  are found in the range of  $5 \text{ ng.ml}^{-1}$  (Scott *et al.*, 1980) up to  $50 \text{ ng.ml}^{-1}$  (Whitehead *et al.*, 1979). Intermediate results were noted in the brown trout (*Salmo trutta*) with  $E_2$  maximum values ranging between  $12$  and  $20 \text{ ng.ml}^{-1}$  (Lambert *et al.*, 1978; Norberg *et al.*, 1989). In Cyprinids, the  $E_2$  levels are similar from  $1.1 \text{ ng.ml}^{-1}$  in *Acheilognathus rhombea* up to  $11.5 \text{ ng.ml}^{-1}$  in common carp, *Cyprinus carpio* (Aida, 1988). Mean  $E_2$  values noted in marine fish are generally lower than these.

They only reach 0.59 ng.ml<sup>-1</sup> in the red sea bream, *Pagrus major* (Matsuyama et al., 1988) or 2.0 ng.ml<sup>-1</sup> in the striped bass, *Morone saxatilis* (Berlinsky and Specker, 1990).

Concerning the VTG levels, similar results were observed between marine and freshwater fish. Norberg et al. (1989) demonstrated that the same species (*Salmo trutta*) had different VTG levels depending of their environment. In cultured fish (freshwater) they registered 120 mg.ml<sup>-1</sup> but only 28 mg.ml<sup>-1</sup> in the wild sea individuals. In the same way Bon et al. (1997) have found a maximum of 60 mg.ml<sup>-1</sup> for rainbow trout (*Oncorhynchus mykiss*) reared under natural photoperiod, increasing until 120 mg.ml<sup>-1</sup> for a population reared under photoperiod conditions leading to a shortening of the sexual cycle. Nuñez Rodriguez et al. (1989) found 13 mg.ml<sup>-1</sup> as plasma VTG maximum value in wild sole, *Solea vulgaris*, while in cultured sea bass maximum levels were only around 3 mg.ml<sup>-1</sup> (Mañanos et al., 1994a). Our plasma E<sub>2</sub> and VTG analysis provided results comparable to those related by most of these studies. E<sub>2</sub> levels ranging from 0.9 to 9.2 ng.ml<sup>-1</sup> were in concordance with results generally stated in marine fishes. The mean VTG levels obtained in skipjack tuna were in the lower part of the range reported in the literature previously cited. Nevertheless it is interesting to observe in a tropical fish character-

ized by multiple spawns throughout the year that the seasonal changes for GSI, E<sub>2</sub> and VTG plasma levels present synchronous profile variations.

We have previously reported (Stéguert, 1976; Stéguert and Ramcharrun, 1995, 1996) that the sexual maturation of the population from the northwestern part of the Mozambique Channel occurred only during the two main climatic seasons (north monsoon from November to March, and south monsoon from June to September). The inter-monsoon seasons (April-May and September-October) corresponded to the post-spawning seasons or resting periods. During the warm season monsoon (north monsoon) a longer period of sexual maturation was recorded while only a shorter period was noted in the cold season (east monsoon). The present study confirms these findings, nevertheless it can be pointed out that the more unfavorable period is the north intermonsoon season corresponding to decreasing water temperatures which is probably the more stressing environmental factor in this area. Finally, it is important to note that even if maximum sexual maturation is observed during two different periods of the year, it is possible to observe a few fish with ripe ovaries which are able to spawn soon all year-round while most of the other fish, constituting the monthly population, are in resting conditions.

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