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NEW RESULTS ON THE REPRODUCTIVE BIOLOGY OF THE BLUEFIN TUNA (*THUNNUS THYNNUS*) IN THE MEDITERRANEAN

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SUMMARY

*To improve knowledge of the reproductive biology of the Mediterranean bluefin tuna (*Thunnus thynnus*), plasma concentrations of the steroid hormones Testosterone (T), 11-Ketotestosterone (11-KT) and 17ß-Estradiol (E_2) and the yolk precursor Vitellogenin (Vtg) have been determined and correlated with gonadal maturity stage. In female bluefin we found rising levels of E_2, T and Vtg approaching the spawning period. In male bluefin, T and 11-KT increase together with gonadal maturation. Characteristic sex hormone profiles for different sex and reproductive maturity stage have been observed. 11-KT has been detected in low levels in females and does not seems to play any role in ovary maturation; on the other hand E_2 and Vtg have no role in male sexual maturation. These findings can be summarised in the formula [(E_2/11-KT)]/[T], which can be used, in addition to the detection of Vtg, to distinguish between females and males and to estimate sexual maturity stage.*

RÉSUMÉ

En vue d'améliorer les connaissances sur la biologie de la reproduction du thon rouge (Thunnus thynnus) *de la Méditerranée, les concentrations de plasma des hormones testostérone (T), 11-ketotestosterone (11-KT) et 17$-oestradiol ($E_2$) et du précurseur du jaune d'oeuf vitellogine (VTG) ont été déterminées et mise en corrélation avec le stade de maturité des gonades. Chez les thons rouges femelles, on observe un niveau croissant de E_2, t et de Vtg lorsque l'on approche de période de frai. Chez les thons rouges mâles, la T et la 11-KT augmentent de pair selon la maturation des gonades. On a observé un profil caractéristique des hormones sexuelles selon les sexes et les différents stades de maturité reproductrice. La 11-KT a été détecté en faible quantité chez les femelles et ne semble pas jouer de rôle dans la maturation des ovaires; en revanche, le E_2 et la Vtg n'ont rien à voir avec la maturation sexuelle des mâles. Ces résultats peuvent être résumés par la formule [(E_2/11-KT]/[T], qui peut être utilisée, en plus de la Vtg détectée, pour distinguer les femelles des mâles et pour estimer le stade de maturité sexuelle.*

RESUMEN

*Para mejorar el conocimiento sobre la biología reproductora del atún rojo del Mediterráneo (*Thunnus thynnus*), se han determinado y relacionado con la etapa de madurez gonadal, concentraciones de plasma de las hormonas esteroides Testosterona (T), 11-Cetotestosterona (11-KT) y 17ß-Estradiol (E_2) y el precursor de yema vitelogenino (Vtg). En la hembras de atún rojo, se encontraron niveles crecientes de E_2,T y Vtg al aproximarse al periodo de desove. En los machos de atún rojo, T y 11-KT aumentan junto con la maduración de las gónadas. Se han observado perfiles característicos de las hormonas sexuales según los sexos y las diferentes etapas de madurez reproductora. En las hembras se han detectado bajos niveles de 11-KT, que no parece desempeñar ningún papel en la maduración de los ovarios; por otra parte, E_2, y Vtg no desempeñan ningún papel en la madurez sexual de los machos. Estos descubrimientos pueden resumirse en la fórmula [(E_2/11-KT)]/[T], que puede utilizarse, junto con la detección de Vtg, para distinguir entre hembras y machos y para estimar la etapa de madurez sexual.*

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KEYWORDS

Thunnus thynnus; Tuna fisheries; Sexual maturity; Sex ratio, Sex determination; Reproductive cycle; Proteins, Juveniles; Animal reproductive organs; Fish physiology

1. INTRODUCTION

In spite of the economic importance of the bluefin tuna (BFT), the knowledge of its reproductive biology is limited to research based on the distribution of eggs and larvae (Piccinetti *et al.*, 1977, 1997; Cavallaro *et al.*, 1997; Nishida *et al.*, 1997), on seasonal variations of the gonadosomatic index (I_G) (de la Serna & Alot, 1992), and on macroscopic classification of gonad maturity stage (Rodríguez-Roda, 1964, 1967). A histological study of Western Atlantic BFT ovaries has been carried out by Baglin (1982).

Since knowledge of reproductive biology is an extremely important tool for determining a correct policy for the management of fish stocks, in our laboratories studies on the correlation between endocrinological patterns and gonad maturity are in progress. Here the results obtained till now are presented, underlining the possible utility of the techniques used for the determination of sex, maturity stage and size at first sexual maturity

2. MATERIAL AND METHODS

2.1. Specimen collection

Blood, tissue and gonad samples were obtained, during the months of March, May, beginning of June and August 1998, from fish caught commercially by longlines and drift nets in South Adriatic, North Ionian (Gulf of Taranto) and Ligurian seas and by traditional traps (Tonnare) operating in Carloforte and Portoscuso (Sardinia, Italy). Soon after the capture, fish fork length (L_F) was measured and sex was determined by macroscopic observation of gonads. Blood was collected, centrifuged at 5000 rpm for 15 min, and plasma collected and stored at -20°C. Fragments of ovaries were fixed in Bouin‘s solution.

2.2. Sex steroid and Vtg measurement

11-Ketotestosterone (11-KT), Testosterone (T) and 17ß-Estradiol (E_2) have been extracted with Dichloromethane and the concentration measured by ELISA as described by Cuisset *et al.* (1994). In our laboratories we have established ELISA's with a sensivity of 1 pg/ml for 11-KT and T and 3 pg/ml for E_2 in the assay.

BFT vitellogenin (BFT-Vtg) has been purified and an ELISA established as reported for other fish species (Bon *et al.*, 1997 and Mosconi *et al.*, 1998). Dot blots analysis have been made using plasma diluted > 1:2000. The membrane has been blocked with 3% powdered skimmed milk then the anti BFT-Vtg serum applied, washed and the secondary antibody labelled with alkaline phosphatase added. Visualisation was performed enzymatically.

2.3. Histology of gonads and immunohistochemical detection of Vtg-like material

Gonad samples were embedded in paraffin wax. Sections (5 µm thick) were stained with haematoxylin – eosin. Immunohistochemical detection of anti-VTG positive material was performed on ovary sections using the abBFT-Vtg. The immunoreaction was revealed by the avidin-biotin-peroxidase complex method.

2.4. Statistical analysis

Plasma levels of steroids, vitellogenin and percentage of vitellogenic oocytes in Figure 3 have been expressed as mean +/- SEM and significant changes have been assessed by Students t-Test. All statistical analysis has been made with the software package Sigma Stat (Jandel Scientific, Erkrath, Germany). Statistical relevance was accepted at $p < 0.05$. Statistical analysis of the identity of two linear regressions in Figure 1A has been performed by F-test using the formula:

$$F = s^2_{y \cdot x\, verb} / s^2_{y \cdot x\, p},$$

$s^2_{y \cdot x\, verb}$= the difference of the sum of square of residues around the regression lines;

$s^2_{y \cdot x\, p}$= pool of the variance around both regression lines.

3. RESULTS

3.1. Sex and sexual maturity stage estimation

Testosterone (T) plasma levels rise in both sexes approaching reproductive period. The T concentration begins to decrease before spawning as reported for many other fishes (Kime, 1993). In female BFT the rise of T plasma level is followed by the increase of E_2; then both decrease just before spawning. After spawning the E_2 level is higher than T. In the reproductive season 11-Ketotestosterone (11-KT) is present in very low levels (0.68 ± 0.10 ng/ml; N = 38) in the plasma of adult females. In males 11-KT rises approaching spawning to high levels in plasma (up to 40 ng/ml) and follows the T peak as for E_2 in female. In spent males 11-KT is present in higher concentrations than T. E_2 in male is present in relatively low concentrations (0.56 ± 0.11 ng/ml; N = 60) and does not seem to play any role in sexual maturation.

We converted the different sex steroid concentrations into a mathematical relationship applying the Steroid Sex ratio Formula (SSF): $[(E_2/11\text{-KT})]/[T]$. We plotted SSF vs. T concentration on a logarithmic scale, Testosterone representing a marker for sexual maturation for both males and females (Fig. 1A). We found 2 statistically different regression lines ($p < 0.001$, assessed by F-test for the comparison of linear regression). The regression lines show that SSF values for different sexes, result from specific steroid profiles in the different maturation phases. The regression line with the higher SSF values has been calculated for females, the one with lower values for males. Fig. 1B shows, for 4 males and 4 females, the correlation between steroid profile and gonad maturity stage evaluated histologically.

The limit of the ability of the SSF to determine sex and sexual maturity stage is represented by immature fish or post-spawning fish because of their low levels of plasma T. In some case quantitative Vtg estimation (by ELISA) or simply qualitative VTG analysis (by dot blot) can clarify the sex and maturity stage (Fig. 2). In fact, Vtg can be detected in ripening, mature and post-spawning females, while it is nearly undetectable in males.

3.2. Size at first sexual maturity

The results obtained correlating BFT-Vtg and E_2 plasma levels and histological analysis by female are summarised in Figure 2.

In the recrudescence period (April) there is no significant difference in sexual maturation by different size classes. In the ripening Period (May) fish with L_F ranging from 110 and 120 cm showed a significant increase of E_2 and Vtg plasma concentrations (12.98 ± 1.53 ng/ml and 25.70 ± 6.86 mg/ml respectively). The mean oocyte diameter increased significantly, in respect to the previous length class (from 55 to 90 µm), and the histological analysis demonstrated the presence of oocytes in late vitellogenic stage. Fish with $L_F > 120$ cm showed an increase of Vtg plasma level and mean

oocyte diameter (32.54 ± 8.36 mg/ml and 106 ± 10.12 µm respectively) in respect to the previous length class and a significant increase of percentage of vitellogenic oocytes (from 5 ± 1.2 % of the previous class to 11.2 ± 3.67 %).

The immunohistochemical staining of ovaries with anti BFT-Vtg serum revealed the presence of Vtg-like material in oocyte having a minimum diameter of 220 µm. Immunopositive oocytes were observed only in ovaries of specimens caught in May with $L_F \geq 110$ cm.

4. DISCUSSION

Determination of reproductive status is a key requirement for any fisheries management programme. Vtg has been widespread used in aquaculture species as an indicator of sexual maturity (Bon *et al.*, 1997 and Mosconi *et al.*, 1998).

In this study we showed the successful use of both steroid and VTG plasma levels for sex determination and estimation of sexual maturity stage of adult bluefin tunas. Sex determination of fish with nearly undetectable steroid and VTG levels, such as juveniles or quiescent fishes, remain to be resolved.

The next objective of our researches is the use of tissues for the sex determination and maturation stage estimation. Since tissue steroid levels are very low, it is important to establish good steroid extraction methods and sensitive detection systems. The ELISA's established are very sensitive and reliable. If we confirm on tissue the results obtained in this study we will be able in future to assess reproductive status and sex ratio using not more than 200 mg of tissue. This seems an important objective if considering that plasma and gonads from fishes caught commercially are very difficult to obtain.

The preliminary study on the first sexual maturity indicates that in our sample no female BFT with $L_F < 110$ cm could be considered to be adult. This seems to be in agreement with Rodríguez-Roda (1967) who, by means of macroscopic evaluation of gonad maturity stage, found 100% Eastern Atlantic BFT females mature over 120 cm (L_F). This findings need to be confirmed in a larger fish sample including sampling at the different phases of the reproductive cycle. It is notable that pre-adult females (L_F ranging from 100 to 110 cm) showed a slight gonadal development, characterised by the appearance of oocytes in lipid stage. This finding occurred contemporaneously to both the appearance of E_2 and Vtg in the plasma. The simulation of gonadal development has already been reported by Baglin (1982) in sexual immature Western Atlantic BFT females.

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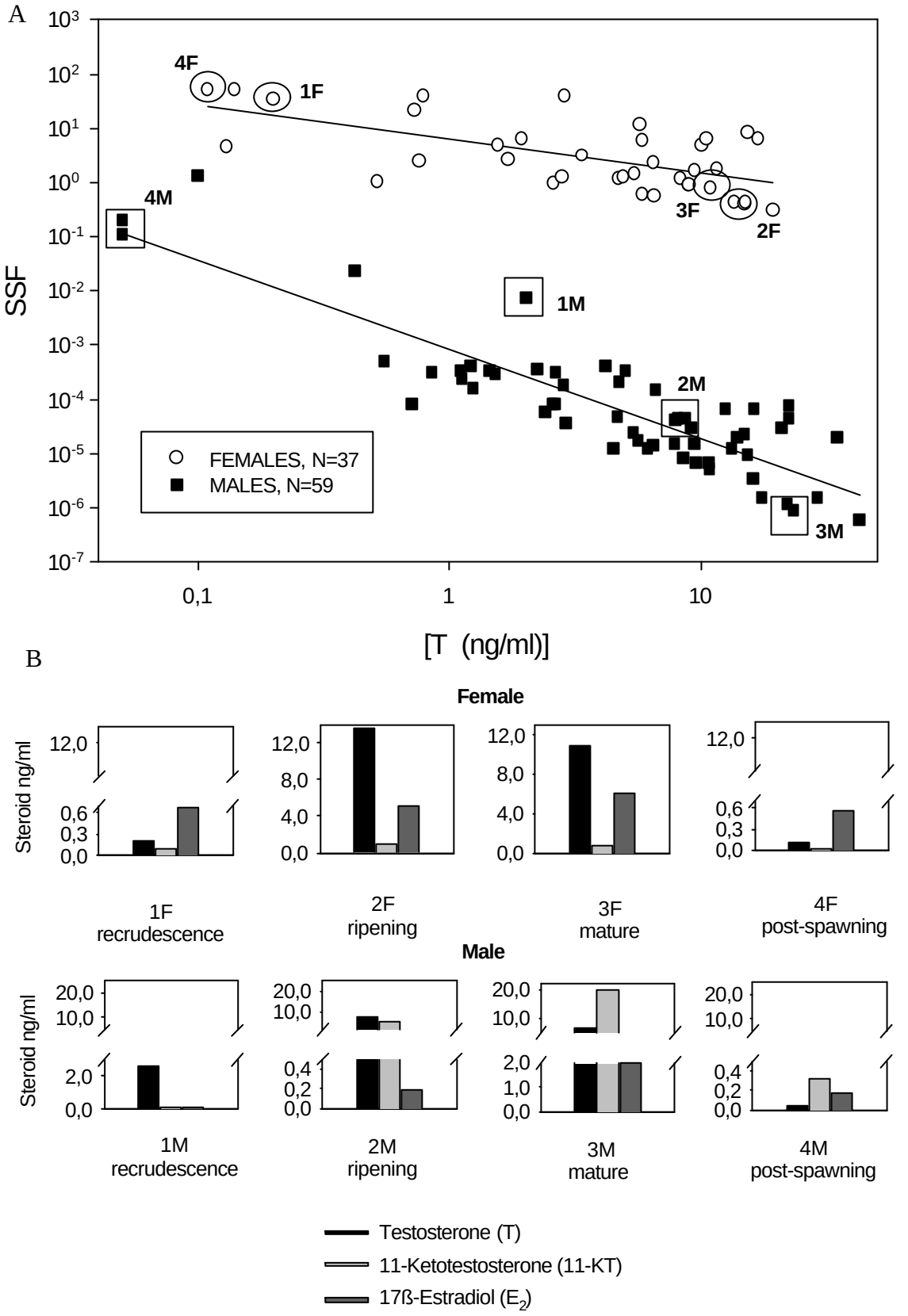

Fig. 1: Sex determination and maturity stage estimation by adult BFT. A) Depending from the SSF (Steroid Sexratio Formula, $[(E_2/11\text{-KT})]/[T]$) value and the concentration of Testosterone (T) females and mals can be clearly distiguished in a logarithmic plot. B) Different maturity stages from single individuals show specific steroid profile with a specific SSF value evidenced in A.

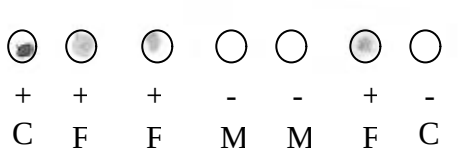

Fig. 2: Use of dot blot analysis for plasma BFT-Vtg to determine the sex. Positive reaction (+) for Vtg is detected by females in reproductive status (F). By males (M) the test is negative (-). This simply test can be used for sex determination when the measurement of steroids gives no precise results. Highly specific is the western blot analysis or ELISA measurement (not shown). CV = positive control, 1 µg BFT-Vtg; C0 = negative control, 1 µg bovine serum albumin (BSA).

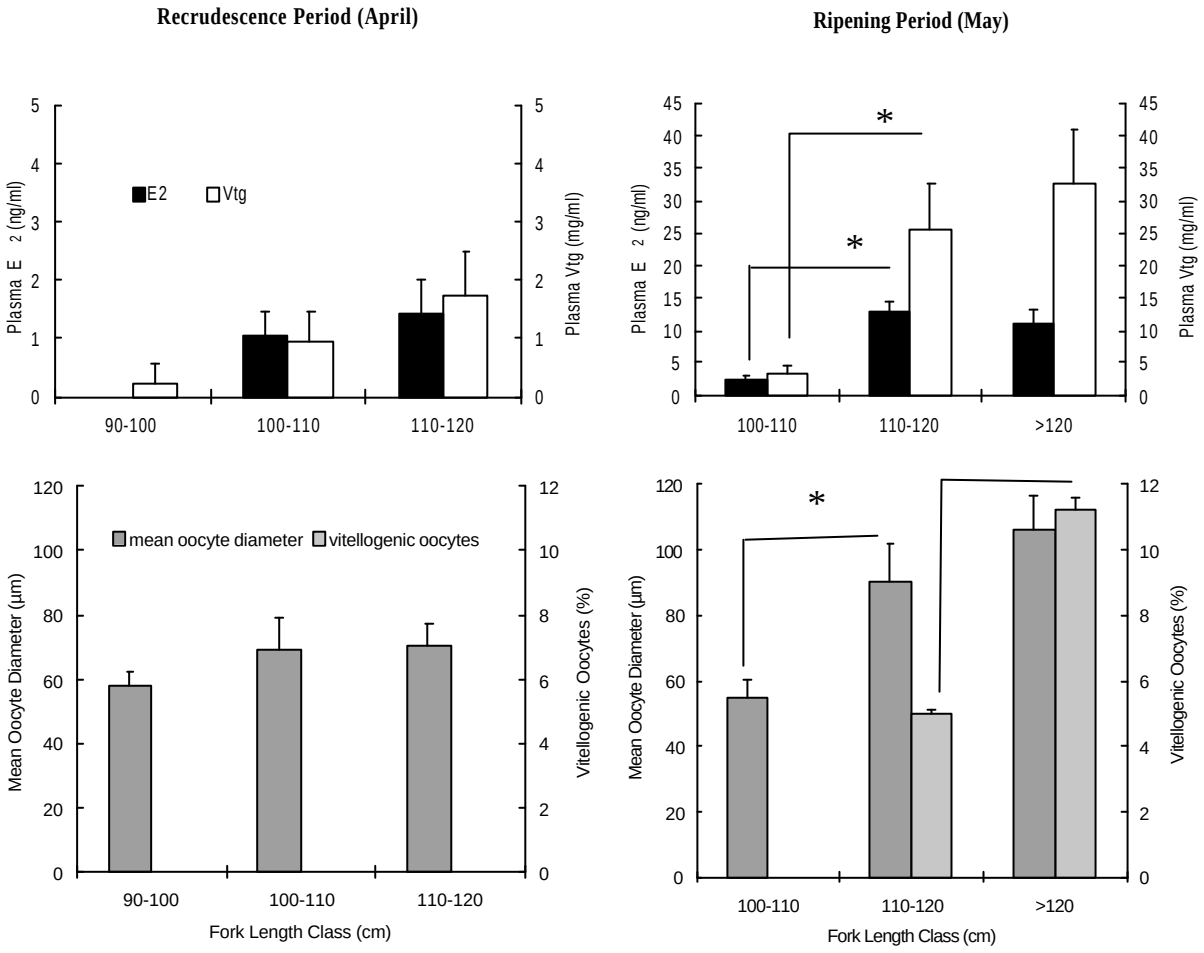

Fig. 3: Determination of the size at first sexual maturity of female BFT using changes in plasma levels of 17ß-Estradiol (E_2), Vitellogenin (Vtg), mean oocyte diameter and percentage of vitellogenic oocytes in two different periods of the female BFT reproductive cycle: recrudescence (April) and ripening (May). Significant differences in the parameters considered are observed in the ripening period (May) between the length classes 100-110 and 110-120. Asterisks represent statistical significance ($p<0.05$) assessed by t-Test, N = 7.