

## A NEW MUSCLE BIOPSY TECHNIQUE FOR SEX AND SEXUAL MATURITY DETERMINATION IN LARGE PELAGIC FISHES

by

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### SUMMARY

*In many fisheries fish are either landed already gutted or the value of the flesh prohibits ventral opening and the determination of sex and sexual maturation. The objective of the present studies was to provide new molecular endocrinological techniques to make this possible and assist stock assessment and management. A muscle biopsy punch which can be used in the field or at the market to obtain muscle biopsy samples from live or dead Bluefin Tuna or Swordfish has been developed. These samples can then be assayed using standard ELISA methods for sex hormones and vitellogenin. A correlation was found between plasma and muscle sample levels for steroids in Tuna. The future role of a muscle biopsy is discussed together with "molecular markers".*

*Keywords: bluefin tuna, swordfish, muscle biopsy, steroid hormones, reproductive cycle, sex determination, sex ratio, sexual maturity, tuna fisheries*

### 1. INTRODUCTION

In the large, socio-economically important, Mediterranean marine fisheries for the Bluefin Tuna (*Thunnus thynnus*) and the Swordfish (*Xiphias gladius*) the determination of sex and gonadal maturity of fishes at capture are important requisites for stock assessment and the controlled regulation of the fishery. In many cases sex determination and gonadal maturity of the swordfish cannot be determined due to the lack of external sexual dimorphisms between the sexes or the catch is either "gutted" at sea making sex and gonadal maturation determinations on the landing of the catch impossible. Therefore this data is mainly lacking from present stock assessment exercises. The use of fisheries observers "on board" may be costly, time-consuming and ineffective when the fishermen due to the high market value of intact fish forbid major invasive sampling.

In search of a more efficient, cost effective and less invasive assessment method of sex and gonadal maturation an innovative enzyme-linked immunosorbent assay (ELISA) mediated muscle biopsy sample (MBS) is being developed to establish the levels of the sex hormones 17 $\beta$ -estradiol (E<sub>2</sub>), 17 $\alpha$ -20 $\beta$ -dihydroxy-4-pregnen-3-one (17,20 $\beta$  P), 11-ketotestosterone (11-KT) together with the lipoprotein vitellogenin (Vtg) from Tuna and Swordfish muscle biopsy samples (MBS). They have until now only been measured in the plasma of other fish species. These substances can be used as specific indicators of sex and spawning state of the fish. The changes in steroid hormones and vitellogenin can normally be correlated with GSI and oocyte diameter. The concentration of 17,20 $\beta$  P can be used to determine the time course of both egg and sperm release. The objective of the present study has been the further development of established plasma ELISA methods for, E<sub>2</sub>, 17,20 $\beta$  -P and 11-KT for use in muscle tissue and the development of a new ELISA test for Vtg in both swordfish plasma and muscle. These will then be correlated with morphological parameters such as gonadal index, gonadal type and oocyte maturation stage. Through this correlation a standardised model with tables of values will be developed to identify either male or female fish and the reproductive state of the gonads i.e. pre-spawning, post spawning. At the same time the correlation between plasma and muscle samples is being established and the stability of both steroid hormones and vitellogenin levels in muscle and plasma under field sampling conditions on board ship and at the market determined. The new MBS method will not only be relevant for large pelagic species but also for other marine commercial species

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in general. It will also be a valuable management tool to oversee conservation measures such as closed seasons by identifying fish in the market place in terms of species, sex and gonadal maturity from single muscle biopsy samples. The present working document outlines some of the present results and suggestions for future directions

## **2. MATERIAL AND METHODS**

BFT and swordfish muscle and plasma samples were obtained by sampling during the Mattanza in the Tonnara's of Porto Scuso and Stintino, Sardinia. Blood was collected and kept on ice until the plasma samples could be frozen at  $-20^{\circ}\text{C}$ . Muscle samples were directly frozen. Steroid hormones were measured by ELISA as described by Cuisset (1994). Muscle samples were homogenised and steroids extracted with Dichloromethane and then measured using ELISA techniques (Susca et al., 2000; 2001). An automatic biopsy needle has been developed in our workshops that can be used at the market or on live fish via a harpoon attachment to obtain the muscle samples.

## **3. RESULTS**

In figure 1 some typical results are shown of the concentrations of steroid hormones in various swordfish tissues. Depending upon season and the sex maturation of the fish maximum levels are observed in the gonads compared with muscle and plasma. Figure 2 indicates similar data for BFT but these fish were pre or post spawning with much lower levels in the gonads. It is clear however that measurable amounts of steroid hormones can be determined in tissue samples.

Figures 3 and 4 indicate the  $\log ([T / 11\text{-KT}])$  plotted against testosterone concentration for BFT plasma and muscle respectively that can be used to assess the sex of a particular fish. (See previous paper by Susca et al). Various sex steroid ratios can be used to define both the maturity and the sex of a given fish.

To be sure that plasma and muscle levels are correlated steroid levels were measured in muscle and plasma from the same fish. Figure 5 shows these values for both male and female plasma and muscle samples. It is clear that there is a good correlation with seasonal changes in the Bluefin Tuna and these values can be correlated with morphological and biometrics data taken at the same time.

At the present time an automatic biopsy sampling needle (prototype available), which is capable of taking a 100-150 mg sample from live or dead fish, is undergoing field trials in the West Atlantic BFT fishery and in Mediterranean waters for BFT and Swordfish.

ELISA protocols are now available for all sex steroid hormones and for BFT vitellogenin. A swordfish vitellogenin ELISA will be ready by the end of this year.

## **4. CONCLUSIONS**

### **4.1. Sex Determination**

From a single muscle biopsy sex determination can be ascertained through three different routes:

1. Testosterone/Ketotestosterone Ratio; non species-specific ELISA possible for all species.
2. Presence or absence of Estradiol indicative of female or male; non species-specific ELISA possible for all species.
3. Presence or absence of Vitellogenin indicative of female or male fish; ELISA relatively species-specific but with some cross reactions between similar species. This is possible at the moment for BFT and for Swordfish.

### **4.2. Muscle Sample Stability and ELISA Sensitivity**

1. Steroids are stable at room temperature for several weeks therefore sampling at the market is not a problem.
2. Vitellogenin samples must be kept at low temperatures
3. ELISA sensitivity is high as seen from figure 6 and lies in the pg range for an assay.

The Mattanza provides basic data for the correlation between hormone levels in both plasma and muscle and the morphological maturation stage of the gonads. An ELISA mediated biopsy can determine both sex and sexual maturity even at the market, therefore this is very important for "Law enforcement" such as a closed season.

### **4.3. Future Problem Research Areas for “Molecular markers” Studies**

- 4.3. a. Sex determination in fish migration „tagging“ studies (At present being tested in Western Atlantic).
  - 1. Are migration patterns and behaviour sex specific?
  - 2. Are migration patterns and behaviour dependent on sexual maturity status?
- 4.3. b. Aquaculture Studies
  - 1. Determining sex and maturation status of captive fish?
- 4.3. c. Age and spawning history in wild fish
  - 1. How old and how often has a fish spawned?
- 4.3. d. Future work and solutions
  - 1. Use a non-lethal automatic muscle biopsy sampler.
  - 2. Develop molecular biology techniques using ELISA to detect low levels of markers.
  - 3. Search for molecular fingerprints from which age and spawning can be determined.

### **5. REFERENCES**

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## SWORDFISH

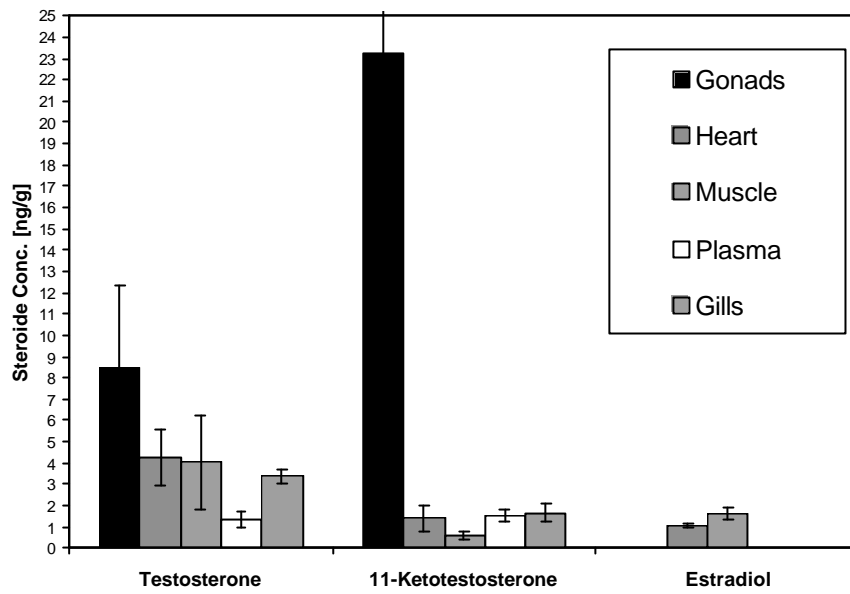


Figure 1. Steroid hormone concentrations in various tissues of *Xiphias gladius* measured with standard ELISA techniques

## BLUEFIN TUNA

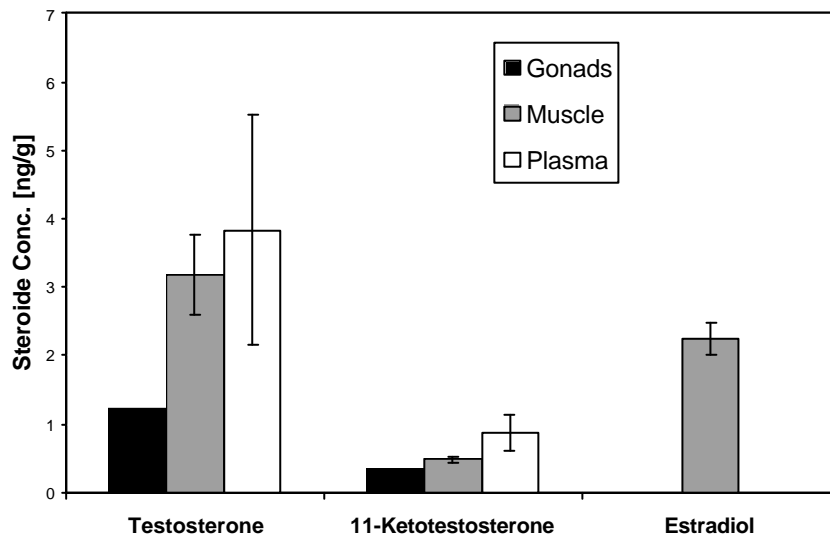


Figure 2. Steroid hormone concentrations in the various tissues of the Bluefin Tuna

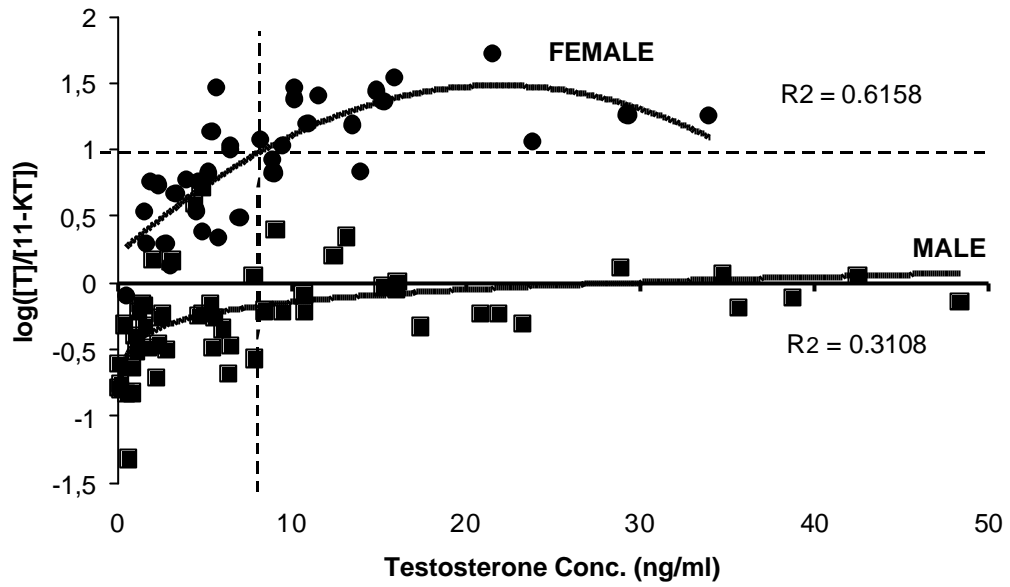


Figure 3 Testosterone/ 11-Ketotestosterone ratio for Bluefin Tuna Plasma (Taken from Susca et al, 2000)

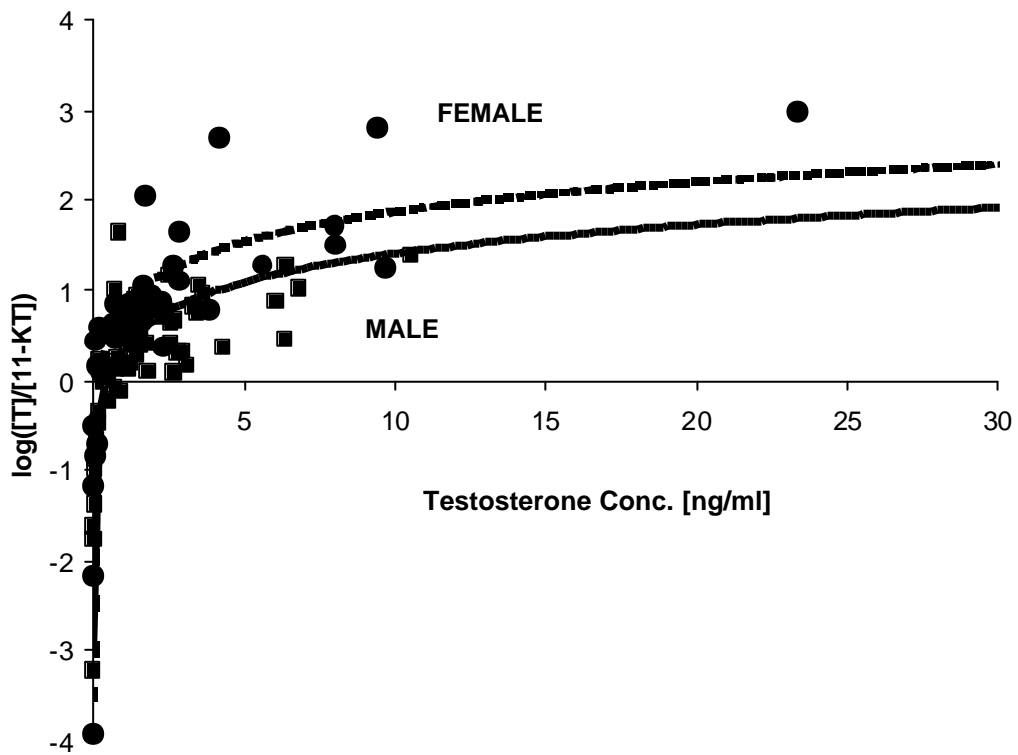


Figure 4. Testosterone / 11-Ketotestosterone ratio for Bluefin Tuna muscle

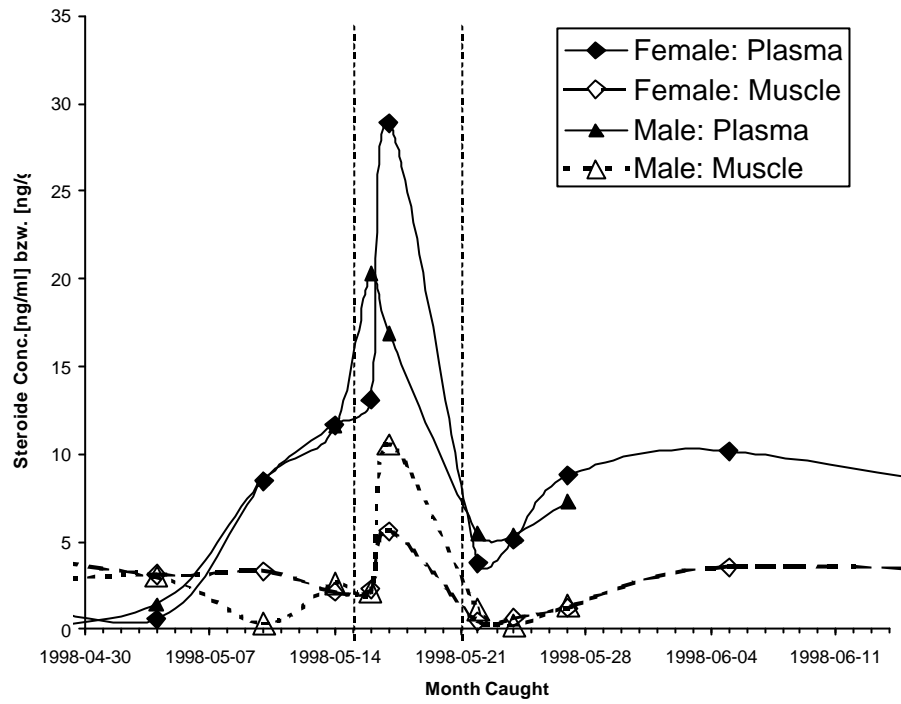


Figure 5. Relationship between plasma and muscle steroids for bluefin tuna from the Mediterranean.

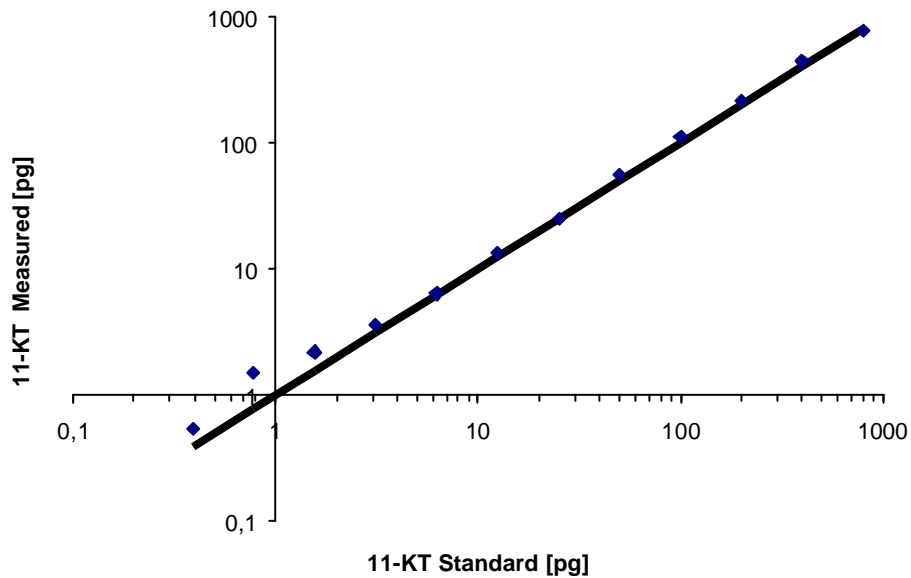


Figure 6. Standard regression line for a typical steroid hormone determinations.