

**JIMAR, PFRP ANNUAL PROGRESS REPORT
FY 2003**

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Project Proposal Title: Developing Methods to Assess Sex and Maturational Stage of Bigeye Tuna (*Thunnus obesus*) and Swordfish (*Xiphias gladius*).

Funding Agency: JIMAR

1. Purpose of the project and indicative results.

A comprehensive understanding of the reproductive biology and spawning activity of the tuna is necessary for sound management of this complex and important fishery. Current methods for assessing maturity schedules of tunas is based on the conventional approach of collecting gonads for detailed histological examination. While this method has proven to be accurate and provides valuable information, it is labor-intensive, expensive and lethal for the fish. In addition, this conventional approach is completely unsuitable to fishery-dependent assessments, since gonadal samples are often unavailable. The most challenging feature of the histological approach is that it is time consuming, thus making it impractical to evaluate the population annually. As a result, management models are static and fail to reflect 'real-time' effects of fishing pressure or environmental perturbations.

The central aim of this proposal is to develop accurate, simple to use and economical tests to determine sex and maturational stage of Bigeye Tuna and Swordfish so that populations can be monitored on a regular basis and at low cost. We proposed the following objectives to achieve this goal:

- i) Develop and modify existing biotechnology to identify the sex and maturational status of individual fish in two species: bigeye tuna (*Thunnus obesus*) and swordfish (*Xiphias gladius*). Our approach will focus on sex determination by gonadal steroids and maturation specific compounds present in blood and muscle tissue samples.
- ii) We will use yellowfin tuna (*Thunnus albacares*) as a model. We will develop highly sensitive radioimmunoassays to identify sex-specific hormones and proteins present in blood and muscle samples. Antisera and specific immunoassays will be created for tuna vitellogenin, the egg-yolk precursor specific to maturing female fish.
- iii) Validate the accuracy of this approach by 'ground truthing' the results with the standard method of staging maturity based on histological examination of the gonads. We will also try to identify sex-determining gene in the tuna.
- iv) Transfer the new technologies to fishery biologists throughout the Pacific for use in constructing and monitoring maturity schedules for bigeye tuna and swordfish.

2. Progress during FY 2002. Provide a thorough discussion of accomplishments and problems.

Field Collections

A major problem encountered during the initial part of FY 2002, and as a carryover from previous years, was obtaining high quality blood serum samples to use in validating and establishing the radioimmunoassay for this project. Our initial attempt to use blood collected from fish previously caught by fishermen and sampled at the pier produced disappointing results. We discovered that high quality serum could only be obtained from fresh animals. Fresh blood can only be obtained from fish sampled immediately after being caught. This eliminated the possibility of obtaining samples from fishermen and forced us to put more time, energy and money into collecting our own fresh fish for sampling. A cooperative agreement was established with the Medical Foundation for the Study of the Environment (MFSE) and, with the assistance of JIMAR funds, several fishing expeditions were successfully conducted in FY 2001.

Plasma and tissue levels of estradiol

As mentioned above, the current methods for assessing sexual maturity in tunas is reliant on collecting gonads for detailed histological examination. This method is accurate but is lethal to the fish. We undertook studies in FY 2002 to determine sexual maturity by measuring levels of estradiol in the plasma and muscle of tunas. During the previous fiscal year we analyzed blood and muscle tissue for plasma. As reported in FY 2002 progress report we found significant amounts of E2 in the plasma and muscle in both female and male Bigeye Tuna, Swordfish and Yellowfin Tuna.

Our observation that male tunas have significant levels of E2 prompted us to look at another species, the tilapia, to ascertain if males in other species of fish have significant amounts of E2 in the plasma.

Plasma concentrations of E2 in male tilapia were determined by RIA using a commercially available kit with modifications (Immuchem Double Antibody Direct Estradiol RIA kit; ICN Biochemicals). The validity of the assay was assessed by demonstrating that parallel displacement curves are generated with serial dilutions of plasma samples and also with stripped plasma. In the preliminary experiment, dilution of neat plasma from the male tilapia was not parallel with E₂ standards. The plasma was then extracted with ether, following the procedure established for the angelfish plasma (Collier et al., 2003). Briefly, 2 ml of ethyl ether (Anhydrous, Fisher Scientific, Pittsburgh, PA) was added to plasma sample. The tubes were vortexed and frozen at -80°C for 10 min, and the aqueous organic layer was decanted into a new glass tube. The ether extract was evaporated to dryness in a water bath at 40°C for 1 hour, and then placed under nitrogen for 5 min to ensure complete evaporation. Extracts were then reconstituted with 50 µl assay buffer. As shown in Figure 1, serial dilution of the ether-extracted plasma was parallel with estradiol standard indicating the presence of estradiol in the male fish.

In order to confirm further the presence of estradiol in the male fish, estradiol was extracted from 200 µl of plasma from the male tilapia with Sep-Pak C-18 Light cartridge (Waters, Milford, MA). In a preliminary experiment, we have confirmed that ³H-estradiol was eluted in fractions between 25-35% acetonitrile through Sep-Pack C18

cartridge (Figure 2). Briefly, the cartridge, preconditioned with 2 ml of 2-propanol and 2 ml of 0.1% aqueous trifluoroacetic acid (TFA), was loaded with the plasma sample. The cartridge was washed with 2 ml of 0.1% TFA, followed by 2 ml of 25% acetonitrile in 0.1%TFA. Estradiol was eluted with 1.5 ml 40% acetonitrile in 0.1% TFA, and evaporated to dryness using Automatic SpeedVac (Savant, Athens, GA). The dried residue was reconstituted with 250 μ l of assay buffer. In the preliminary experiment, 10 μ Ci of 3 H-estradiol ([2, 4, 6, 7, 16, 17- 3 H] oestradiol, Amersham, Piscataway, NJ) in 10 μ l ethanol was diluted with 250 μ l assay buffer, and added to the cartridge. As shown in Figure 2, estradiol was eluted in fractions between 25% and 35% acetonitrile.

Finally, we demonstrated that acetonitrile extracted plasma was parallel with estradiol standard (Figure 1), further indicating the presence of estradiol in the male tilapia.

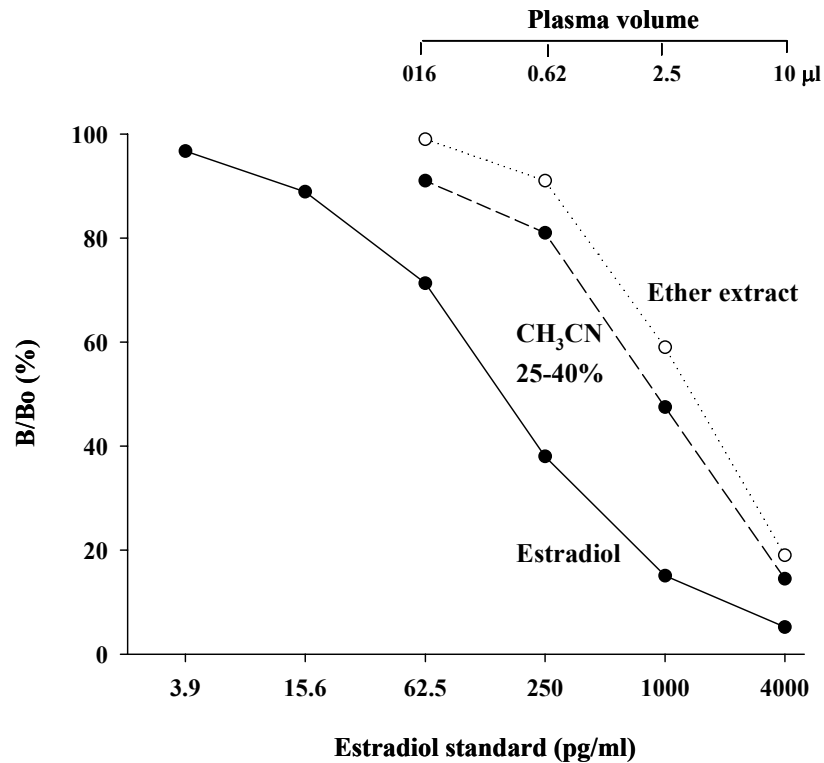


Figure 1. Displacement curves for estradiol and serial dilutions of plasma from the male tilapia. Diamonds represent estradiol-17 β standards. Open circles represent diluted plasma after ether extraction. Closed circles represent diluted plasma after acetonitrile extraction Sep-Pak C18 cartridge purification.

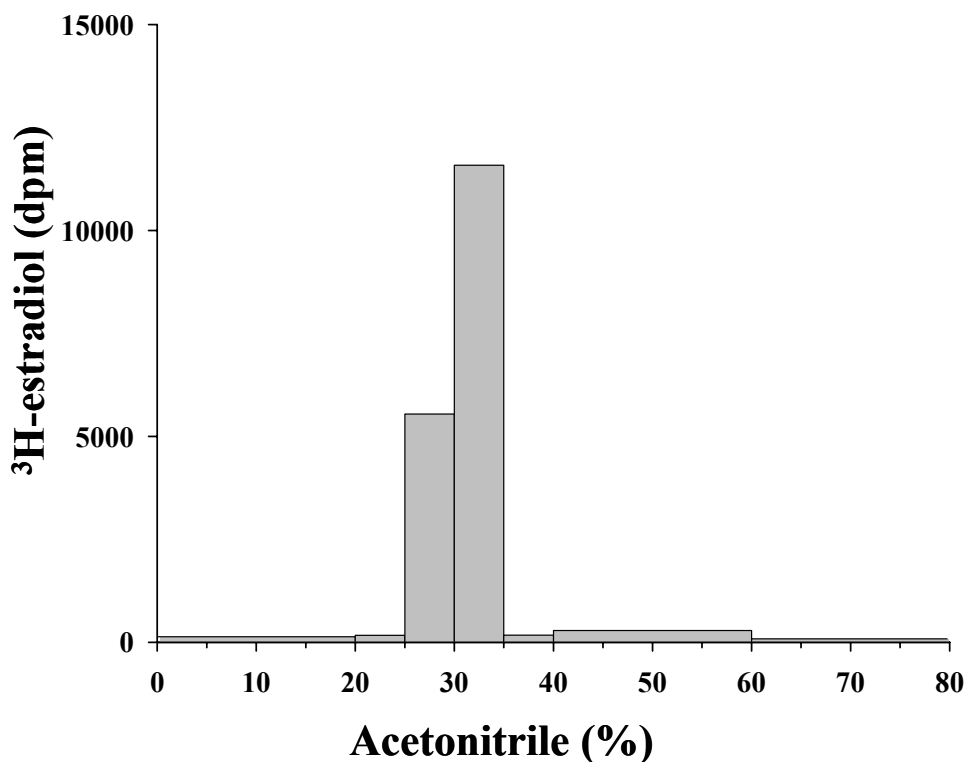


Figure 2. Elution profile of ³H-estradiol through Sep-Pak C18 cartridge. 10 μ Ci ³H-estradiol in 10 μ l ethanol was diluted with 250 μ l assay buffer and added to the cartridge.

Plasma Vitellogenin

One of the problems we encountered this pass year is the difficulty in inducing vitellogenin (VTG) production in tunas. Efforts are on going to experimentally induce VTG in juvenile tuna as a standard for the enzyme-linked immunosorbant assay (ELISA). The challenge lies in capturing and maintaining juvenile fish for the duration of the experimental treatment (5-7 days). We decided to use the tilapia as a model to establish the VTG ELISA in our laboratory. We recently established a homologous VTG ELISA using a “universal” antibody for fish vitellogenin. We have found that male tilapia contain low levels of VTG in the plasma (0.008 mg/ml). These levels however, are 400-500 times lower than that observed in females (3.6 mg/ml). Similar findings have been observed in other teleosts species. The reason why males appear to have detectable levels of plasma VTG has not been investigated.

Plans for the next fiscal year.

1) Sex Determination by Gonadal Steroids

Plasma concentrations of gonadal steroid hormones vary seasonally with sex and maturation in all teleost fish studied to date, and can serve as indicators for age at maturity and sex ratio in a population. As described above, we found significant amounts of E2 not only in the plasma and muscle of the female fish but also in the male plasma and muscle. Thus, it remains unclear whether E2 can be used to determine females. We

shall establish and optimized a radioimmunoassay for the measurement of plasma and muscle concentrations of 11-KT. We anticipate that concentrations of E2 and 11-KT in the plasma and muscle will be correlated with the sexual maturity, and that the sex could be determined by the ratio of E2/11-KT. Detailed histological examination of the gonads will also be conducted whenever gonadal samples are available to identify definitively the sex and state of maturation in individual fish.

2) *Maturity Assessment by Vitellogenin*

We will continue to pursue the establishment of the ELISA to measure plasma VTG levels in female tunas.

3) *Search for Sex-Determining Gene*

We will try to identify and characterize a sex-determining molecular marker that can distinguish the gender of a fish regardless of age and maturational stages. Our collaborator, Dr. Y. Nagahama of National Institute of Basic Biology has recently identified sex-determining gene (PG17) in the medaka (*Oryzias latipes*) for the first time in non-mammalian vertebrates. This sex determining gene does not appear to be present in other teleosts. Dr. Nagahama has been unable to locate this gene in the tilapia and in the zebrafish. Although it appears that the sex-determining gene is different from species to species, we shall try to identify PG17 in the tuna tissues, using a probe provided by Dr. Nagahama. If PG17 were also present on Y chromosome of the tuna, we should be able to identify only one copy of the gene by Southern blot analysis. If successful, this approach will allow us to assess rapidly the gender of a fish from a muscle biopsy, and create a new possibility that the gender of any teleost fish can be determined non lethally

4. List of papers published in refereed journals during FY 2003.

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5. Other papers, technical reports, meeting presentations, etc.

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6. Names of students graduating with MS or Ph.D. degrees during FY 2003. Include title of thesis or dissertation.

7. For multi-year projects, provide budget for the next year on a separate page.