

**JIMAR, PFRP ANNUAL PROGRESS REPORT  
FY 2002**

**P.I. Name:** E. Gordon Grau

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

**Funding Agency:** JIMAR

**Project Purpose 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 are 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 maturation 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 maturation 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 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 a 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.

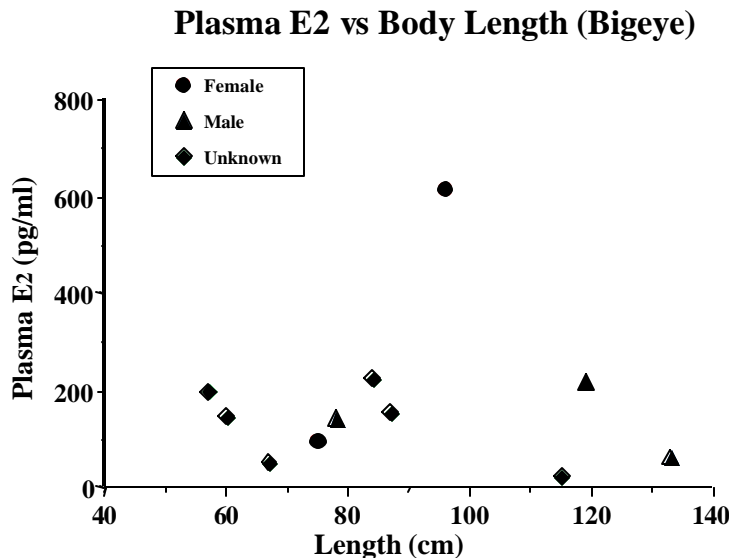
## Project Activities and Progress during FY 2002:

### 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. We have analyzed blood and tissues of 13 bigeye tuna, 19 swordfish and 17 yellowfin tuna.

### Plasma and tissue levels of estradiol and cortisol

In FY 2001, we have optimized an estradiol radioimmunoassay (E2-RIA) for all three species, bigeye tuna, yellowfin tuna and swordfish. Representative results of plasma levels of E2 for Bigeye tuna are shown below.

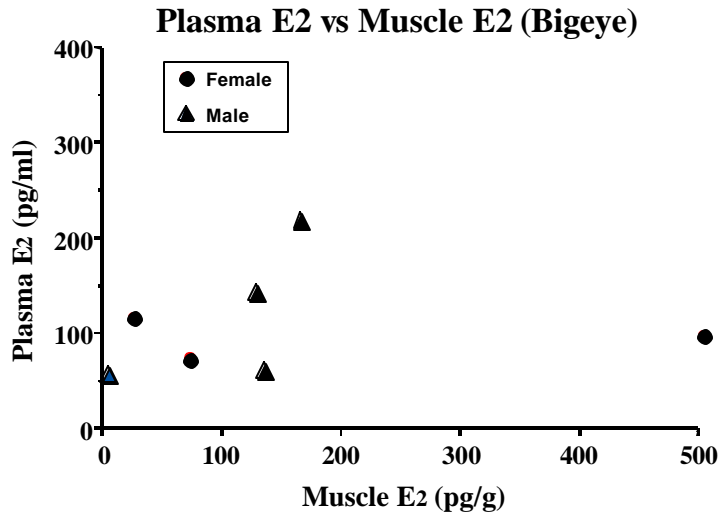


Levels of estradiol (picograms per ml of plasma) are plotted against the length (in centimeters) of each animal sampled. Females are represented as closed circles; males are represented as triangles and undetermined sex (juveniles) is represented as closed squares. Size at maturity for bigeye tuna is 110cm (D. Itano, personal communication). As is clear in the figure, significant amounts of E2 were found in plasma from both female and male fish, and there was no significant difference between the sexes.

Similar results were obtained with the plasma samples of swordtail and yellowfin tuna. It remains unclear, however, whether estradiol can be used to determine the sex of either

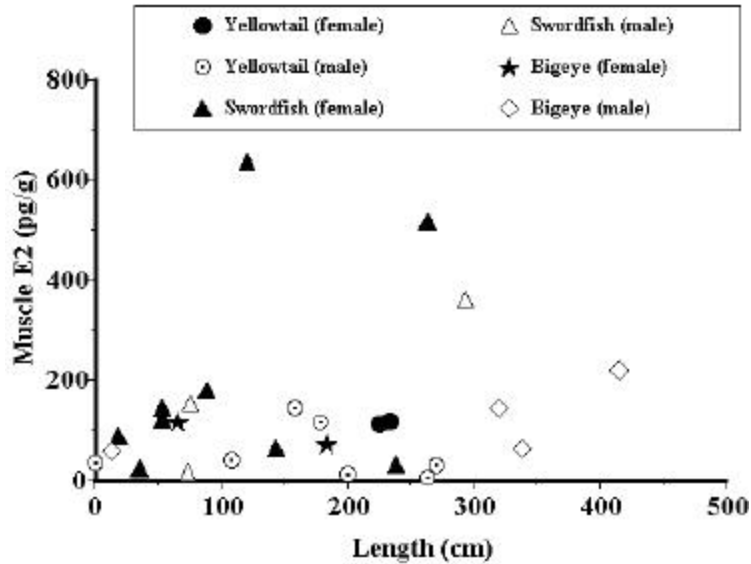
Bigeye Tuna or Yellowfin Tuna, since males express significant levels as compared to females. Additional samples of both mature male and female fish will need to be included before concluding the usefulness of estradiol in determining maturational stage of swordfish, bigeye or yellowfin tuna.

This year, we have established RIA for muscle concentrations of E2 using a commercially available kit with modification (Immuchem Double Antibody Direct Estradiol RIA Kit; ICN Biochemicals). Frozen muscle was thawed, and homogenized with 5 ml/g of 10 mM phosphate buffer (pH 7.4). Glass tubes (12 x 75 mm) were used, and to each were added 300 µl of the homogenate and 3 ml of ether. The contents of the tubes were vortexed and frozen at -80°C for 10 minutes, 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 minutes to ensure complete evaporation. Extracts were then reconstituted with 100 µl assay buffer, and 250 µl of <sup>125</sup>I estradiol and 250 µl of anti-estradiol (provided with kit) were added. After incubation at 37°C for 90 minutes, 250 µl of precipitant solution (provided with the kit) was added. After centrifugation at 2000 x g for 20 minutes at 4°C, the tubes were aspirated and counted in a gamma counter (Cobra II, Packard, Meriden, CT). The validity of the assay was assessed from the parallel displacement curves obtained with serial dilutions of muscle samples. Cross-reactivity was unaffected by heat deactivation, whereas no cross-reaction was seen in the stripped plasma.

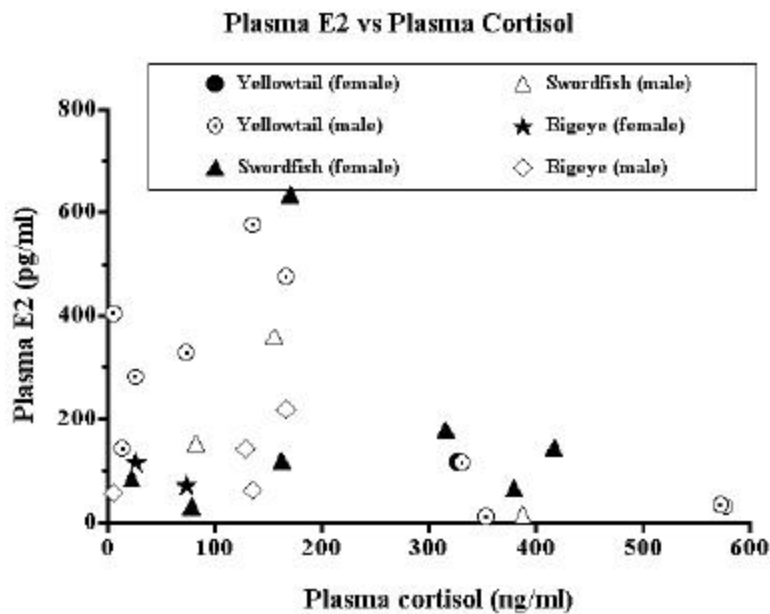


Representative results of plasma levels of E2 for Bigeye tuna are shown above. Significant amounts of E2 were detected in both females and males, and there was no difference between the sexes.

As shown in the following figure, E2 was detected in the muscle of all three species, regardless of their sex. Although 2 female swordfish exhibited high levels of muscle E2, there was no significant correlation between muscle E2 and body size.



We have also established RIA for the measurement of plasma levels of cortisol, a hormone released from interrenal gland, equivalent to adrenal in higher vertebrates, in response to stress. Normal range of plasma cortisol varies from species to species. For example, plasma cortisol levels of tilapia are around 50 ng/ml in unstressed fish, and increase to 200-300 ng when stressed. As shown in the following figure, plasma cortisol levels of all three species of tuna were surprisingly low, considering the fact that the blood samples were obtained from the fish caught by a long line. At any rate, there was no significant correlation between plasma levels of E2 and cortisol in any of the species.



## **Planned Project Activities for FY 2003:**

### *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 optimize 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 have recently established enzyme-linked immunosorbent assay (ELISA) for tilapia vitellogenin. We have obtained “universal” antibody for fish vitellogenin from our collaborator, Dr. N. Denslow of University of Florida. Dr. A. Takemura of University of the Ryukyus offered us the vitellogenin from the greater amberjack. Efforts are ongoing to experimentally induce vitellogenin in juvenile yellowfin tuna, swordfish or amberjack as a standard for ELISA. The challenge lies in capturing and maintaining juvenile fish for the duration of the experimental treatment (5-7 days). A pitfall of this approach is that vitellogenin may be detected in blood plasma and muscle of the male fish, and may not be suitable for sex determination, because small but significant amounts of E2 were found also in males. Nevertheless, this could still be a useful tool to identify maturing females. To confirm this possibility, we are in great need to obtain the yellowfin tuna during the breeding season from April through September/October in Hawaiian waters.

### *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. Dr. Y. Nagahama of National Institute of Basic Biology has recently identified a sex-determining gene (PG17) in the medaka (*Oryzias latipes*) for the first time in non-mammalian vertebrates. PG17 has DM domain, which is in common with sex-determining gene of *Drosophilla* (Double sex) and *C. elegans* (MB3), and is localized only on the Y chromosome (Nagahama *et al.*, unpublished observation). Although it is possible 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 the 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 nonlethally.

**Papers Published in Journals During FY 2002: None.**

**Other Papers, Reports, and Presentations During FY 2002: None.**

**Graduating Students with MS or Ph.D. Degrees During FY 2002:**

*Thomas A Leedom (Department of Animal Sciences, University of Hawaii at Manoa). Title of MS thesis, "Effect of blood withdrawal and angiotensin II on prolactin release in the tilapia, Oreochromis mossambicus".*