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**NOTE**

# ENZYME-LINKED IMMUNOSORBENT ASSAYS AND BILLFISH GENDER: TESTING MUSCLE TISSUE AND SURFACE MUCUS IN TAGGING STUDIES

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Recent advances in enzyme-linked immunosorbent assay (ELISA) technology (Crowther, 2001) have facilitated studies of the gender factors of billfishes (Tan et al., 2006). In this paper, appropriate methodologies and ELISAs were investigated for non-lethal sex identification in billfish tagging studies, where gender is typically unknown (Wilson et al., 1991). Tan et al. (2006) developed an ELISA to quantify vitellogenin-derived yolk proteins (VDYP) in muscle tissue biopsy samples from Atlantic blue marlin, *Makaira nigricans* Lacépède, 1802, and also used a commercially-available ELISA for 11-ketotestosterone (11-KT). Here, we show that the same assays can be applied to three other istiophorid billfish species: white marlin, *Tetrapturus albidus* Poey, 1860, longbill spearfish, *Tetrapturus pfluegeri* Robins and de Sylva, 1963, and sailfish, *Istiophorus platypterus* (Shaw in Shaw and Nodder, 1792). Samples from two different groups of sailfish were tested by ELISA: the first with extracts of muscle tissue, the second, with surface mucus. Use of surface mucus represents a less invasive and more practical alternative than either muscle biopsy or blood serum collection (Gordon et al., 1984; Kishida et al., 1992; Moncaut et al., 2003; Schultz et al., in press), especially when dealing with large, live animals at boatside (Graves et al., 2002).

## Materials and Methods

Billfish muscle tissue was collected from 9 blue marlin, 20 white marlin, 25 longbill spearfish, and 17 sailfish. The fish were captured at various stages of maturity by longline, purse seine, and rod-and-reel from several locations in the western North Atlantic. The gender of all 71 individuals was validated at dockside via visual examination of gonadal tissue, and when necessary, with the aid of a dissecting microscope. Forty-three were males and 28 were females. Also, surface mucus was collected with polyurethane sponges from 15 additional sailfish that received satellite tags and were subsequently released in waters off the Florida Keys, U.S.A. Their surface mucus was used to quantify gender factors following the methods of Schultz et al. (2005).

Muscle tissue and surface mucus from the above fish was extracted as described by Tan et al. (2006) and Schultz et al. (2005), respectively, and assayed by ELISA for VDYP and 11-KT. The direct sandwich ELISA (Bayer and Wilchek, 1992; Crowther, 2001) for blue marlin VDYP was carried out as described by Tan et al. (2006). The values of the VDYP were reported as ng VDYP/mg total soluble protein (TSP). The assay for 11-KT was an enzyme immunoassay EIA kit (Cayman Chemical Co., Ann Arbor, MI) following instructions of the manufacturer as modified by Schultz et al. (2005); values were reported as pg 11-KT/mg TSP.

## Results and Discussion


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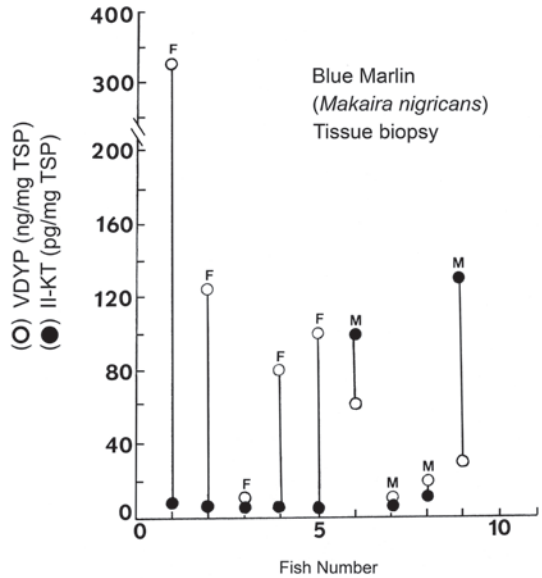


Figure 1. Quantitation of VDYP and 11-KT by ELISA in samples of muscle tissue from five female and four male Atlantic blue marlin captured by longline and collected from various sites in the western North Atlantic Ocean in the summer months, 2002–2003. The sex of fish 3, 7, and 8 was known from visual inspection at the time of collection because gender identification would not be possible based only on the immunoassays. The three fish would be classified as sexually quiescent/immature males or females. F, female, M, male, TSP, total soluble protein.

Results from ELISAs of VDYP and 11-KT from individual tissue extracts from five adult female and four adult male Atlantic blue marlin that were captured during the reproductive season (May–September, 2002–2003) revealed four sexually mature females (Fish 1, 2, 4, and 5), two sexually mature males (Fish 6, 9), and three sexually quiescent or immature fishes (Fish 3, 7, 8). The data from immunoassays of VDYP and 11-KT of blue marlin muscle tissue extracts from 27 additional fish in Tan et al. (2006) produced similar results (Fig. 1). Quantification of the two gender factors in muscle tissue extracts from 20 white marlin demonstrated that the ELISAs for Atlantic blue marlin muscle tissue extracts were also feasible for sexing this species, revealing nine sexually-mature females, one sexually quiescent/immature female (Fish 7), and 10 sexually quiescent/ immature males (Fig. 2). Of the 18 longbill spearfish quantified for the two gender factors in muscle tissue extracts, four sexually mature fish were evident: Two fish had high values of gender factors (Fish 1 and 4), two had lower values (Fish 2 and 3), and nine males were sexually quiescent/immature (Fig. 3). Quantifying extracts of muscle tissue from 17 sailfish by ELISA and confirmation by gonad inspection indicated 12 males and five females. All males were inferred to be sexually quiescent/immature by the levels of 11-KT, and it would not have been possible to determine gender without gonad inspection. The five females had levels of VDYP ranging from 67 to 247 ng and 11-KT ranging from 2.6 to 8.4 pg.

A scatter plot (not shown) of ng VDYP/mg TSP on the x-axis and pg 11-KT/mg TSP on the y-axis was generated using all the gender factor data described above (i.e., 71 gender-validated individuals, species combined). While, on average, males had 2.2 times the quantity of 11-KT compared to females, no clear gender separation was

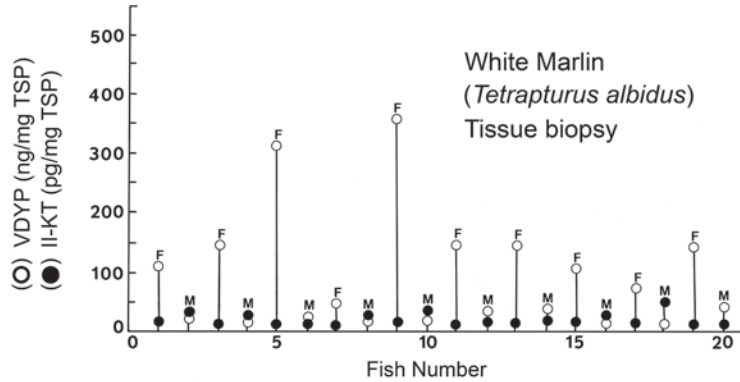


Figure 2. Quantitation of VDYP and 11-KT by ELISA in samples of muscle tissue from ten female and ten male white marlin captured by longline and collected from various sites in the western North Atlantic Ocean in the summer months, 2002–2003. The results demonstrate that the ELISA developed for blue marlin VDYP is cross-reactive with white marlin VDYP. All males were classified as sexually quiescent/immature, but nine of the fish were classified as females based on the assay results. Fish 7 was classified as a sexually quiescent/immature male or female. F, female, M, male.

evident from individual 11-KT values. However, the plot revealed that 80% of the females had levels ≥ 50 ng VDYP/mg TSP, and 90% of the males had levels < 50 ng VDYP/mg TSP. Based on the ≥ 50 ng VDYP/mg TSP criterion, surface mucus samples from 15 sailfish revealed 13 as females and the remainder (Fish 3, 15) as male, immature females, and/or sexually quiescent females (Fig. 4). We were fortunate that

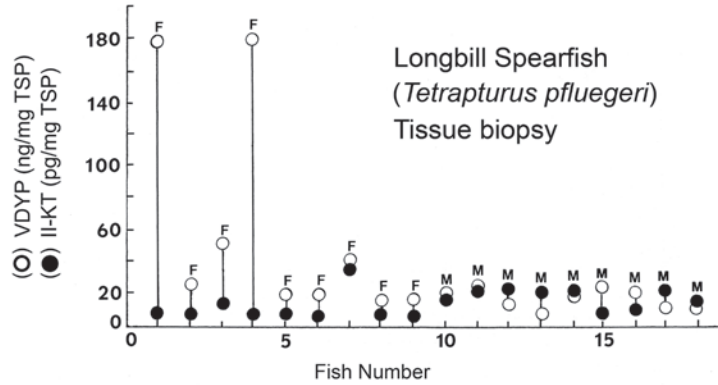


Figure 3. Quantitation of VDYP and 11-KT by ELISA in samples of muscle tissues from nine female and nine male longbill spearfish captured by longline and collected from various sites in the western North Atlantic Ocean in the summer months, 2002–2003. The results demonstrate that the ELISA developed for blue marlin VDYP is cross-reactive with longbill spearfish VDYP. Fish 1–4 were classified as females based on the assay results, and the remaining, female or male, were classified as sexually quiescent/immature.

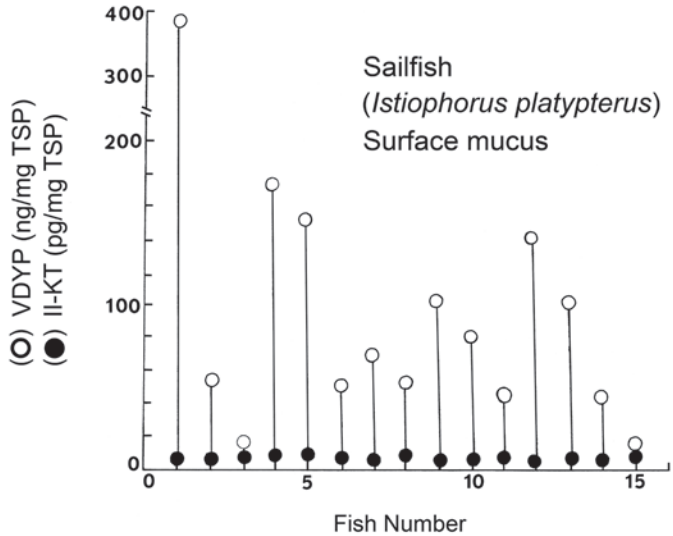


Figure 4. Quantitation of VDYP and 11-KT by ELISA in samples of surface mucus collected from 15 Atlantic sailfish during attachment of pop-off satellite tags. The fish were captured in waters of the western Atlantic Ocean between Key West and Key Biscayne, Florida. The results demonstrate that the ELISA developed for blue marlin VDYP is cross-reactive with sailfish VDYP. Based on the immunoassays, all fish were classified as female except fish 3 and 15, which were classified as sexually quiescent/immature males or females. Fish 5 was recaptured and its sex verified as a female (see text for details).

one of the satellite-tagged sailfish (Fish 5) was landed intact by a sport fisherman on December 15, 2005 and that we were able to examine this specimen directly. Gonad examination by one of us (D.S.) revealed the fish to be a female, as we would have predicted from its VDYP level (i.e., 155 ng VDYP/mg TSP).

Therefore, the ELISA for VDYP from individual billfish was helpful for separating genders of sexually mature animals for all four billfish species. The exceptions were immature or quiescent individuals, which cannot be sexed from the data of the immunoassays used here. We selected billfish captured during the spawning season (May–September) (de Sylva and Breder, 1997; Luthy, 2004) to maximize the probability of detecting sexually mature individuals, however, levels of 11-KT were not a dependable gender factor for distinguishing the sexes.

The detection of VDYP in males and 11-KT in females has been described in a number of other fishes (Sumpter, 1985; Copeland and Thomas, 1988; Ding et al., 1989; Goodwin et al., 1992; Schultz et al., 2005; Tan et al., 2006; Schultz et al., in press). Vitellogenin induction in male fish in some studies has been reported as evidence of endocrine disruption because of sewage effluent in streams and exposure to exogenous estrogens or estrogen mimics (Purdom, 1994; Heppell et al., 1995; Folmar, 1996). Because billfish have not been maintained in tanks, it has not been possible to investigate the effects of either natural or artificial regulation (e.g., water temperature, nutrition, estrogen mimics) on levels of gender factors (Devlin and Nagahama, 2002).

Because of the limitations of ELISAs used in this study, experiments have been initiated with Y chromosomal DNA markers and the polymerase chain reaction-

technology to probe the possibilities of using sex-linked DNA markers to determine genetic sex (Devlin and Nagahama, 2002). If successful, the gender of tagged billfishes could be determined at any time of the year, independent of the reproductive season, and presumably, would be effective on immature specimens. These types of experiments have been successfully carried out with chinook salmon [*Oncorhynchus tshawytscha* (Walbaum, 1792)] (Devlin et al., 1991) and medaka [*Oryzias latipes* (Temminck and Schlegel, 1846)] (Matsuda et al., 2002).

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