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Use of Morphology and Endocrinology to Predict Sex in California Sheephead: Evidence of Altered Timing of Sex Change at Santa Catalina Island, California

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Abstract

To estimate the health of the California sheephead *Semicossyphus pulcher* fishery, a 2004 stock assessment used available biological data that were collected decades prior to an increase in fishing pressure. However, a recent study has found that sex ratios, growth rates, survivorship, and average sizes of females and males have changed in response to size-selective fishing in some California sheephead populations. To better understand the potential changes in protogynous California sheephead, this study sought to determine (1) whether external morphology was still an accurate method of predicting sex in sexually dimorphic California sheephead at Santa Catalina Island, California, and (2) whether nonlethal blood sampling and plasma hormone analysis could be used to predict sex for future stock assessments. Sex was determined using gonadal histology and compared with several specific external morphological characters. Estradiol and 11-ketotestosterone plasma concentrations were also compared across sexed individuals. The rate of error when using external morphology alone to predict sex was 58%. In contrast, sex steroid concentrations varied significantly across sexes; estradiol concentrations were significantly greater in females than in transitioning and male individuals during the breeding season, and 11-ketotestosterone concentrations were significantly lower among females. Gonadal histology showed that 21% of the fish caught during the breeding season were classified as transitional, in stark contrast to historical data. The inability to accurately predict sex using external morphology alone suggests that commonly used methods of surveying California sheephead populations (e.g., diver surveys) may be inaccurate. Nonlethal blood sampling and subsequent plasma hormone analysis may offer an alternative method for assessing sex in California sheephead. Because California sheephead are not reproductively functional during transition and because we found such a large proportion in transition during the breeding season at Santa Catalina Island, we believe there is a need for continued assessment of the reproductive potential in this population of California sheephead.

In response to concerns for the sustainability of the California sheephead *Semicossyphus pulcher* fishery in the 1990s, a statewide stock assessment was completed in 2004 (Alonzo et al. 2004). The stock assessment used biological characteristics from Warner's (1975) and

Cowen's (1990) previous studies that were conducted prior to the commercial fishery boom in the 1990s. However, because increased fishing pressure has the potential to alter life history characteristics, it is critical to examine possible effects of this pressure to accurately determine the status and sustainability of the stock. Indeed, a recent study suggests that at Santa Catalina and San Nicolas islands, California (the study locations used by Warner 1975 and Cowen 1990), size-selective fishing pressure has resulted in altered sex ratios and reduced growth rates, survivorship, and average size of females and males compared with historical studies (Hamilton et al. 2007). Other important aspects of California sheephead biology, such as predictability of sex based on morphological characteristics and timing of sexual transition, have not yet been compared with Warner's (1975) historical findings and are also likely to have been altered. Understanding how these biological characteristics of California sheephead have changed since the studies by Warner (1975) and Cowen (1990) will allow for better estimations of sustainability and may provide greater insight into the impact of fishing on California sheephead.

California sheephead are protogynous hermaphrodites that are sexually dimorphic in both color and in body shape. During sex change, female ovaries regress and male testicular tissue develops (Warner 1975; Sundberg et al. 2009). As part of a comprehensive study of California sheephead biology, Warner (1975) collected 20–30 individuals/month for more than 1 year by using hand spears. He predicted the sex of individual California sheephead based on morphology, and he used histological analysis to determine the gonadal state of individuals. Warner (1975) described morphological criteria to externally identify California sheephead by sex (female, male, or transitional) at Santa Catalina Island. He found that sex was predictable based on external morphology with a low rate of error; only 3.5% of individuals that appeared female had male gonads, and it was proposed that those individuals had recently transitioned from female to male (Warner 1975). Females and males were present during the summer breeding season, and females, males, and transitional individuals were identified during the nonbreeding season (Warner 1975). Based on these observations, fisheries biologists and commercial and sport fishers have used morphology to identify the sex of California sheephead (Cowen 1990; Adreani et al. 2004; Topping et al. 2006; Hamilton et al. 2007). However, the idea that California sheephead can be accurately sexed using external morphology has not been confirmed in the current populations with potentially altered population structures as a result of increased fishing pressure.

The objective of this study was to determine (1) whether California sheephead sex could be accurately predicted based on external morphology in the current population at Santa Catalina Island and (2) whether other nonlethal methods of sexing, such as analyzing blood plasma concentrations of sex steroid hormones, could be employed during the breeding season. We hypothesized that the ability to accurately predict sex of California sheephead based on external morphology during the breeding season has decreased in this exploited population and that sex steroid plasma concentrations may prove to be a more accurate predictor of sex.

Methods

Fish and study site

All California sheephead were collected by hook and line or by trap on the north side of Santa Catalina Island within 2.5 km of Two Harbors, California (33°26'41"N, 118°29'07"W; $n = 108$). Breeding and nonbreeding season samples were collected between 17 September 2004 and 28 October 2005, and additional breeding season samples were taken in the summer of 2006 between 26 June and 1 September. After capture, swim bladders were vented using a hypodermic needle in individuals suffering from the effects of barotrauma. Two trained observers described each individual as female, transitional, or male based on their external morphology according to criteria from Warner (1975). Within 5 h of capture, live fish were transported to a laboratory facility, where blood samples were taken via the caudal vein (Sundberg et al. 2009). Blood samples were centrifuged at 5,000 revolutions/min for 5 min in heparinized tubes, and plasma was separated and stored at -80°C . Digital images were taken of each individual, with a ruler placed beside the fish for scale in morphological analyses. Portions of both lobes of the bilobate gonads were removed and placed in 10% neutral buffered formalin for 10 d before transferring the samples to 70% ethanol, where they were stored prior to histological analysis.

Gonadal histology

Gonads were removed from ethanol, washed in a phosphate-buffered saline solution, and dehydrated in a series of ethanol washes. Small pieces from throughout the gonad were then removed, processed, and embedded in paraffin wax. To confirm sex, embedded gonads were cut into 6- μm -thick sections and mounted onto Superfrost-Plus microscope slides (Fisher Scientific, Pittsburgh, Pennsylvania). Tissues were rehydrated through a series of xylene and ethanol washes and then stained using hematoxylin and eosin. Gonadal tissues were identified and characterized as female, transitional, or male by trained observers based on previous work (Warner 1975; Sundberg et al. 2009). As a conservative measure, late-stage transitional individuals (stage 7; Sundberg et al. 2009) were grouped with males.

Morphometric analysis

Morphometric measurements were taken using Scion Image software (Scion Corporation, Frederick, Maryland) by two trained independent observers. A series of measurements—standard length, body depth, forehead depth, forehead length, head length, and head area (Figure 1)—was taken from digital images of each individual. Each image was calibrated using the ruler included in the image. The two observers practiced both together and independently before actually taking the measurements. Measurements from the observers were averaged and compared across sexes to determine the reliability of morphometric measurements for predicting sex.

Hormone analysis

Plasma estradiol concentrations were measured using DSL-4800 Ultra-Sensitive Estradiol double-antibody ^{125}I radioimmunoassay kits (Diagnostic Systems Laboratories, Inc., Webster, Texas) from all fish with sufficient plasma samples (total $n = 97$; females: $n = 34$;

males: $n = 31$; transitional fish: $n = 32$). A majority of the blood plasma hormone data used in this study came from fish that were previously analyzed during a complementary study in our laboratory (Sundberg et al. 2009). Samples were assayed in duplicate, and radioactivity was measured using a Perkin-Cobra II gamma counter (Packard Instruments Co., Boston, Massachusetts). SigmaPlot version 8.0 software (SPSS, Inc., Chicago, Illinois) was used to generate a standard curve in the four-parameter logistic curve function and thereby determine hormone concentrations. Assay standards and controls were within the normal limits (Schmidt and Kelley 2001; Moffatt-Blue et al. 2006), with a 2.2-pg/mL lower limit of detection and low (0.64–2.40%) cross-reactions to other steroids.

Plasma 11-ketotestosterone concentrations were measured from all fish with sufficient plasma samples (total $n = 62$; females: $n = 13$; males: $n = 26$; transitional fish: $n = 23$) using competitive enzyme immunoassay kits (ACE kits; Cayman Chemical Co., Ann Arbor, Michigan). Each sample was assayed in duplicate, and two dilutions of each sample with B/B_0 (the ratio of sample absorbance to that of a maximum binding control) values between 20% and 80% were averaged. Plates were read using a Powerwave XS Bio-Tek microplate spectrophotometer at 412 nm. Raw data (absorbances) were exported to a Microsoft Excel spreadsheet and analyzed using 2006 Cayman Chemical Enzyme Immunoassay Tools software. The average intra-assay variability was 8.3%, and the lower limit of detection was 5.3 pg/mL.

Analysis of hormone data

Plasma 17 β -estradiol concentrations were compared using a Friedman's two-way nonparametric test with sexual type and season as factors. A nonparametric test was necessary because the assumptions of a parametric analysis of variance (ANOVA) could not be met. In addition, Kruskal–Wallis tests were used post hoc to compare estradiol concentrations between sexes and within seasons (breeding and nonbreeding), and a Mann–Whitney test was used to compare estradiol concentrations in females between breeding and nonbreeding seasons.

Concentrations of 11-ketotestosterone were compared among sexes and seasons using a two-way ANOVA. Nonnormal data were log transformed if possible. Bonferroni post hoc tests were used after ANOVA to identify significant differences among groups.

Results

Length Frequency Distribution

California sheephead ($n = 108$) ranged in size from 120 to 420 mm standard length (Figure 2). Immature females ranged in size from 188 and 270 mm. Mature females were between 207 and 329 mm. The fish underwent transition between 210 and 385 mm. A single mature male was 126 mm, while the other males ranged in size from 219 to 420 mm. All California sheephead collected in this study were within the same size range as those measured by Warner (1975).

Sex Prediction Based on External Morphology

Initial predictions of sex based solely on external morphology were compared with functional sex determined using histological analysis of the gonads for each individual. Females (mature and immature) were the most accurately identifiable based on external morphology; of the 48 individuals with female gonads, 39 were accurately identified as female in the field. The other nine individuals with apparent female morphology had transitional gonads (Figure 3). Histological analysis of gonads determined that 34 of the fish were undergoing transition at the time of capture; however, only 11 could accurately be identified as transitional based on morphology. Six of the transitioning individuals had female morphology, and the other 17 transitioning fish had male morphology. Twenty-six individuals were confirmed to have male gonads, but only 10 of these fish were correctly identified as males based on morphology. Instead, 13 confirmed male fish were erroneously identified as transitional based on external morphology, and three of the males were incorrectly identified as females. Overall, the error rate for predicting sex based on external morphology was 58%; however, when only breeding season data were analyzed, the error rate decreased to 38% (Figure 3).

Timing of Transition

A total of 39 California sheephead were captured during the peak months of the summer breeding season (July–September). Of those, 21% (8 of 39 fish) were found to be in sexual transition based on histological analysis (Figure 4). During the nonbreeding season (October–June), the percentage of individuals undergoing transition increased to 35% (24 of 69 fish).

Sex Determination from Morphometric Measurements

Morphometric measurements (body depth, forehead depth, forehead length, head length, and head area; Figure 1) were plotted against standard length and compared across confirmed sexual types to determine whether any of these measurements could be used to accurately determine the sex of individuals (Figure 5). No significant changes in allometry of head morphology relative to body size or sex were identified using any of the morphometric measures, including those indicating the development of the nuchal hump (Figure 5).

Plasma Hormone Concentrations

There was a significant difference in estradiol concentrations among sexual types (female, transitional, and male; $F = 83.21$, $df = 2$, 95 , $P < 0.0001$; Figure 6) and between seasons (breeding and nonbreeding; $F = 5.55$, $df = 1$, 95 , $P = 0.0053$). Females had higher concentrations of estradiol than did transitional and male individuals during both the breeding season (Kruskal–Wallis test: $H = 12.72$, $df = 2$, $P = 0.002$) and the nonbreeding season ($H = 10.88$, $df = 2$, $P = 0.004$), and estradiol concentrations were significantly greater in females during the breeding season than during the nonbreeding season (Mann–Whitney test: $W = 275$, $P = 0.0045$). There was no difference in estradiol concentrations between males and transitional individuals during the breeding season (Mann–Whitney test: $W = 41$, $P = 0.20$) or the nonbreeding season ($W = 634.5$, $P = 0.90$).

There was a significant difference in 11-ketotestosterone concentrations among sexual types (female, transitional, and male; $F = 66.02$, $df = 2, 56$, $P < 0.0001$) but not between seasons (breeding and nonbreeding; $F = 2.31$, $df = 1, 56$, $P = 0.13$; Figure 7). During the breeding and nonbreeding seasons, females had significantly lower concentrations of 11-ketotestosterone than did transitional individuals (Bonferroni post hoc test, breeding: $t = 6.73$, $P < 0.001$; nonbreeding: $t = 7.42$, $P < 0.001$) and males (Bonferroni post hoc test, breeding: $t = 6.65$, $P < 0.001$; nonbreeding: $t = 7.61$, $P < 0.001$).

Discussion

Our data show that the probability of misidentifying individual California sheephead as male and female from Santa Catalina Island has increased since the study conducted by Warner (1975). This is driven primarily by the increased rate of sexual transition in the population during the summer breeding season because individuals undergoing transition are the most morphologically ambiguous. Warner's (1975) study found that only 3.5% of fish with the color pattern of females and immature individuals ("uniform color") actually had male gonads as determined by histology. He also found that California sheephead that had male morphology ("bicolored") were exclusively male or late transitional. In contrast, we found that 12.5% of the individuals we collected that appeared to be female were actually undergoing transition and 6% had male gonads. This increased error in predicting sex of California sheephead at Santa Catalina Island shows that morphology is a poor indicator of sex in this population, and this should be considered in future studies that rely on sex prediction in this species.

Although we were unable to derive any morphometric measure that could be used to quickly discern sex and although overall external morphology proved to be an unreliable indicator of reproductive function in California sheephead at Santa Catalina Island, our data suggest that breeding season hormone concentrations can instead be used to predict California sheephead sex. Plasma estradiol concentrations were elevated only in mature females (those with oocytes containing yolk globules; Sundberg et al. 2009) during the breeding season, while all other sexes had no measurable levels of estradiol (Figure 6). There was also a significant difference in the concentration of 11-ketotestosterone between females and males (Figure 7), and although there was a high degree of variability, estradiol and 11-ketotestosterone assays together may provide a nonlethal method for distinguishing reproductively active females from males, especially in populations that lack transitional fish during the summer breeding season. While plasma estradiol and 11-ketotestosterone in California sheephead may be used as a nonlethal method for identifying functional females during the breeding season, no studies to date have examined this indicator of gonadal function outside of the Santa Catalina Island population.

Histological assessment of gonad form and function indicates a shift in the timing of sex change. Interestingly, while Warner's (1975) study at Santa Catalina Island found no individuals transitioning during the summer breeding season, our study found that 21% of fish (8 of 39) sampled during the peak months of the summer breeding season (July–September) were transitioning (Figure 3). Although Warner's (1975) study implemented different field sampling methods (hand spear versus trap or hook and line), it is unlikely that

our different sampling methods (hook and line and trap) could have targeted any one sexual type, especially since the size range of our samples fell within the size range sampled by Warner (1975; Figure 2). Although most individual California sheephead undergoing sexual transition in this study were collected during the nonbreeding season (30 of 39), the high proportion of transitioning individuals in the summer breeding season catch implies that sex ratios and the timing of transition have shifted at Santa Catalina Island. Prior to the collection and analysis of the present data and because of Warner's (1975) findings, it was believed that California sheephead did not change sex during the breeding season (Alonzo et al. 2004). The small size of transitioning gonads and the absence of hydrated or vitellogenic oocytes or fully developed sperm crypts suggest that there is a period of reproductive inactivity during sex change (Sundberg et al. 2009). It is reproductively inefficient for individuals of a species that must endure a period of reproductive inactivity due to sex transition to do so during the breeding season. Therefore, transitioning during the summer breeding season could negatively affect the reproductive potential of the California sheephead population.

The ramifications of transitioning during the breeding season have not been fully elucidated in California sheephead, and a more complete survey of the timing of transition in California sheephead populations across the species' range is required. Although the time required for sexual transition in California sheephead is not known, other sex-changing teleosts reportedly take from 2–3 weeks to several months to complete the transition (Robertson 1972; Sadovy and Shapiro 1987; Mackie 2003, McBride and Johnson 2006). Presently, it is not clear whether females that transition during the summer begin transitioning (1) when they have a fully developed ovary or (2) before the breeding season and become stalled in transition. If the transition occurs during the summer in females that have fully developed ovaries, then a serious energetic cost is likely to be involved in transitioning since developed female gonads are large and the extent of ovarian regression and testis development would be great. If the increased proportion of transitioning fish during the summer breeding season is a widespread phenomenon that is occurring in other populations at the same rate as in the Santa Catalina Island population, then the effects could be detrimental to California sheephead populations and may imply that the 2004 stock assessment overestimated the reproductive potential of the stock. Identification of functional sex based on plasma hormone concentrations, gonadal histology assessment, or both is needed in populations across the California sheephead's range to determine accurate and current sex ratios for optimal management of this species. If sex cannot be accurately determined by visual observation in California sheephead populations outside of Santa Catalina Island, then there is no reliable unobtrusive method for determining the most basic of reproductive characteristics (e.g., sex ratios, size at sex change, and transition rate) that are currently used.

A previous study found that size-selective fishing may be responsible for the removal of large male California sheephead at Santa Catalina Island (Hamilton et al. 2007). In addition, Hamilton et al. (2007) described the plasticity of California sheephead and found that populations subjected to increased fishing pressure showed decreased survivability. Females also matured earlier and changed sex earlier and at smaller sizes. The removal of large males at Santa Catalina Island could put pressure on small California sheephead to transition

earlier and could result in sex change occurring at younger ages and smaller sizes on average. Hamilton et al. (2007) noted the negative effects on the reproductive potential of the population; however, our study suggests that the effects may be worse than imagined if fishing pressure is also driving a shift in the timing of sex change. Such a shift in timing suggests that individuals are experiencing environmental or social feedback that causes them to forego reproductive activity during the breeding season, at least for some period of time. It also implies that the reproductive potential of an individual California sheephead must be greater when that individual is a male than when the individual is a female, as predicted by the size advantage hypothesis (Ghiselin 1969; Warner 1988; Cowen 1990), even though transitioning may involve an energetically expensive process and a period of reproductive inactivity.

Plasticity of life history characteristics in teleosts in response to altered environmental conditions and to fishing pressure is commonly observed (Law 2000; Grift et al. 2003; Munday et al. 2006). This plasticity may serve as a defense mechanism and may help California sheephead survive extreme changes from natural and human influences (Jennings et al. 1998); however, altered reproductive characteristics could have negative effects that result in reduced overall reproductive potential in certain populations. A greater understanding of the extent of those effects in site-specific California sheephead populations will help develop better stock assessment models and, in turn, will help fisheries managers to implement practical regulations for maintaining a sustainable California sheephead fishery.

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Figure 1.

Morphometric measurements obtained by using image analysis of California sheephead collected at Santa Catalina Island, California, between June and August 2005. Six measurements were taken and compared for each individual: (A) standard length, (B) body depth (at straight part of operculum), (C) forehead depth (measured from anterior part of preopercle behind the eye), (D) forehead length (upper jaw to first dorsal spine), (E) head length (perimeter of head from base of pelvic fin to first dorsal spine), and (F) head area (area within the perimeter of the head).

Figure 2. Length frequency histograms (standard length shown in 20-mm size-classes) of California sheephead sampled by Warner (1975) at Santa Catalina Island and those sampled in the present study at the same location.

Figure 3.

Matrix comparing sex identification of California sheephead based on initial examination of external morphology (e.g., coloration and head shape) with verification of sex using histological analysis of gonads (gonadal sex). Numbers in the upper left portion of each box represent summer samples only. Numbers in the lower right portion of each box represent the total number of individuals collected throughout the year. Total sample sizes for each sexual type are indicated across the bottom of the grid.

Figure 4. Percentages of immature, female, transitional, and male California sheephead caught during the nonbreeding season (black bars) and breeding season (gray bars).

Figure 5. Digital morphometric measurements (defined in Figure 1) versus standard length in a subset of immature, female, transitional, and male California sheephead collected at Santa Catalina Island.

Figure 6. Average blood plasma estradiol concentrations in mature female, transitional, and male California sheephead collected at Santa Catalina Island during the nonbreeding season (black bars) and breeding season (gray bars). Number over each bar indicates sample size. Different uppercase letters over bars indicate post hoc test results showing significant differences ($P < 0.05$).

Figure 7. Average blood plasma 11-ketotestosterone concentrations in mature female, transitional, and male California sheephead collected at Santa Catalina Island during the nonbreeding season (black bars) and breeding season (gray bars). Number over each bar indicates sample size. Different uppercase letters over bars indicate post hoc test results showing significant differences ($p < 0.05$).