B-Mode ultrasonographic evaluation of the testis in relation to serum testosterone concentration in male Yangtze finless porpoise (Neophocaena phocaenoides asiaeorientalis) during the breeding season

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Abstract

The use of ultrasonography as a noninvasive tool for assessing the reproductive status of the male Yangtze finless porpoise (YFP; Neophocaena phocaenoides asiaeorientalis) was validated by correlating ultrasonographically determined testicular volume (TV) and testicular parenchyma pixel intensity (PI) with serum testosterone (T) concentration. The testes of 13 free-ranging male YFPs from the Tian-e-Zhou Reserve and three captive animals from the Baiji Dolphinarium (Wuhan, China) were examined ultrasonographically during April 2008. Testis volume was determined using Lambert’s formula for an ellipsoid. Testicular parenchyma PI was evaluated by analyzing testicular ultrasonograms using pixel analysis software (Image J). Serum T concentrations were determined using a single-antibody radioimmunoassay. The TV, PI, and serum T concentration were low and similar in animals with body length <133 cm, highest in those with body length ≥142 cm, and highly variable in those with body length from 133 to 141 cm. Both TV and PI were closely correlated with serum T concentration (r = 0.91 and r = 0.85, respectively; P < 0.01), indicating a consistent association between structural and functional development of the testis. In conclusion, we inferred that puberty onset in male YFPs occurred when TV was >150 cm³ and PI was >60 during the breeding season and that testicular ultrasonography and pixel analysis was an efficient, noninvasive, real-time tool to evaluate testicular function of live male YFPs.

Keywords: Pixel intensity; Testis volume; Testosterone; Ultrasonography; Yangtze finless porpoise

1. Introduction

Yangtze finless porpoise (Neophocaena phocaenoides asiaeorientalis) is a unique freshwater subspecies of the genus Neophocaena. It lives only in the middle and lower regions of the Yangtze River and the nearby Poyang and Dongting lakes in China [1]. The Yangtze finless porpoise (YFP) is considered the most
threatened subpopulation of the species: The IUCN Red List of Threatened Species has recognized the YFP as endangered since 1996 [2]. The population of the YFP was estimated at ~2700 in 1991 [3] but has dropped dramatically in recent years, due to the negative impacts of various human activities, such as illegal fishing, shipping, pollution, water projects, and so forth [4]. At present, the population contains approximately 1800 individuals [5]. Effective protective measures must be rapidly enacted to protect the YFP from extinction.

Scientific research institutions have sought to conserve this species by conducting research on these animals, both in their native habitat and in captivity. Knowledge regarding the reproductive biology of male YFPs should assist development of appropriate strategies for population recruitment and management. In previous studies, most information regarding the reproductive physiology of YFPs has been derived from examining reproductive tissues of dead animals or from analysis of reproductive hormone concentrations in blood samples taken from living animals [6–11]. By plotting the weight of the left testis against body length of collected dead specimens, Gao and Zhou estimated that male YFPs attained sexual maturity at body lengths between 130 cm and 140 cm [8]. Similarly, Hao et al. deduced that males attained sexual maturity at a body length between 134 cm and 138 cm by plotting the serum testosterone (T) concentrations against body length of YFPs living in the wild [11]. Although these findings are meaningful for population studies, they are not applicable when attempting to evaluate the reproductive status of individuals within a population. Thus, research biologists and wildlife managers making decisions regarding possible relocation of animals based on their current reproductive potential require methods that allow them to rapidly determine the reproductive status of an individual.

Real-time B-mode ultrasonography is a noninvasive, highly sophisticated diagnostic tool that allows researchers not only to assess the shape and size of the testes but also to collect valuable information on the function of reproductive organs by using pixel-intensity analysis on the ultrasonogram [12,13]. In past decades, the clinical application of ultrasonography in veterinary medicine and animal reproduction has expanded dramatically, and the value of this technique in breeding both domestic and nondomestic species has been well documented [14–19]. As animals are trained to cooperate, ultrasonographic examinations have aided in determining the reproductive status of captive cetaceans [20–23]. Nevertheless, no previous research has used this approach to evaluate the reproductive physiology of YFPs.

The objectives of the current study were to (1) describe the normal ultrasonographic image features associated with the testis of male YFPs of various body lengths; (2) further investigate the structural and functional development of the testes by computer-assisted determination of the pixel intensity (PI) of testicular ultrasonograms; and (3) validate ultrasonography as a tool for rapid assessment of reproductive status in free-ranging male YFPs by correlating ultrasonographic features with serum T concentrations.

2. Materials and methods

2.1. Animals and physical examination

To protect the YFP from extinction, a national seminatural reserve was established in 1992 in Tian-e-Zhou Oxbow (29°46’N to 29°51’N, 112°31’E to 112°36’E). The oxbow was cut off from the main stream of the Yangtze River and formed naturally in 1972. It is approximately 1 km wide and 21 km long. As early as 1990, five YFPs were introduced into the reserve, and two or three calves have been born annually in the reserve in recent years [4]. When the current study was initiated, there were 22 animals living in the reserve. In April 2008, all animals in the reserve were captured for physical examination. For all captured male YFPs, we obtained morphologic data (body length, body weight, and other morphologic end points) and used ultrasonography to examine the reproductive organs. A blood sample (10 mL) was collected from the central vein of the tail fluke using a 10-mL nonheparinized disposable syringe. The sample was centrifuged (1500 g, 15 min), and serum was placed in frost-free plastic tubes and temporarily stored in liquid nitrogen. Samples were transferred to the laboratory and stored at −25 °C for subsequent hormone assays. In addition, three captive male YFPs living in the Baiji Dolphinarium (Wuhan, China) were subsequently subjected to similar procedures during April 2008. For each animal, age was roughly estimated by the formula $Y = 114.4458X^{0.1410}$ ($Y$ = body length, $X$ = age), obtained from calculating the relationship between body length and age of male YFP specimens [7]. Basic data are shown in Table 1.

2.2. Ultrasonographic examinations and PI analysis

All examinations were performed with a LOGIQ Book XP ultrasound unit (General Electric Co., Schenectady, NY, USA) in conjunction with a broadband (3–5 MHz) linear-array transducer. The ultrasound
settings were standardized to a 4 MHz frequency, 48 overall gain, and 17 cm scanning depth. The settings for near and far gain were consistent throughout the study. The entire unit was protected from water exposure by a plastic waterproof cover when conducting the examinations. All data were stored on the built-in hard disk.

Ultrasound measurements of the testes were conducted using methods previously described by Brook et al. [23]. Briefly, the examination was performed with the animals lying laterally on a wet sponge mattress. Each testis was viewed both vertically and horizontally, yielding longitudinal and cross-sectional images, respectively. The length, width, and depth of each testis were measured independently (the epididymis was excluded). All cross-sectional measurements of the testes were done directly from the image display, using the built-in electronic caliper functions. However, the maximum width of the ultrasonographic field of view was only 10 cm, making it impossible to measure the length of the testes of mature males directly from the monitor image. Although the total image could be determined using the film function, the accuracy of this approach was regarded as less accurate than the indirect measurement method. By locating the ends of each testis and noting these points on the surface of the skin, it was possible to make indirect measurements of testicular length. The transducer was placed perpendicular to the flank, at the level of the genital slit, and moved dorsally or cranially until the testis was located. The proximal and caudal ends of the testis were identified, and the distance between them was determined with a measuring tape. The same orientation of the transducer was maintained throughout the procedure, and the measurement was completed between inhalations to minimize any error induced by the movement of the animal. The longitudinal section of the entire testis was then examined, and the testicular parenchyma was carefully assessed. At the widest part of the testis, the transducer was rotated 90 degrees to produce a cross-sectional image. After capturing this image on the monitor, the width and depth of the testis were measured directly from the image with electronic calipers. The volume of each testis was determined using Lambert’s formula for an ellipsoid, volume (cm$^3$) = length / width / depth / 0.71, which had been employed similarly in a previous study of bottlenose dolphins (Tursiops truncatus) [23].

For each animal, ultrasonograms of the testes were transferred from the built-in hard disk of the ultrasonography device to a personal computer. Testicular parenchyma PI was calculated for each animal based on the longitudinal ultrasonogram of the left testis. Numerical pixel values (gray-scale values of individual picture elements ranged from zero to 255; 0 represented black and 255 represented white) were determined by placing six circular points at random on the portion of the testis located near the blubber layer. Sample regions encompassed only testis parenchyma, avoiding surrounding tissues. The gray-scale value (mean of all pixels within the measuring point) was measured by using the histogram function of the Image J software, a public-domain Java image processing program [24].

### Table 1

<table>
<thead>
<tr>
<th>Animal</th>
<th>Body length (cm)</th>
<th>Body weight (kg)</th>
<th>Left testis length (cm)</th>
<th>Right testis length (cm)</th>
<th>Estimated age (yr)*</th>
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</table>

*The ages of the animals were roughly estimated by their body lengths according to the relationship between body length and age described by Zhang [7].

$^1$TT, AB, and AF are captive animals in Wuhan Baiji Dolphinarium.
images were analyzed in a random order by researchers blinded to the corresponding body length of the animal.

2.3. Testosterone assay

Commercially available radioimmunoassay (RIA) kits for human testosterone have been validated for use with YFPs in previous studies [11,25]. The single-antibody $^{125}$I RIA kits for humans used in this study were also validated by running serial dilutions (1:2–1:64) of a serum sample of a mature male YFP and comparing the slope of the curve with that of a standard curve. The antiserum used was a rabbit anti-human testosterone. Testosterone standards ranged from 0 to 2000 ng/dL. The detection limit was 2 ng/dL. The cross-reactions of the serum testosterone RIA kit were 100% with testosterone; 0.03% with 5α-dihydrotestosterone; 0.06% with 11α-hydroxyprogesterone; 0.012% with androstenedione; and < 0.001% with other hormones. The intra-assay coefficient of variance was < 10% (n = 15), and the interassay variance was < 15% (n = 15). All assays were performed in duplicate and followed the manufacturer’s instructions (Kemei-Dongyga Biotechnological Company, Inc., Beijing, China).

2.4. Data analysis

The data were analyzed using the software SPSS 13.0 for Windows (SPSS Inc., Chicago, IL, USA). Left testis volume and right testis volume were compared using the paired-samples Student’s t-test. Differences were considered significant at $P < 0.05$. The mean PI of the left testis and the mean TV of each animal were calculated using descriptive statistics. Scatter plots of the mean TV, PI, and serum T concentrations against body length were correlated with testis development trends. Pearson correlation coefficient was calculated between testicular volume (TV), PI, serum T concentration, and body length, between TV, PI, and serum T concentration, and between TV and PI. All data are presented as mean ± SEM.

3. Results

3.1. Ultrasonographic appearance of the testis

Similar to other cetaceans, YFPs have internal testes located near the kidney [23,26]. The testes are cylindrical in shape, comparatively large, and easily visualized with transabdominal ultrasonography. Although juveniles have comparatively smaller testes, they are relatively superficial, tucked high in the dorsolateral aspect of the abdomen, lying in the angle formed by the hypaxialis lumbarum and rectus abdominus. Hence, the testes are easily identified when the flank is scanned perpendicularly at the level of the genital slit.

Ultrasoundograms of the testes of YFPs of various body lengths are shown in Figs. 1–3. There were no major differences in shape or appearance between the right and left testes. In longitudinal section, the testes are elongated, and the border of each testis was clearly demarcated from the surrounding tunica, which was visualized as a single

Fig. 1. Longitudinal ultrasonogram of the testis in a juvenile male YFP. The caudal pole of the testis is located to the left of this image. Arrow a indicates the testicular parenchyma, Arrow b indicates the head of epididymis, and Arrow c indicates testicular tunica. Scale marks are in centimeters.

Fig. 2. Longitudinal ultrasonogram of the testis seen in a pubescent male YFP. The caudal pole of the testis is located to the left of this image. Arrow a indicates the testicular parenchyma, Arrow b indicates the testicular mediastinum, and Arrow c indicates testicular tunica. Scale marks are in centimeters.
hyperechoic border to the parenchyma (Figs. 1–3). In cross section, the testes were circular and could still be clearly distinguished from the demarcated surrounding tunica (Fig. 4). The testicular mediastinum was visible as a prominent hyperechoic linear structure, extending the full length of the testis (Figs. 2 and 5). The echo pattern of the testicular parenchyma varied among individuals. Juvenile testes had a homogeneously hypoechoic pattern, whereas pubescent testes showed obviously intensified echodensity. In mature males, the testis generally had a homogeneous and strong echo pattern. Two individuals with body lengths >160 cm were the exception to this generality. These two individuals had a testicular parenchyma with coarse and moderate echo patterns (Fig. 5).

3.2. Variation of TV and parenchyma PI with body length

Testicular length ranged from 4.2 to 29.0 cm (Table 1). Testicular volume ranged from 15.65 to 1203.03 cm³ (Fig. 6), and no differences were noted between the right and left testes (n = 16, P > 0.05; Table 1). Pixel intensity of the left testicular parenchyma ranged from 41.33 to 140.85 (Fig. 7).
With body length <133 cm, the TV was similar among animals (15.40 ± 1.28 cm³). Although large variation was seen in animals with body lengths between 133 and 141 cm (57.82 ± 78.65 cm³), the greatest TV was in animals with body length ≥142 cm (809.00 ± 203.37 cm³; Fig. 6). Similar to the correlation with TV, the PI of the left testis was less than 60 for animals with body length <133 cm, and it ranged from 60 to 80 for animals with body length between 133 and 141 cm. Pixel intensity was 103.37 ± 24.20 (range, 79.64 to 140.85) in animals with body length ≥142 cm. However, the two individuals with body length >160 cm had moderate PI values (~ 80; Fig. 7).

3.3. Variation of serum T concentration with body length

Scatterplots of serum T concentrations plotted against body lengths of male YFPs are shown in Fig. 8. Concentrations ranged from 21.57 ng/dL to 1320.12 ng/dL. In animals with body length <133 cm, serum T concentrations were consistently low (33.03 ± 9.34 ng/dL) and had no clear correlation with body length. However, individuals with body lengths between 133 cm and 141 cm had higher serum T concentrations and greater variation (370.80 ± 337.17 ng/dL). Individuals with body lengths ≥142 cm consistently had the greatest concentrations and the lowest variation (1149.94 ± 141.43 ng/dL).

3.4. Correlations of TV, PI, and serum T concentration

In general, TV, PI, and serum T concentration were all positively correlated with the body length of males. Moreover, we observed TV and PI closely correlated with serum T concentration (r = 0.91 and r = 0.85, respectively; P < 0.01). Additionally, PI was highly correlated with TV (r = 0.80, P < 0.01).

4. Discussion

Though located within the abdomen, the testes of YFPs are large and relatively superficial and are thus easily accessible for ultrasonographic evaluation. The internal structure of the testes was clearly demonstrated ultrasonographically; those images were quite consis-
tent with anatomic descriptions [26]. In addition, testicular dimensions could be accurately measured by ultrasonography, again similar to the measurements of the testis length in previous anatomic studies [7–10]. Chang and Zhou reported that the lengths of dissected testes in YFPs ranged from 5.57 to 25.5 cm [9]. In comparison, the testicular lengths observed in our study ranged between 4.2 and 29.0 cm. Although the epididymis could be clearly distinguished from the testis (Fig. 1), we failed to identify the cross-sectional diameter of individual tubules in the tail of the epididymis. Our data were collected at the onset of the breeding season, which may have affected the diameter of the tubules. Furthermore, the resolution of our portable ultrasound instruments may also have affected our measurements. Nevertheless, ultrasonography appeared to be a useful, convenient, and noninvasive technique for assessing the reproductive organs of these endangered cetaceans.

Computer-assisted analysis of image attributes is a natural extension of the technologic advances in diagnostic ultrasonography. It is becoming an important biomedical tool for ultrasonographic image analysis [27,28]. In our study, the echodensity of the testes of YFPs were determined directly using software-based analyses of numerical PI. These analyses showed a consecutive complex echo pattern (Fig. 7). These changes may reflect the structural variation of the testicular parenchyma substructures, such as cellular proliferation and fluid production. Homogeneous and hypoechoic echo patterns (PI ranges from 40 to 60) may indicate inactive cellular differentiation and nonproliferated testes in juveniles, whereas increased PI (PI ranges 60 to 80) may signal the onset of division of the seminiferous tubule and the gradual formation of more mature cell types, with the onset of spermatogenesis in pubescent males [10]. Moreover, a homogeneous and highly intensified echo pattern (PI >80) may suggest an increase in both the diameter of the seminiferous tubules and the number of maturing spermatocytes, which normally occurs in mature males. The enlarged seminiferous tubules containing dense concentrations of spermatocytes would cause an increased acoustic interface between the tissues surrounding the testis and the testicular parenchyma, resulting in increased echodensity. The presence of free spermatozoa may also increase echodensity. Indeed, the homogeneous and highly echodense pattern was characteristic of mature testes in many mammals, including bottlenose dolphins [23,29]. Nevertheless, in two YFP individuals with body length >160 cm, the testicular parenchyma had a coarse and moderate echo pattern (Fig. 5). This may have been due to unsynchronized cellular atrophy and decreasing diameter of the seminiferous tubules. Furthermore, the TV was clearly decreased in these two animals. These changes in the ultrasonographic appearance of this structure seemed consistent with previous gross anatomic descriptions of testes that were compatible with reproductive senescence [10]. Nevertheless, none of the previous studies compared the ultrasound images with histologic images. As a result, we cannot definitively state that ultrasonographic imaging reflects exact changes in such instances.

The reproductive status of various animals, including rams, rabbits, and cetaceans, has been evaluated using testicular echodensity [23,30,31]. Even though tissue densities and tissue characteristics were assessed ultrasonographically, these characteristics were not readily quantifiable. Consequently, previous research could only comparatively distinguish three echo patterns in the testes of male bottlenose dolphins by comparing them with the echodensity of the hypaxialis lumborum muscle, which is located near the testes [23]. The PI of this muscle is thought to remain consistent in all individuals, regardless of size or age [23]. In our study, relative changes of PI between the hypaxialis lumborum muscle and the testes were not affected by adjusting gain settings on the ultrasound scanner. However, we had difficulty simultaneously capturing a cross-sectional image of the hypaxialis lumborum muscle and testis when the testis was so large that it occupied most of the ultrasonogram. Therefore, we standardized all settings for the ultrasonographic examination and determined PI values using computer-assisted pixel analysis. The variation pattern of the PI compared with body length was quite consistent with that of the TV. The reproductive status of the animal could be quantitatively distinguished into three categories based on the PI values achieved in this season: immature (40 to 60), pubescent (60 to 80), and mature (>80). A recent study demonstrated that both cross-sectional testicular area and echo patterns of the testes have significant seasonal variations in Pacific white-sided dolphin (Lagenorhynchus obliquidens) [32]. However, in YFPs, there is still no clear evidence showing that testis shape and testicular parenchyma echo patterns varied with seasonal profiles [10]. Perhaps testis structure varies seasonally, and thus these categories should not be extrapolated to determine the reproductive maturity of animals in different seasons. Furthermore, because of the inevitable measurement variations produced with different types of ultrasonographic scanners, we recommend that a cutoff value of 20 be used to distinguish between the reproductive status (immature, puberty, and mature) of the animals in the breeding season.
In addition, serum T concentration has been used in previous studies as a reliable end point for assaying reproductive biology. Serum T concentrations correlated closely with body length in male YFPs during the breeding season [11,33], and our results corroborated these findings. The significant positive correlations between TV, PI, and serum T concentration demonstrated the consistency of the structural and functional development of the testis. Furthermore, TV, PI, and serum T concentration were directly correlated with body length. Therefore, we are confident that ultrasonographic evaluation, combined with hormone analysis, may provide a comprehensive understanding of the morphology, structure, function, and status of the reproductive organs in live male YFPs.

We have demonstrated that ultrasonography can be used to examine the testes of living YFPs and that TV and PI were closely associated with body length and serum T concentration. Based on the ultrasonographic evaluation and variations in serum T concentration, we inferred that the male YFPs with TV > 150 cm³ and PI > 60 could be classified as at the onset of puberty, whereas TV > 650 cm³ and PI > 80 during the breeding season indicated sexual maturity. According to these criteria, seven of the male YFPs assessed were classified as juveniles, seven were determined to be mature, and two were classified as pubescent. However, because of the limitations imposed by the sampling season and the number of individuals assessed, we are cautious about making comprehensive conclusions regarding the reproductive physiology of the YFPs. Further studies are required before comprehensive guidelines can be established for assessing the reproductive status of male YFPs.

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References


