

Effect of Photoperiod Extension on the Testicular Sonographic Appearance and Sexual Behavior of Captive Yangtze Finless porpoise (*Neophocaena asiaeorientalis asiaeorientalis*)

Xueying Yu^{1,2}, Yujiang Hao^{1,*}, Brian CW Kot³, and Ding Wang¹

¹The Key Laboratory of Aquatic Biodiversity and Conservation of Chinese Academy of Sciences, Institute of Hydrobiology of the Chinese Academy of Sciences, Wuhan, China. E-mail: wangd@ihb.ac.cn

²College of Ocean, Qinzhou university, Qinzhou, China. E-mail: junzhi45@163.com

³Department of Applied Biology and Chemical Technology, The Hong Kong Polytechnic University, Hung Hom, Kowloon, Hong Kong SAR, China. E-mail: briankot@yahoo.co.uk

(Received 11 February 2015; Accepted 4 March 2016)

Xueying Yu, Yujiang Hao, Brian CW Kot, and Ding Wang (2016) Photoperiod plays an important role in the seasonal regulation of reproduction in mammals. In the present study, we examined the effect of an extended photoperiod (light/dark: 14 h/10 h) during the usual sexually inactive phase (Jan-Mar) on testicular sonographic appearance and sexual behaviors of two captive Yangtze finless porpoise (*Neophocaena asiaeorientalis asiaeorientalis*, YFP). During the extended photoperiod treatment, the parenchyma pixel intensity, cross-sectional area of testicular sonographic images, as well as the frequency of sexual behaviors of the two male porpoises increased significantly compared to the control treatment. Parenchyma pixel intensity, the cross-sectional area of testicular sonographic images and the frequency of sexual behaviors of the two captive males all increased in response to extended photoperiod. These results may suggest that photoperiod is a potent factor regulating the reproductive seasonality in YFPs.

Key words: Photoperiod, Seasonal reproduction, Testicular changes, Ultrasonography, Sexual behavior, Yangtze finless porpoise.

BACKGROUND

The Yangtze finless porpoise (*Neophocaena asiaeorientalis asiaeorientalis*, YFP) is found exclusively in the middle and lower reaches of the Yangtze River and adjacent Poyang and Dongting Lakes (Wang 1992). Due to increasing deterioration of the Yangtze River ecosystem (due to illegal and over fishing, boat traffic, massive dam-building and pollution etc.), the free-ranging population of YFP is severely threatened and has suffered a dramatic decline over the past few decades. Based on a survey conducted in 2012, Mei et al. (2014) estimated approximately 1000 individuals to remain, with annual population rates of decline as

high as 13.7% (Mei et al. 2014). The species has subsequently been declared critically endangered by the International Union for the Conservation of Nature (IUCN) (Wang et al. 2013).

Considering the endangered status of YFPs, captive breeding has been recognized as one of the most important and urgent measures to conserve this cetacean subspecies (Wang et al. 2005). A basic knowledge of reproduction of this species is crucial for successful captive breeding (Dierauf and Gulland 2001). It is quite clear now that the YFP is a seasonal breeder according to histological, behavioral and physiological evidence. For example, Jiang (1998) revealed that the diameter of seminiferous tubules of YFP

*Correspondence: Present address: Institute of Hydrobiology, Chinese Academy of Sciences, 7th South Donghu Road, Wuchang District, Wuhan 430072, P.R. China. E-mail: hao.yj@ihb.ac.cn

specimen collected between March and May was much greater than between November and January. Similarly, their sexual behaviors also show obvious seasonal trends based on the observations conducted both in free-ranging and captive porpoise (Yu et al. 2003; Wei et al. 2004; Wu et al. 2010b). By monitoring serum hormones, we also noticed significant seasonal changes in testosterone (Chen et al. 2006; Hao et al. 2007). Furthermore, with the aid of ultrasonographic examinations, we clearly found seasonal trends of testicular volume changes both in free-ranging and captive animals (Wu et al. 2010a, b). An interesting question is whether photoperiod is a regulator of reproductive seasonality in the YFP, particularly considering they spend most of their time in a turbid river system.

Sexually mature individuals of most mammalian species undergo marked physiological changes such as gonadal development and sexual activity when exposed to photoperiod changes (Bradshaw and Holzapfel 2007). For example, photoperiod triggers synchronization of the annual reproductive cycles of experimental (Breed and Clarke 1970; Yellon and Goldman 1984; Hoffmann 1973; Johnston and Zucker 1980; Lincoln and Davidson 1977; Lincoln and Peet 1977), domestic (Robinson et al. 1985; Freedman et al. 1979; Thomson et al. 1977; Clay et al. 1987; Peters et al. 1980; Davis and Meyer 1972) and some exotic species (Beasley and Zucker 1984; McAllan and Dickman 1986; Perret and Aujard 2001). Only a few studies on reproductive responses to photoperiod changes have been conducted with marine mammals, such as Californian sea lions (*Zalophus californianus*) (Temte and Temte 1993), harbor seals (*Phoca vitulina*) (Bigg and Fisher 1975; Temte 1994) and northern fur seals (*Callorhinus ursinus*) (Spotte and Adams 1981; Temte 1985; Tomita et al. 2011). However, there is still no solid experimental evidence showing the effect of photoperiod changes on reproductive activities in cetaceans; mainly due to the difficulties associated with conducting experiments in these

exclusively aquatic animals.

In the present study, we aimed to test whether the reproductive seasonality of male YFP could be altered under experimentally induced conditions of extended photoperiod by conducting testis ultrasound imaging and sexual behavior observation.

MATERIALS AND METHODS

Animals and management

Two mature male YFPs, Tao-Tao (TT) and A-Fu (AF) were investigated in this study. AF was captured and introduced from the Jiayu section of the Yangtze River to the Baiji Dolphinarium in 1996. TT is the offspring of AF and was born in captivity on July 5, 2005. Based on testicular ultrasonographic examinations and blood testosterone results, both TT and AF were sexually mature at the time of the study. Basic animal characteristics are shown in table 1.

The animals were housed in the main rearing hall of the Baiji Dolphinarium, which is a round ceiling building with windows on the roof and walls. There are two connected housing pools, a kidney-shaped pool (20.0 × 7.0 × 3.5 m) and a smaller round pool (Diameter = 10 m, depth = 3.5 m). The two pools are separated by a submerged stainless steel gate. Animals on each side of the gate could still maintain visual and acoustic contact. TT and AF were housed in the kidney-shaped pool while one mature female (Ying Ying, YY) was kept in the small round pool. There are 7 rectangular windows (1.8 × 1.2 m) on the submerged walls of the pools, through which the animals could be clearly observed. A closed-circuit filter system recycled the water four times a day and the water temperatures varied with the natural seasonal changes during the entire course of the experiment (ranged from 7°C to 28°C). The monthly mean water temperature during 2010 and 2011 is shown in figure 1 and no significant difference between

Table 1. Information of the captive Yangtze finless porpoises investigated in this study

Animal	Sex ^a	Length ^b (cm)	Weight ^b (kg)	Age ^c (yr)
TT	M	145	41.5	4.5
AF	M	153	44.8	≈15

^aM: male; ^bBody length and weight at the beginning of the study; ^cAges of AF were roughly estimated according to the relationship between body length and age (Zhang 1992).

years was found for each month (Independent-Samples t test, $P > 0.05$).

The animals were fed approximately 10% of their body weight daily which varied between seasons. Diets consisted of various proportions of thawed whole fish (*Carassius auratus*, *Cyprinus carpio*, *Hemiculter leucisculus*) with vitamin and mineral supplements (Theragran 1 pill/day, Bristol-Myers Squibb, Shanghai).

The study was licensed under special permits, in accordance with domestic law (*Regulations on the Management of Laboratory Animals*) issued by the Ministry of Science and Technology of the People's Republic of China. All procedures were reviewed and approved under China's Wild Animal Protection Law, which regulates the capture and possession of YFPs.

Experimental design

Photoperiod was controlled and regulated by 24 ceiling-mounted fluorescent tubes (500w, Philips, Royal Dutch Philips Electronics Ltd., Shanghai) approximately 8 m above water surface to simulate natural daylight. The average illumination intensity of the water surface was monitored using a photometer (TES digital light meter, Shenzhen) positioned at four sites on the kidney-shaped pool. We turned on the lights at 16:00 when the average illumination intensity of the water surface was lower than 500 lx. The average illumination intensity of the water surface

was about 220 lx with all lights working during the night. The water surface illumination intensity of a 'day' therefore varied from 220 lx (at 22:30 hrs) to about 1900 lx (at 12:00 hrs). During overcast and sunny periods of the day, the illumination intensity outside the rearing hall was about 50-500lux and 1000-50000lux, respectively.

To investigate the effects of photoperiod on the seasonal pattern of testicular development, the animals were exposed to artificial long-day (LD) conditions during a natural short-day period. The animals were exposed to the artificial photoperiod extended to a LD cycle of 14hrs/day (08:30-22:30 hrs day length) from January 2, 2010 until March 12, 2010 (natural photoperiod: 10-11.5 h/day, national time service center, Shanxi) to approximate the maximum photoperiod of 14 h/day during the breeding season within the Yangtze River. Data collected from these two animals in 2011 under natural photoperiod condition were used as a control, while artificial extended illumination was added during the 2010.

Testicular ultrasonographic examination and image analysis

All ultrasonographic examinations were carried out along the poolside. The animals were trained to approach the poolside and position themselves in a dorsal recumbency position, with their flukes supported by a trainer. All examinations were performed with a LOGIQ Book XP ultrasound

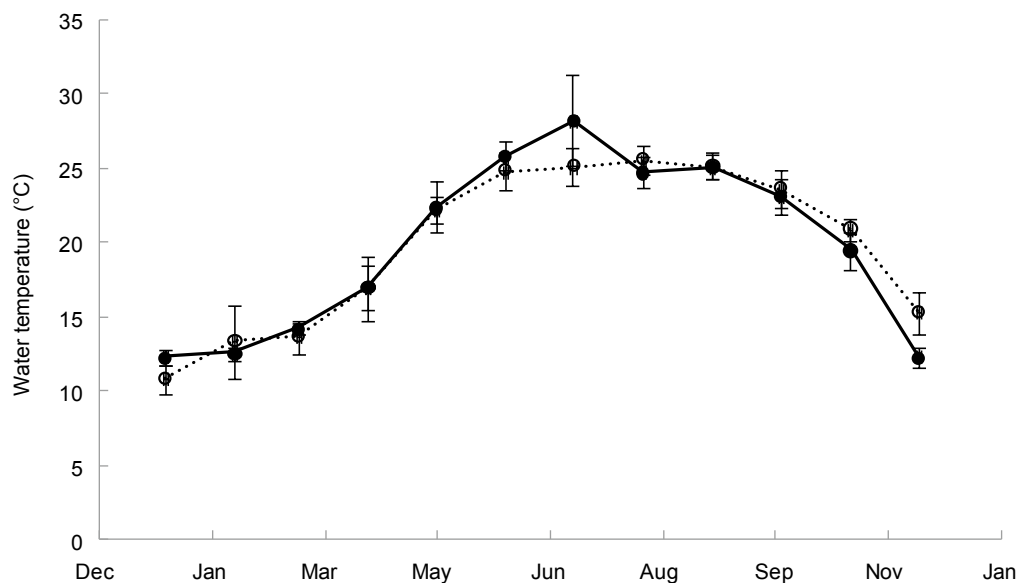


Fig. 1. Variation of water temperature in Baiji Dolphinarium. Monthly mean water temperature in 2010 (closed circles, solid line); Monthly mean water temperature in 2011 (open circles, broken line).

unit (General Electric Co., Schenectady, NY, USA), in conjunction with a 3-5 MHz curvilinear transducer. To minimize movement, the trainer supported the subject's body with his hands at the poolside during the ultrasonographic examination. The protocol for testicular ultrasonography was adopted from Brook et al. (2000). Ultrasonographic examinations were performed once a week for each animal during the extended photoperiod experiment.

In 2011, we performed ultrasonographic examinations under voluntary conditions once every two weeks to minimize potential stress due to being restrained. Since it has been demonstrated that there are no major differences in shape or appearance between the right and left testes in YFPs (Wu et al. 2010a), only the left testis of each animal was examined. All data were stored on the built-in hard disk of the ultrasound unit, and then transferred to computer for further analysis. Testicular sonograms of the YFP are shown in figure 2.

We used the testicular pixel intensity (PI) value and changes in testicular cross-sectional area (CSA) as the indicator of the morphological variation of the testicular parenchyma. Image J software program (National Institute of Mental Health, Bethesda, Maryland, USA) was used to evaluate the testicular echogenicity according to the method previously described by Wu et al. (2010a). PI changes may briefly reflect the structural variation of the testicular parenchyma substructures, such as cellular proliferation and

fluid production. Numerical pixel values (gray-scale values of individual picture elements ranging on a scale from zero to 255 (zero being black and 255 being white)) representing the testicular PI were determined by placing 6 circular points at random on the portion of the longitudinal testicular ultrasonographic image near the blubber layer and sample regions encompassed only the testis parenchyma, avoiding surrounding tissues (Wu et al. 2010a). The PI of the testis for the two animals was calculated as the average of the 6 measurement values from the sonograms of the left testis.

We used CSA to demonstrate testicular morphology development which was validated in the Pacific white-sided dolphin (*Lagenorhynchus obliquidens*) (Robeck et al. 2009). To measure the CSA, the transducer was rotated 90° anti-clockwise at the widest part of the testes identified in the longitudinal scan plane, and the sonogram was recorded for CSA estimation by the measurement tool in Image J (Rasband 1997-2010).

Sexual behavior observation

The monthly mean frequency of sexual behavior (SBF) per 45-minute periods was calculated to represent the intensity of male sexual behavior. To evaluate the sexual behavior in the animals, a scoring system was developed and sexual behavior events were defined as: within one body length range, the focal individual started to arch his genital region with his erect

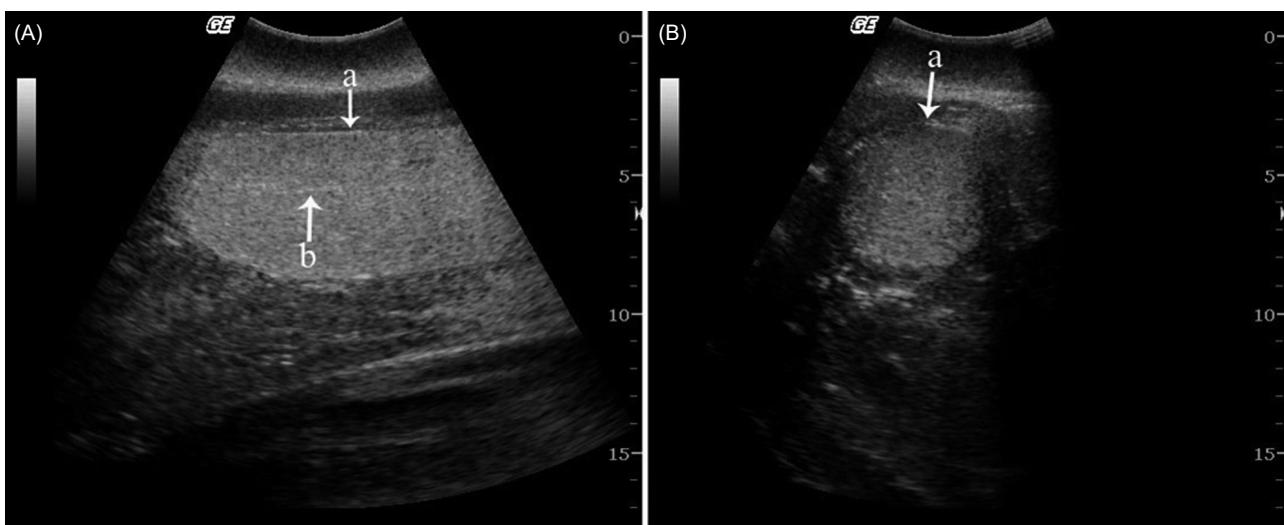


Fig. 2. Testicular ultrasonograms of Yangtze finless porpoise. A. Longitudinal ultrasonogram of testis. B. Transverse ultrasonogram of testis. (a: testicular tunica; b: testicular mediastinum). Scale marks are in centimeters.

penis and touched the receiver’s genital slit until they retreated (Wu et al. 2010b; Xian et al. 2010). Three observation sessions with 15 minutes each, during 09:45-10:30 hrs, 12:45-13:30 hrs and 15:30-16:15 hrs were conducted. Each animal was observed twice a week throughout the course of this experiment (Wu et al. 2010b).

Statistical analysis

The software SPSS19.0 for Windows (SPSS Inc., Chicago, IL, USA) was used for data analysis. The mean testicular PI and the mean CSA of the left testis were calculated using descriptive statistics. All data were tested for normal distribution using the Shapiro-Wilk test and for homogeneity of variances using the Levene’s test. If the data were normally distributed and of equal variances, then one-way analysis of variance (ANOVA) was performed to compare means among all measured variables. If the data did not have similar variances, the non-parametric Wilcoxon Rank Sum Test for comparing the median was applied. Correlation coefficients between testicular PI, CSA and sexual behavior frequency were also calculated by Spearman correlation analysis. All values are given as mean ± standard error (SE), and values of $P < 0.05$ were considered significant for all statistical tests in this study.

RESULTS

Changes in Testicular CSA and PI

The testicular PI value for AF and TT measured during the extended photoperiod and natural photoperiod is shown in table 2. AF was

diagnosed with gastric distress from late March to October 2010, thus only the data collected from December 2009 to February 2010 were used for analysis. Before the extended photoperiod experiment, the testicular PI during December 2009 in AF and TT were 68.25 ± 6.75 and 72.25 ± 1.71 , respectively. After approximately two weeks during exposure to the 14 hrs/day LD photoperiod, the testicular PI increased noticeably. In January and February 2010, the testicular PI values increased significantly in both animals compared to the data collected at the same time in 2011 ($P_{AF} < 0.05$, $P_{TT} < 0.05$).

During extended photoperiods, the testicular size (measured as CSA) of both males increased significantly compared to the data collected in the same period in 2011 (Table 3) ($P_{AF} < 0.05$, $P_{TT} < 0.05$). In 2011 the testicular CSA of TT and AF showed a similar trend during the control period. The testicular CSA of the 2 males remained small from January to February, and started to increase during March, and continued to grow from April to June.

To compare the changes of the testicular size and PI of the two males in 2010 with those in 2011, we use monthly mean difference values of the two years to show the effect of extended photoperiod on testicular changes in early 2010 (Fig. 3). In both animals, the differences in PI quickly reached a peak in January, and decreased slightly in February, but still elevated above baseline. However, the differences of PI in TT gradually diminished over the next few months (Fig. 3A). Comparatively, the differences in testicular CSA of the two male porpoises gradually increased from January and continued increasing throughout February in AF and peaked in March in TT, which were one to two months later than the significant

Table 2. Changes of monthly mean testicular PI of AF and TT under different photoperiodic exposures

Month	PI of AF (Mean ± SEM)		PI of TT (Mean ± SEM)	
	Experiment (2009.12-2010.6)	Control (2010.12-2011.6)	Experiment (2009.12-2010.6)	Control (2010.12-2011.6)
Dec	68.25 ± 6.75 (n = 4)	70.50 ± 6.36 (n = 2)	72.25 ± 1.71 (n = 4)	72.50 ± 0.71 (n = 2)
Jan*	103.50 ± 5.69 (n = 4)	77.00 ± 5.66 (n = 2)	106.00 ± 11.05 (n = 4)	74.00 ± 0.00 (n = 2)
Feb*	101.75 ± 8.02 (n = 4)	87.00 ± 2.83 (n = 2)	112.00 ± 4.40 (n = 4)	89.50 ± 0.70 (n = 2)
Mar*		118.50 ± 0.71 (n = 2)	119.25 ± 2.87 (n = 4)	107.00 ± 0.00 (n = 2)
Apr		115.00 ± 2.83 (n = 2)	108.00 ± 5.35 (n = 4)	116.00 ± 0.00 (n = 2)
May		116.00 ± 2.83 (n = 2)	117.75 ± 2.63 (n = 4)	114.00 ± 1.40 (n = 2)
June		113.00 ± 2.83 (n = 2)	118.00 ± 3.37 (n = 4)	114.00 ± 2.83 (n = 2)

*the months for light extension experiment in 2010.

Table 3. Changes of monthly mean testicular CSA of AF and TT under different photoperiodic exposures

Month	CSA of AF (Mean ± SEM) (cm ²)		CSA of TT (Mean ± SEM) (cm ²)	
	2009.12-2010.6	2010.12-2011.6	2009.12-2010.6	2010.12-2011.6
Dec	16.5 ± 0.91 (n = 4)	15.03 ± 1.01 (n = 2)	12.09 ± 0.36 (n = 4)	11.40 ± 0.20 (n = 2)
Jan*	27.31 ± 7.43 (n = 4)	15.66 ± 1.62 (n = 2)	20.66 ± 5.81 (n = 4)	12.74 ± 0.41 (n = 2)
Feb*	48.12 ± 6.67 (n = 4)	16.89 ± 1.10 (n = 2)	25.22 ± 6.05 (n = 4)	13.14 ± 0.12 (n = 2)
Mar*		21.98 ± 2.49 (n = 2)	37.69 ± 1.67 (n = 4)	18.98 ± 0.19 (n = 2)
Apr		38.71 ± 0.94 (n = 2)	35.97 ± 2.54 (n = 4)	30.40 ± 0.76 (n = 2)
May		39.34 ± 0.93 (n = 2)	43.26 ± 5.25 (n = 4)	30.80 ± 0.32 (n = 2)
June		40.67 ± 0.94 (n = 2)	34.65 ± 1.030 (n = 4)	31.82 ± 2.41 (n = 2)

*the months for light extension experiment in 2010.

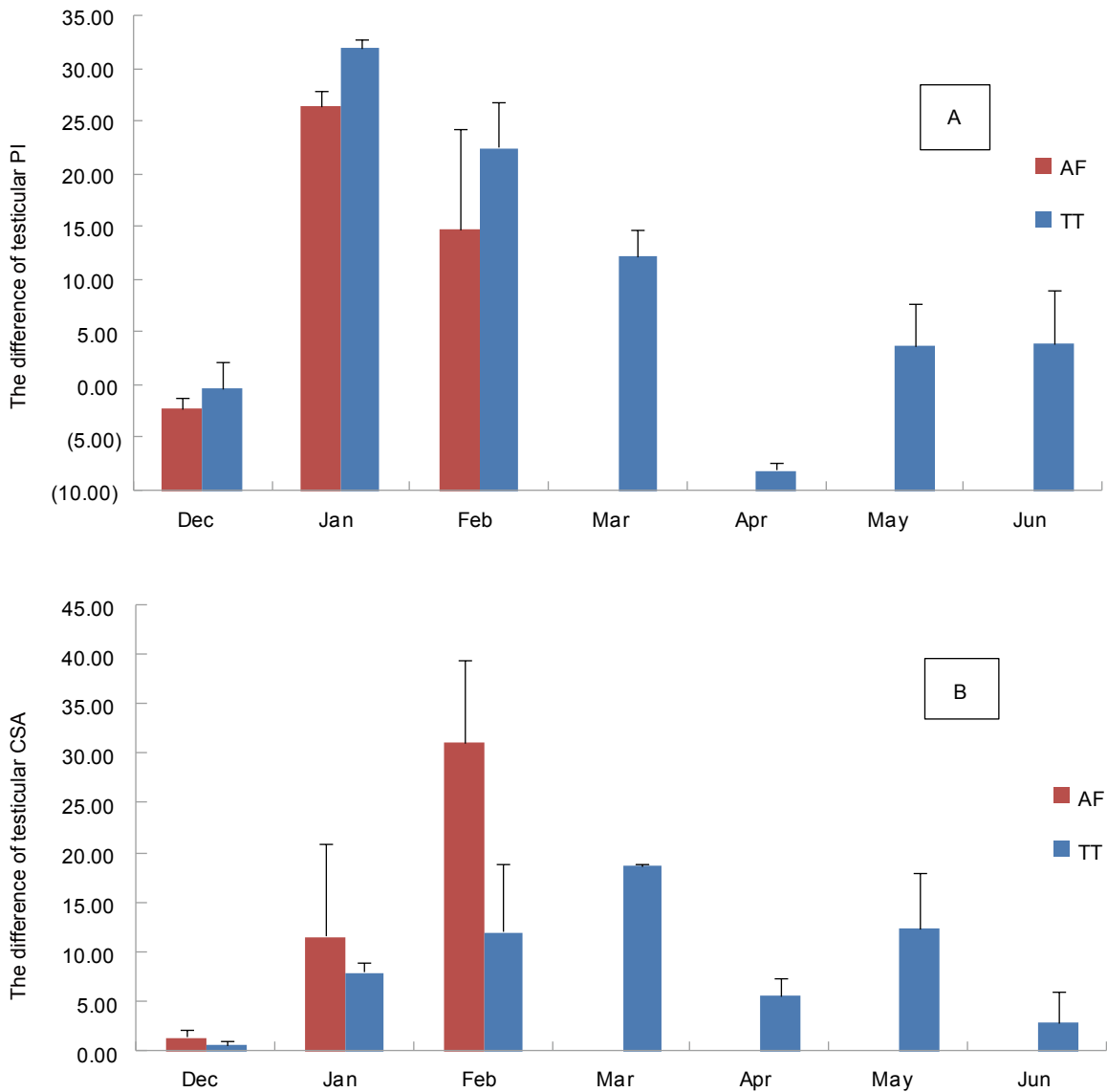


Fig. 3. (A) Difference of testicular pixel-intensity (PI) between 2010 (Experiment) and 2011 (Control). The difference in testicular PI between 2010 and 2011. (B) Difference of cross-sectional area (CSA) between 2010 (Experiment) and 2011 (Control). The difference in testicular CSA between 2010 and 2011. (AF, red bar; TT, blue bar).

differences in the testicular PI. The differences in PI decreased over the next few months in TT even though there were some fluctuations (Fig. 3B).

Sexual behavior

TT and AF showed similar seasonal profiles of sexual behavior frequency (SBF) during the control in 2011, but the SBF of TT was much higher than that of AF during all seasons, and its SBF also varied drastically compared to AF (Table 4). The SBF of the two males increased during the extended photoperiod treatment in early 2010, when compared to the same period of time in 2011 ($P_{AF} < 0.01$; $P_{TT} < 0.01$).

The difference value was also used to demonstrate the effect of extended photoperiod on the sexual behaviors of the two male porpoises.

The difference value of the SBF increased significantly between January and February compared with that of December 2009 both in AF and TT. The monthly mean difference value of SBF for TT gradually decreased over the following months when the extended photoperiod had been removed since mid-March, even though the difference in SBF for TT was still noticeable between 2010 and 2011, respectively (Fig. 4).

In addition, the two males both responded quickly to the changes of day length. Even on the second day after the extended photoperiod experiment started, the SBF of AF and TT increased to 10 times/45 min and 25 times/45 min, respectively (Table 4). However, interestingly, the SBF for AF was strongly correlated to the testicular PI changes ($P_{AF} < 0.01$, $r_{AF} = 0.717$) and the testicular CSA variations ($P_{AF} < 0.05$, $r_{AF} = 0.625$),

Table 4. The monthly Frequency of sexual behavior counts (SBF) of AF and TT

Month	SBF of AF (Mean ± SEM) (times/45 min)		SBF of TT (Mean ± SEM) (times/45 min)	
	2009.12-2010.6	2010.12-2011.6	2009.12-2010.6	2010.12-2011.6
Dec	2.38 ± 0.90 (n = 8)	2.25 ± 1.00 (n = 4)	14.75 ± 2.60 (n = 8)	15.00 ± 2.60 (n = 4)
Jan*	6.50 ± 2.00 (n = 8)	2.00 ± 0.82 (n = 4)	20.50 ± 3.55 (n = 8)	11.50 ± 1.00 (n = 4)
Feb*	9.25 ± 3.81 (n = 8)	3.00 ± 0.82 (n = 4)	24.25 ± 3.73 (n = 8)	20.00 ± 1.63 (n = 4)
Mar*		9.75 ± 2.63 (n = 4)	25.75 ± 4.37 (n = 8)	22.50 ± 3.42 (n = 4)
Apr		10.00 ± 0.82 (n = 4)	23.50 ± 4.38 (n = 8)	27.00 ± 1.41 (n = 4)
May		8.00 ± 2.58 (n = 4)	16.25 ± 5.92 (n = 8)	16.25 ± 0.96 (n = 4)
June		8.50 ± 2.38 (n = 4)	17.00 ± 1.31 (n = 8)	15.50 ± 1.29 (n = 4)

*the months for light extension experiment in 2010.

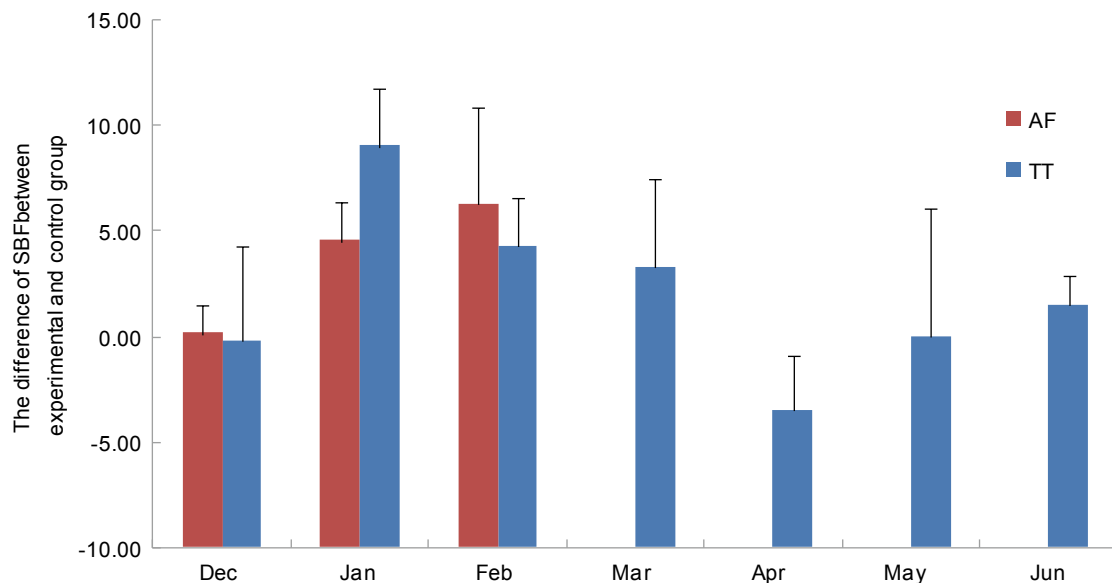


Fig. 4. Difference of SBF (counts/45minutues) between 2010 (Experiment) and 2011 (Control). (AF, red bar; TT, blue bar).

and there was no significant correlation between SBF and testicular changes observed in TT during the photoperiod extended experiment ($P_{AF} > 0.05$).

DISCUSSION

Previous studies have shown that Yangtze finless porpoises are seasonal breeders (Wei et al. 2004; Chen et al. 2006; Hao et al. 2007; Wu et al. 2010b). Previous work has shown that the testicular volume of an adult male porpoise normally begins to increase in March, and plateaus in May through to June, and the PI of a testicular ultrasound image shows a similar profile, but rather the PI usually begins to increase one month earlier than the testicular size (Wu et al. 2010b). In this study, we used maximum cross-sectional area (CSA) instead of testicular volume to measure the changes of testicular size. This method has been used and validated by previous studies in other cetacean species (Robeck et al. 2009). The CSA changes in the two male porpoises showed quite similar seasonal profiles with that observed in other studies (Wu et al. 2010b), which may imply that CSA could be used as a reliable indicator for testicular growth in Yangtze finless porpoise.

From the difference value of monthly mean testicular PI and CSA between 2010 and 2011, the testicular changes under the photoperiod-extended experiment were clearly seen in winter. The testicular CSA of the two males started to increase in January, but the greatest differences were noticed in February in AF and in March in TT. Due to the gastric problem of AF that occurred in early March, there was not sufficient data for AF from that month for analysis (Fig. 3B). We therefore used the data from TT to infer the regular pattern of the testicular changes. The significant differences in testicular CSA occurred in March, as demonstrated by the abrupt growth of the testicular size of the male porpoise under the extended photoperiod treatment compared with the control; thereby suggesting the potent effect of photoperiod on the testicular growth in this animal.

The greatest differences in testicular PI between 2010 and 2011 were all noticed in January both in AF and TT (Fig. 3A), and this difference gradually decreased after February in both animals. This is expected since the testicular PI reflects the echo intensity of testes of YFP, which could be interpreted to reflect the advanced histological structure changes of the testicular parenchyma, such as cellular proliferation and

fluid production (Wu et al. 2010a). We therefore conclude that the development of the testes was sensitive to the extended photoperiod treatment, and then the testicular size gradually increased with the cellular proliferation and fluid production.

The differences of sexual behavior frequency (SBF) between the two years were also significantly higher during the extended photoperiod treatment, which showed a similar trend as the PI variations. This is expected since it has been demonstrated that the sexual behavior of male captive YFP fluctuated normally with the variation of sexual hormones (testosterone) (Chen et al. 2006), which is a result of the testicular parenchyma development. However, our findings suggest that the SBF were more sensitive and responded more quickly to the extended photoperiod than the PI changes. We speculate that it might be due to the fact that the testicular parenchyma development is relatively difficult to be detected by the ultrasound scanning during early stages. Therefore, considering the similar pattern of testicular PI and SBF, we suspect that the elevated SBF during the extended photoperiod treatment was the result of testicular parenchyma development.

Since there were no female kept together with the males during the extended photoperiod experiment, all sexual behaviors observed in this study could be seen as 'homosexual' behaviors. Sexual behaviors of dolphins are commonplace and may have a social role, as well as a reproductive role (Boyd et al. 1999; Brook 1997). Nevertheless, it is not easy to distinguish between reproductive and social sexual behaviors in nature. We therefore speculate that even the non-reproductive homosexual behavior frequency of male YFP still reflects the libido of socio-sexual communication (Xian et al. 2010). Moreover, the significant difference of SBF between AF and TT would be considered as individual differences. The cause of this difference may be age, but could not be concluded in this study. To further understand this difference, a larger sample size is needed in the future work.

In general, based on our data of testicular changes and sexual behavior, the present study provides the first evidence to demonstrate that male YFP were reproductively responsive to extended photoperiods, which also suggests day length to be an important environmental factor in regulating the seasonality of reproduction in YFP.

There are many previous experiments conducted on terrestrial mammals about photoperiod and

seasonal changes in reproductive functions (Farner 1961). For example, the testicular size of hamsters (*Phodopus sungorus*) experienced a considerable increase when they were exposed to LD 16hrs/day (Hoffman and Reiter 1965). A similar pattern was also reported in some marine mammals, for example, estrus in harbour seals occurred earlier than usual when exposed to extended photoperiods, and was delayed during shorter photoperiods, and the timing of parturition in pinnipeds was also regulated by photoperiod (Bigg and Fisher 1975; Temte 1985). The seasonality and circadian rhythms of vertebrates is believed to be regulated by melatonin secreted by the pineal gland, and entrained by photoperiods (Erlich and Apuzzo 1985; Malpoux et al. 1999). However, the presence of a distinct pineal gland in cetaceans is still controversial, although a few unconfirmed reports describe the organ in some cetacean species (Panin et al. 2012). Extra-pineal melatonin production by the retina, the Harderian gland, and the gut has been quantitatively assessed in bottlenose dolphins (*Tursiops truncatus*), although no pineal gland was found in their examination on a series of brains of the same species (Panin et al. 2012). Since there is still no work conducted concerning the presence of the pineal gland in finless porpoises, we are cautious to discuss further the mechanism of this seasonality of reproduction in the YFP. Nevertheless, the present study provides evidence of the positive response of gonadal and behavioral changes to extended photoperiods, which may imply the potent regulation by melatonin secreted by the pineal or extra-pineal glands and warrants further investigation.

Due to the training difficulties, we failed to collect useful samples to investigate the effect of photoperiod on the reproductive cycles in female porpoises. Tomita et al. (2011) suggested that photoperiodic control of the male reproductive cycle was similar to that of female reproductive cycle in Northern fur seals. We speculate that reproductive cycles may also be altered under the extended photoperiod if the females share the same mechanism for reproductive seasonality regulation. This assumption, however, can only be tested when training for ultrasound scanning and urine sample collection (for hormone assay) can be achieved.

Apart from photoperiod, temperature, local food supply or climate are other environmental factors that can also influence the reproductive physiology of mammals (Bronson 1985; Boyd

1991). In this present study, there were no obvious water temperature differences, nor differing variations in temperature, between 2010 and 2011 due to a consistent water regulation regime (Fig. 1). Moreover, food supply in terms of fish species and consumption were all similar during the same season between years, and thus it is unreasonable to explain the difference by these factors. We are therefore confident that seasonal photoperiod variation is also used in the YFP as a major environmental regulator of reproductive cycles.

Captive breeding has been recognized as one of three conservation strategies for the YFP by Chinese researchers and authorities. A captive population has been established since 1996 within the Baiji Aquarium. However, there is only one male finless porpoise successfully born in this captive population since 2005 (Wang et al. 2005; 2009). There are still some technical bottlenecks that need to be ironed out for a successful captive program. The present study may not only help us to understand the reproductive seasonality of this species, but also provide evidence for potential possibilities to regulate or even extend the reproductive seasons of the YFP in captive facilities. Definitely, this could only be achieved by further understanding more on the testosterone changes, sperm production in males, and ovarian cycles in females under artificial photoperiod treatments. There is no doubt that the present research and possible further works in this field will enhance the captive breeding programs for conservation of this endangered freshwater cetacean species.

CONCLUSIONS

This study has demonstrated that the testes of two captive male YFPs showed significant morphological growth, and altered sexual behaviors in response to extended photoperiods, which provides new insights for understanding the role of photoperiod in the regulation of reproductive cycles in aquatic mammals. This study suggests that extended photoperiods during the non-breeding season can trigger an elevation in reproductive physiological activities in captive male YFP. Therefore, we speculate that length of day during the breeding season may be key in "switching on" the testicular activity, thereby playing an important role in manipulating the reproductive cycles in male YFP.

List of abbreviations

YFP: Yangtze finless porpoise
 PI: Pixel intensity
 CSA: Cross-sectional area
 SBF: Frequency of sexual behavior

Acknowledgments: We thank the training staff at Baiji Dolphinarium for their assistance in data collection during all experiments. Thanks are sincerely extended to the anonymous reviewers for their suggestions and comments. We also thank Dr. Matt Pine for proofreading the MS. This study was funded by the Knowledge Innovation Program of Chinese Academy of Sciences (No. KSCX2-EW-Z-4), and the Experimental Platform Program of Chinese Academy of Sciences (No. CZBZX-1).

REFERENCES

- Beasley LJ, Zucker I. 1984. Photoperiod influences the annual reproductive cycle of the male pallid bat (*Antrozous pallidus*). *J Reprod Fertil* **70**(2):567-573.
- Bigg MA, Fisher HD. 1975. Effect of photoperiod on annual reproduction in female harbour seals. *Rapports et procès-verbaux des réunions Conseil International pour l'Exploration de la Mer* **169**:141-144.
- Boyd IL. 1991. Environmental and physiological factors controlling the reproductive cycles of pinnipeds. *Can J Zool* **69**(5):1135-1148.
- Boyd IL, Lockyer C, Marsh HD. 1999. Reproduction in marine mammals. *In*: Reynolds JE, Rommel SA (eds) *Biology of marine mammals*. Smithsonian Institution Press, Washington, pp. 218-286.
- Bradshaw WE, Holzapfel CM. 2007. Evolution of animal photoperiodism. *Annu Rev Ecol Evol Syst* **38**:1-25.
- Breed WG, Clarke JR. 1970. Effect of photoperiod on ovarian function in the vole, *Microtus agrestis*. *J Reprod Fertil* **23**(1):189-192.
- Bronson FH. 1985. Mammalian reproduction: an ecological perspective. *Biol Reprod* **32**(1):1-26.
- Brook FM. 1997. The use of diagnostic ultrasound in assessment of the reproductive status of the bottlenose dolphin, *Tursiops aduncas*, in captivity and applications in management of a controlled breeding programme. The Hong Kong Polytechnic University, Hong Kong.
- Brook FM, Kinoshita R, Brown B, Metreweli C. 2000. Ultrasonographic imaging of the testis and epididymis of the bottlenose dolphin, *Tursiops truncatus aduncas*. *J Reprod Fertil* **119**(2):233-240.
- Chen DQ, Hao YJ, Zhao QZ, Wang D. 2006. Reproductive seasonality and maturity of male *Neophocaena phocaenoides asiaeorientalis* in captivity: A case study based on the hormone evidence. *Mar Freshw Behav Phy* **39**(2):163-173.
- Clay CM, Squires EL, Amann RP, Pickett BW. 1987. Influences of season and artificial photoperiod on stallions: Testicular size, seminal characteristics and sexual behavior. *J Anim Sci* **64**(2):517-525.
- Davis GJ, Meyer RK. 1972. The effect of daylength on pituitary FSH and LH and gonadal development of snowshoe hares. *Biol Reprod* **6**(2):264-269.
- Dierauf LA, Gulland F. 2001. *CRC handbook of marine mammal medicine*. CRC Press, Boca Raton.
- Erlich SS, Apuzzo ML. 1985. The pineal gland: anatomy, physiology, and clinical significance. *J. Neurosurg* **63**(3):321-341.
- Farner DS. 1961. Comparative physiology: photoperiodicity. *Annu Rev Physiol* **23**:71-96.
- Freedman LJ, Garcia MC, Ginther OJ. 1979. Influence of photoperiod and ovaries on seasonal reproductive activity in mares. *Biol Reprod* **20**(3):567-574.
- Hao YJ, Chen DQ, Zhao QZ, Wang D. 2007. Serum concentrations of gonadotropins and steroid hormones of *Neophocaena phocaenoides asiaeorientalis* in middle and lower regions of the Yangtze River. *Theriogenology* **67**(4):673-680.
- Hoffman RA, Reiter RJ. 1965. Rapid pinealectomy in hamsters and other small rodents. *Anat Rec* **153**(1):19-21.
- Hoffmann K. 1973. The influence of photoperiod and melatonin on testis size, body weight, and pelage colour in the Djungarian hamster (*Phodopus sungorus*). *J Comp Physiol* **85**(3):267-282.
- Jiang XF. 1998. Studies on the development and histological characteristics of black finless porpoise, *Neophocaena phocaenoides* in the Yangtze River. *Acta Hydrobiol Sin* **22**:341-345 (in Chinese).
- Johnston PG, Zucker I. 1980. Photoperiodic regulation of the testes of adult white-footed mice (*Peromyscus leucopus*). *Biol Reprod* **23**(4):859-866.
- Lincoln GA, Davidson W. 1977. The relationship between sexual and aggressive behaviour, and pituitary and testicular activity during the seasonal sexual cycle of rams, and the influence of photoperiod. *J Reprod Fertil* **49**(2):267-276.
- Lincoln GA, Peet MJ. 1977. Photoperiodic control of gonadotrophin secretion in the ram: a detailed study of the temporal changes in plasma levels of follicle-stimulating hormone, luteinizing hormone and testosterone following an abrupt switch from long to short days. *J Endocrinol* **74**(3):355-367.
- Malpoux B, Thiéry JC, Chemineau P. 1999. Melatonin and the seasonal control of reproduction. *Reprod Nutr Dev* **39**(3):355-366.
- McAllan BM, Dickman CR. 1986. The role of photoperiod in the timing of reproduction in the dasyurid marsupial *Antechinus stuartii*. *Oecol* **68**(2):259-264.
- Mei ZG, Zhang XQ, Huang SL, Zhao XJ, Hao YJ, Zhang L, Qian ZY, Zheng JS, Wang KX, Wang D. 2014. The Yangtze finless porpoise: On an accelerating path to extinction? *Biol Conserv* **172**:117-123.
- Panin M, Gabai G, Ballarin C, Peruffo A, Cozzi B. 2012. Evidence of melatonin secretion in cetaceans: plasma concentration and extrapineal HIOMT-like presence in the bottlenose dolphin *Tursiops truncatus*. *Gen Comp Endocrinol* **177**(2):238-245.
- Perret M, Aujard F. 2001. Regulation by photoperiod of seasonal changes in body mass and reproductive function in gray mouse lemurs (*Microcebus murinus*): differential responses by sex. *Int J Primatol* **22**(1):5-24.
- Peters RR, Chapin LT, Emery RS, Tucker HA. 1980. Growth and hormonal response of heifers to various photoperiods. *J Anim Sci* **51**(5):1148-1153.

- Rasband WS. 1997-2008. ImageJ. National Institutes of Health, Bethesda. <http://rsb.info.nih.gov/ij/>.
- Robeck TR, Steinman KJ, Greenwell M, Ramirez K, Van Bonn W, Yoshioka M, Katsumata E, Dalton L, Osborn S, O'Brien JK. 2009. Seasonality, estrous cycle characterization, estrus synchronization, semen cryopreservation, and artificial insemination in the Pacific white-sided dolphin (*Lagenorhynchus obliquidens*). *J Soc Reprod Fertil* **138(2)**:391-405.
- Robinson JE, Radford HM, Karsch FJ. 1985. Seasonal changes in pulsatile luteinizing hormone (LH) secretion in the ewe: relationship of frequency of LH pulses to day length and response to estradiol negative feedback. *Biol Reprod* **33(2)**:324-334.
- Spotte S, Adams G. 1981. Photoperiod and reproduction in captive female Northern fur seals. *Mammal Rev* **11(1)**:31-35.
- Temte JL. 1985. Photoperiod and delayed implantation in the northern fur seal (*Callorhinus ursinus*). *J Reprod Fertil* **73(1)**:127-131.
- Temte JL. 1994. Photoperiod control of birth timing in the harbour seal (*Phoca vitulina*). *J Zool* **233(3)**:369-384.
- Temte JL, Temte J. 1993. Photoperiod defines the phenology of birth in captive California sea lions. *Mar Mam Sci* **9(3)**:301-308.
- Thomson DL, Pickett BW, Berndtson WE, Voss JL, Nett TM. 1977. Reproductive physiology of the Stallion. VIII. Artificial photoperiod, collection interval and seminal characteristics, sexual behavior and concentrations of LH and testosterone in serum. *J Anim Sci* **44(4)**:656-664.
- Tomita N, Kohyama K, Koido T, Takemura A. 2011. Effect of photoperiod on gonadal steroid hormone levels and reproductive cycles of northern fur seals (*Callorhinus ursinus*). *Mammal Study* **36(4)**:223-228.
- Wang D. 2009. Population status, threats and conservation of the Yangtze finless porpoise. *Chin Sci Bull* **54(19)**:3473-3484.
- Wang D, Hao Y, Wang K, Zhao Q, Chen D, Wei Z, Zhang X. 2005. Aquatic Resource Conservation. The first Yangtze finless porpoise successfully born in captivity. *Environ Sci Pollut R* **12(5)**:247-250.
- Wang D, Turvey ST, Zhao XJ, Mei ZG. 2013. *Neophocaena asiaeorientalis ssp. asiaeorientalis* in IUCN Red List of Threatened Species. In: The IUCN Species Survival Commission. <http://www.iucnredlist.org/details/full/43205774/0>. Accessed 24 February 2012.
- Wang PL. 1992. Taxonomy of finless porpoise in China. *Fish Sci* **11**:10-14 (in Chinese).
- Wei Z, Wang D, Zhang XF, Wang KX, Chen DQ, Zhao QZ, Kuang XA, Gong WM, Wang XQ. 2004. Observation on some sexual behavior of the Yangtze finless porpoise (*Neophocaena phocaenoides asiaeorientalis*) in captivity. *Acta Theriologica Sin* **24**:98-102.
- Wu HP, Hao YJ, Li X, Zhao QZ, Chen DQ, Kuang XA, Kou ZB, Feng KK, Gong WM, Wang D. 2010a. 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. *Theriogenology* **73(3)**:383-391.
- Wu HP, Hao YJ, Yu XY, Xian YJ, Zhao QZ, Chen DQ, Kuang XA, Kou ZB, Feng KK, Gong WM, Wang D. 2010b. Variation in sexual behaviors in a group of captive male Yangtze finless porpoises (*Neophocaena phocaenoides asiaeorientalis*): motivated by physiological changes? *Theriogenology* **74(8)**:1467-1475.
- Xian YJ, Wang KX, Dong LJ, Hao YJ, Wang D. 2010. Some observations on the sociosexual behavior of a captive male Yangtze finless porpoise calf (*Neophocaena phocaenoides asiaeorientalis*). *Mar Freshw Behav Phy* **43(3)**:221-225.
- Yellon SM, Goldman BD. 1984. Photoperiod control of reproductive development in the male Djungarian hamster (*Phodopus sungorus*). *Endocrinology* **114(2)**:664-670.
- Yu DP, Jiang WH, Huang LX. 2003. Swimming areas and their seasonal variation for Yangtze finless porpoise in the semi-natural conservation district. *Acta Hydrobiologica Sinica* **27(6)**:657-659 (in Chinese).
- Zhang XF. 1992. Studies on the age determination, growth and reproduction of finless porpoise (*Neophocaena phocaenoides*). *Acta Hydrobiologica Sinica* **4**:289-298 (in Chinese).