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Circulating gonadotropins and testicular hormones during sexual maturation and annual changes in male bottlenose dolphins (*Tursiops truncatus*)

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ABSTRACT. To reveal the reproductive biology in male bottlenose dolphins (*Tursiops truncatus*), circulating gonadotropins (follicle stimulating hormone [FSH] and luteinizing hormone [LH]) and testicular hormones (testosterone and inhibin) were monitored for 8–12 years in 2 captive bottlenose dolphins (Mars and Regulus). During the study period, Mars was undergoing sexual maturation, whereas Regulus was already mature at the beginning of the study. Assuming that Mars had reached sexual maturity when the significant increase in circulating testosterone levels was observed, serum concentration of inhibin was higher in the sexually immature stage than in the mature stage, whereas the serum concentration of FSH was higher in the sexually mature stage than in the immature stage. No difference was observed in the LH levels between pre- and post-sexual maturation. There was a significant increase in serum concentration of testosterone during spring in both animals. These results suggest that the mechanism responsible for regulating FSH secretion by inhibin functions during the sexually immature stage in this species.

KEY WORDS: FSH, inhibin, LH, male bottlenose dolphin, testosterone

Bottlenose dolphins (*Tursiops truncatus*) are the most common cetacean species maintained in aquariums. To date, many Japanese aquariums have been purchasing individuals captured from wild populations to maintain the number of animals in captivity. Under recent social circumstances, it is becoming more challenging to obtain animals from the wild cetacean population to bring into aquariums, which has resulted in an increased need to develop *ex-situ* breeding programs.

The female reproductive biology (particularly endocrinology) of bottlenose dolphins is a well-studied topic and basic understanding of reproductive seasonality and the ovulation cycle has been established [1]. With the purpose of artificial insemination of dolphins in captivity, detailed information on serum and urinary follicle stimulating hormone (FSH) and luteinizing hormone (LH) concentrations during estrus have been reported. Being able to detect the exact timing of the LH surge was a breakthrough for successful artificial insemination utilizing the one-time procedure [20, 23].

Reproductive research on male bottlenose dolphins has also focused on artificial insemination, although this has been aimed at semen collection and its cryopreservation rather than basic endocrine monitoring. Additionally, a more recent study utilized sperm sorting technology (i.e., flow cytometry) to better manage the sex ratio of the captive population [21]. An early long-term study on male reproductive physiology using captive bottlenose dolphins was first described by Harrison and Ridgway [5] who reported serum concentration of testosterone with a sensitive competitive protein binding technique. In addition, Katsumata *et al.* [12] reported testosterone dynamics of captive mature male bottlenose dolphins from Japanese waters. These studies revealed testosterone concentrations and seasonality in sexually mature animals. Schroeder [25] also described the relationship between serum testosterone concentrations and semen characteristics. Based on previous studies on male reproductive physiology, research

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has primarily been limited to testosterone concentrations and changing patterns in relation to sexual maturity and season [1]. Only a few studies have focused on the basic dynamics of hormones that regulate the male reproductive system apart from testosterone, such as FSH, LH, and especially inhibin in bottlenose dolphins. Schneyer *et al.* [24] reported on male and female FSH and LH using commercially available radioimmunoassay kits to analyze wild and captive bottlenose dolphins.

In the present study, as basic research for breeding, changes in secretion of gonadotropins (FSH and LH) and testicular hormones (testosterone and inhibin) of male bottlenose dolphins during their sexual maturation process and after sexual maturation were investigated.

MATERIALS AND METHODS

Animals

Two male bottlenose dolphins (*Tursiops truncatus*) named "Mars" and "Regulus" were utilized in the present study. Mars was transported from the wild population in Japan and relocated to Kamogawa Sea World on February 28, 1985. His body length and body weight on the day of transport were 224 cm and 115 kg, respectively, and he was estimated to be 2 years of age. His estimated age was determined based on body length and dental growth layer [11]. The duration of the study concerning Mars was 12 years from age 10 through to age 22 (1993–2004). Regulus was also transported from the wild population in Japan and relocated to Kamogawa Sea World on December 17, 1997. His body length and body weight on the day of acquisition were 296 cm and 273 kg, respectively, and he was estimated to be 10 years of age. He participated in the research for 8 years, from the age of 10 through to 18 (1997–2004). All procedures were carried out in accordance with the ethical guidelines established by the Kamogawa Sea World for marine animals.

Sample collection

Blood samples of 10 m*l* were collected from the tail fluke vessels with trained behavior. The samples were centrifuged at 1,700 × g for 15 min and the serum was maintained at −20°C until assay.

Facilities

During the research, the two animals were housed in separate outdoor pools that contained groups of bottlenose dolphins consisting of a few sexually mature females and immature males. The pools were maintained by natural sea water systems with regular chlorination. The average water temperature was 23.1 (14.8–28.2)°C and the ambient temperature was an average of 19.1 (1.0–36.0)°C.

Hormone assay

Serum concentrations of FSH and LH were determined by heterologous double-antibody radioimmunoassay (RIA). Serum concentrations of LH were measured using an anti-ovine LH serum (YM 18) and purified rat LH (NIDDK-rat LH I-7) as the radioiodination and reference standard (NIDDK-rat LH-RP-2). Serum concentrations of FSH were measured using an anti-human FSH serum (MP91) and purified rat FSH (NIDDK-rat-I-7) as the radioiodination and reference standard (NIDDK-rat FSH-RP-2). Intra- and inter-assay coefficients of variation were 5.9 and 11.5% for LH and 5.5 and 9.9% for FSH, respectively.

Concentrations of testosterone were determined by double-antibody RIA systems using ^{125}I-labeled radioligands as previously described [26]. Anti-sera against testosterone (GDN 250) were applied. The intra- and inter-assay coefficients of variation were 6.3 and 7.2% for testosterone, respectively. The concentration of immunoreactive inhibin was measured using rabbit antiserum against purified bovine inhibin (TNDH 1) and ^{125}I-labeled 32-kDa bovine inhibin as previously described [4]. The results were expressed in terms of 32-kDa bovine inhibin. The intra- and inter-assay coefficients of variation were 7.0 and 11.4%, respectively.

Statistical analyses

All values are expressed as mean ± SEM. One-way analysis of variance (ANOVA), Student's *t*-test, and post hoc tests were applied to detect significant differences in the amounts of hormones. A value of $P<0.05$ was considered an indication of statistical significance.

RESULTS

Changes in circulating testosterone, inhibin, FSH, and LH levels, and body weight of each individual, Mars and Regulus, are shown in Figs. 1 and 2, respectively. In Mars, circulating testosterone remained relatively low during the beginning of the study until 15 years of age. Although a significant increase in serum testosterone was observed at the age of 17 (3.7 *n*g/m*l*), it fluctuated between 1.0 and 18.1 *n*g/m*l* over the study period. At this point, Mars was considered to have reached sexual maturity. His body length was 292 cm and body weight was 317 kg at 17 years of age, when circulating testosterone concentrations increased. Mating that led to pregnancy was observed at 19 and 21 years of age. Body weight remained around 300 (269–338) kg post-sexual maturation (Fig. 1).

Regulus was considered sexually mature at the time of introduction to the aquarium (10 years of age). His circulating testosterone level was 15.0 *n*g/m*l* one month after introduction and ranged between 0.7 and 25.3 *n*g/m*l* during the study period. Mating that led to successful pregnancy was observed at 11 and 14 years of age. His body weight remained around 300 (276–330) kg

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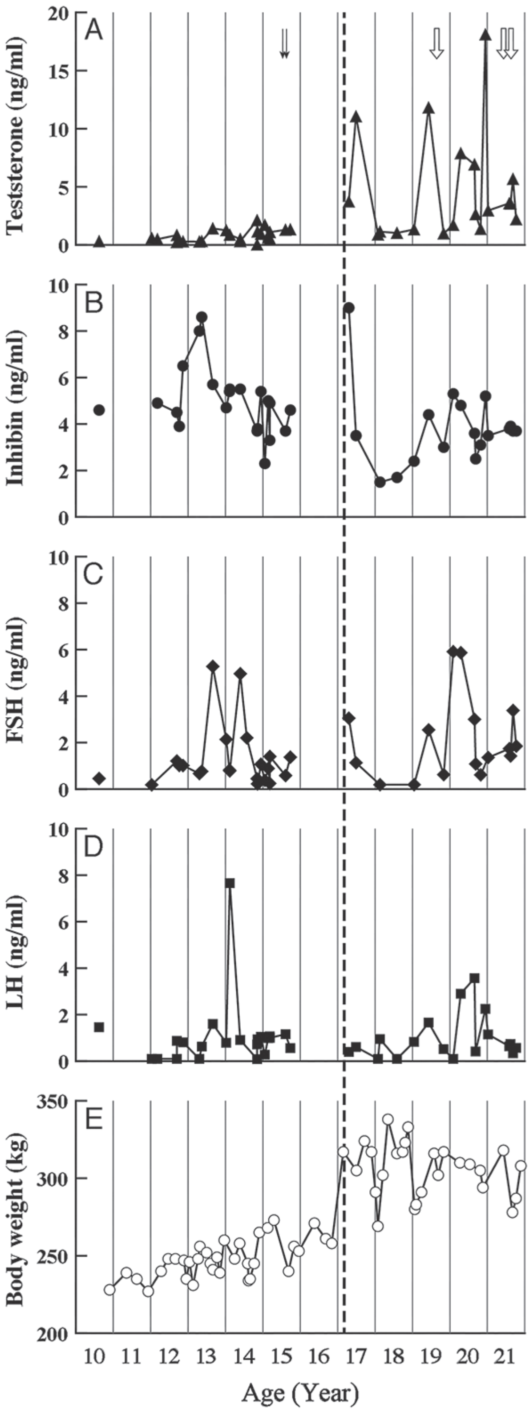

Fig. 1. Changes in serum concentrations of testosterone, inhibin, FSH, and LH levels, and body weight in a male bottlenose dolphin, Mars. The vertical dotted line represents the date showing a clear increase in serum concentration of testosterone representing the age when Mars reached sexual maturity. Small arrows at the age of 15 in June and August represent the mating behavior that did not result in conception, whereas the white arrows at the age of 19 in August and twice at the age of 21 in June and August represent the mating behavior that resulted in conception. Blood samples were collected at the following frequency: once at the age of 10, no sampling at age 11, 4–5 times at age 12, three times at age 13, 7–8 times at age 14, 6 times at age 15, no sampling at 16, twice at 17, 2–4 times at 18, 3 times at 19, 4−5 times at 20, 5 times at 21.

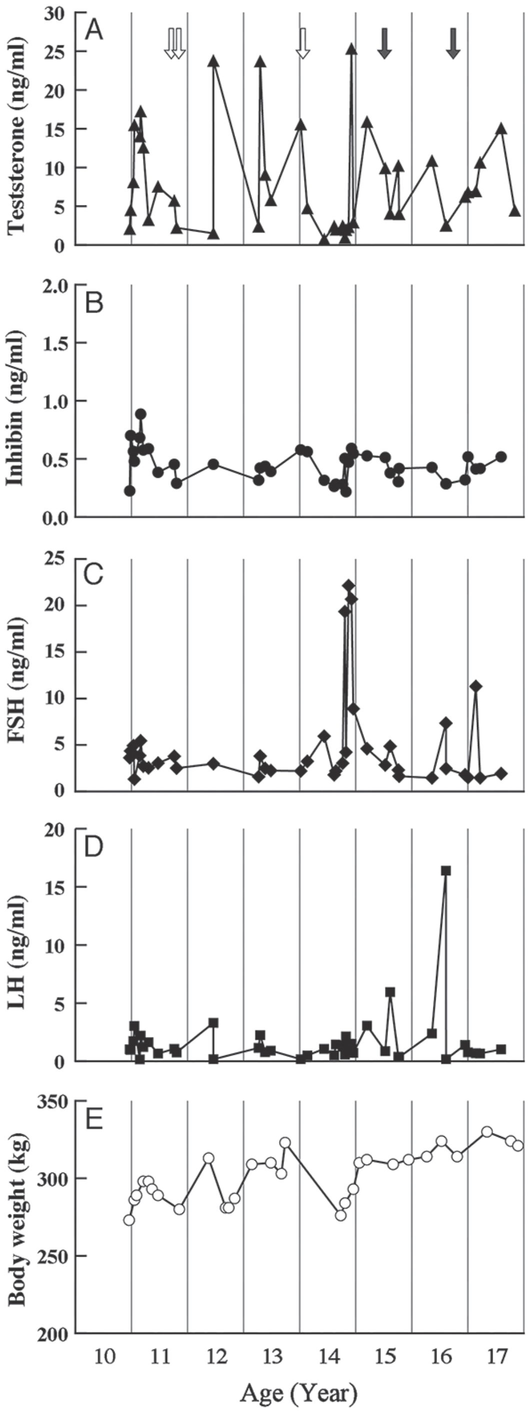

Fig. 2. Changes in serum concentrations of testosterone, inhibin, FSH, LH and body weight in a male bottlenose dolphin, Regulus. A white arrows at the age of 11 in August and October, the age of 14 in January represent day of copulation that resulted in conception and black arrows at the age of 15 in June and the age of 16 in September, represent day of generous amount of semen was collected for artificial insemination respectively. At each age, blood samples were collected at the following frequency: 4 times at the age of 10, 11 times at age 11, 1–2 times at age 12, 4 times at age 13, 13–14 times at age 14, 6 times at age 15, 4 times at age 16 and 4 times at age 17.

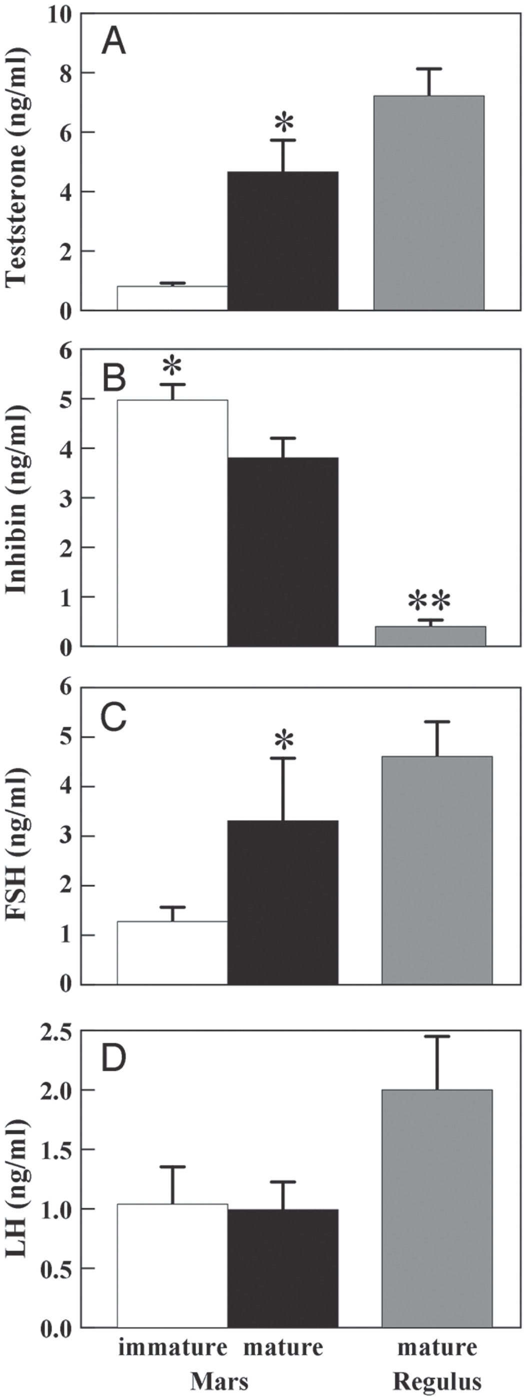

Fig. 3. Concentration of testosterone, inhibin, FSH, and LH at immature and mature stages of the male bottlenose dolphin, Mars. Sexual maturity was determined by the apparent increase in serum concentration of testosterone. Each value represents mean ± SEM, n=21–31, asterisks: $P<0.05$. Comparison between pre and post sexual maturity for Mars is shown in the figure. Data of Regulus was shown as reference value of mature male. Double asterisk: Mars (mature stage) vs Regulus, $P<0.05$.

throughout the period studied (Fig. 2).

Changes in the testosterone, inhibin, FSH, and LH levels during immature and mature stages in Mars and Regulus are shown in Fig. 3. In Mars, circulating testosterone levels before and after the age of 17 were 0.8 ± 0.1 *ng*/m*l* (n=23) and 4.6 ± 1.1 *ng*/m*l* (n=18), respectively, with a significant increase observed after the age of 17 ($P<0.05$). Assuming that the animal had reached sexual maturity when increased testosterone was observed, the following results of inhibin, FSH, and LH levels comparing sexually immature and mature stages based on the testosterone concentrations. The circulating inhibin levels in immature and mature stages were 5.0 ± 0.3 *ng*/m*l* (n=20) and 3.8 ± 0.4 (n=18), respectively, as significantly lower concentrations were seen once the animal reached sexual maturity ($P<0.05$). Conversely, the circulating FSH levels in immature and mature stages were 1.3 ± 0.3 *ng*/m*l* (n=22) and 3.3 ± 1.3 *ng*/m*l* (n=17), respectively, and in the sexually mature stage the concentration increased significantly higher than in the immature stage ($P<0.05$). The circulating LH levels in immature and mature stages were 1.0 ± 0.2 *ng*/m*l* (n=18) and 1.0 ± 0.3 *ng*/m*l* (n=23), respectively. Dissimilar to the other hormones, there were no significant differences between these two stages for LH levels.

Inhibin concentrations of Regulus and Mars in the mature stage were 0.4 ± 0.02 *ng*/m*l* (n=39) and 3.8 ± 0.4 *ng*/m*l* (n=18), respectively, which shows significantly lower values in Regulus than in Mars. There were no differences in circulating testosterone, FSH, and LH levels between Mars and Regulus during the mature stage (Fig. 3).

Seasonal changes of circulating testosterone, inhibin, FSH and LH levels after the increase in testosterone levels are shown in intervals of two months in Fig. 4. Seasonal changes were observed in the circulating testosterone levels of Mars: the level started to increase in March and April and peaked during May and June, with May and June being significantly higher than in the other months. Although there were no significant differences, circulating inhibin and FSH levels reached their maximum in March and April, and circulating LH levels had slight height variations during March through to July. For Regulus, the circulating testosterone levels in March and April were significantly higher than in September and October. Evident seasonal changes in levels of inhibin, FSH and LH were not observed.

DISCUSSION

To date, there are only a few studies that have been dedicated to investigating cetacean inhibin levels. Katsumata *et al.* [13] determined serum testosterone and inhibin levels in two captive belugas (*Delphinapterus leucas*) and demonstrated that these two hormones are negatively correlated and inhibin levels were higher in the sexually immature stage than in the mature stage. In the present study, changes in testicular functions during the growth process of male captive bottlenose dolphins (*Tursiops truncatus*) were revealed by measurements of circulating gonadotropins (FSH and LH) and testicular hormones (testosterone and inhibin) in blood samples taken from two male captive bottlenose dolphins reared at Kamogawa Sea World in Japan for 8 and 12 years. Mars was considered to have reached sexual maturity at 17 years of age when the circulating testosterone level rose to 3.7 *ng*/m*l*. His body length reached 305 cm at 19 years of age and no growth has been observed since then. It is predicted that the sexual maturation age of wild bottlenose dolphins is around 8 to 11 years old, from a previous study by Kasuya [11] who studied bottlenose dolphins in south-west Japanese coastal waters. This author reported that the average body length of individuals over 20 years of age was 305 cm and testis weight increased steadily from 8 to 11 years of age and

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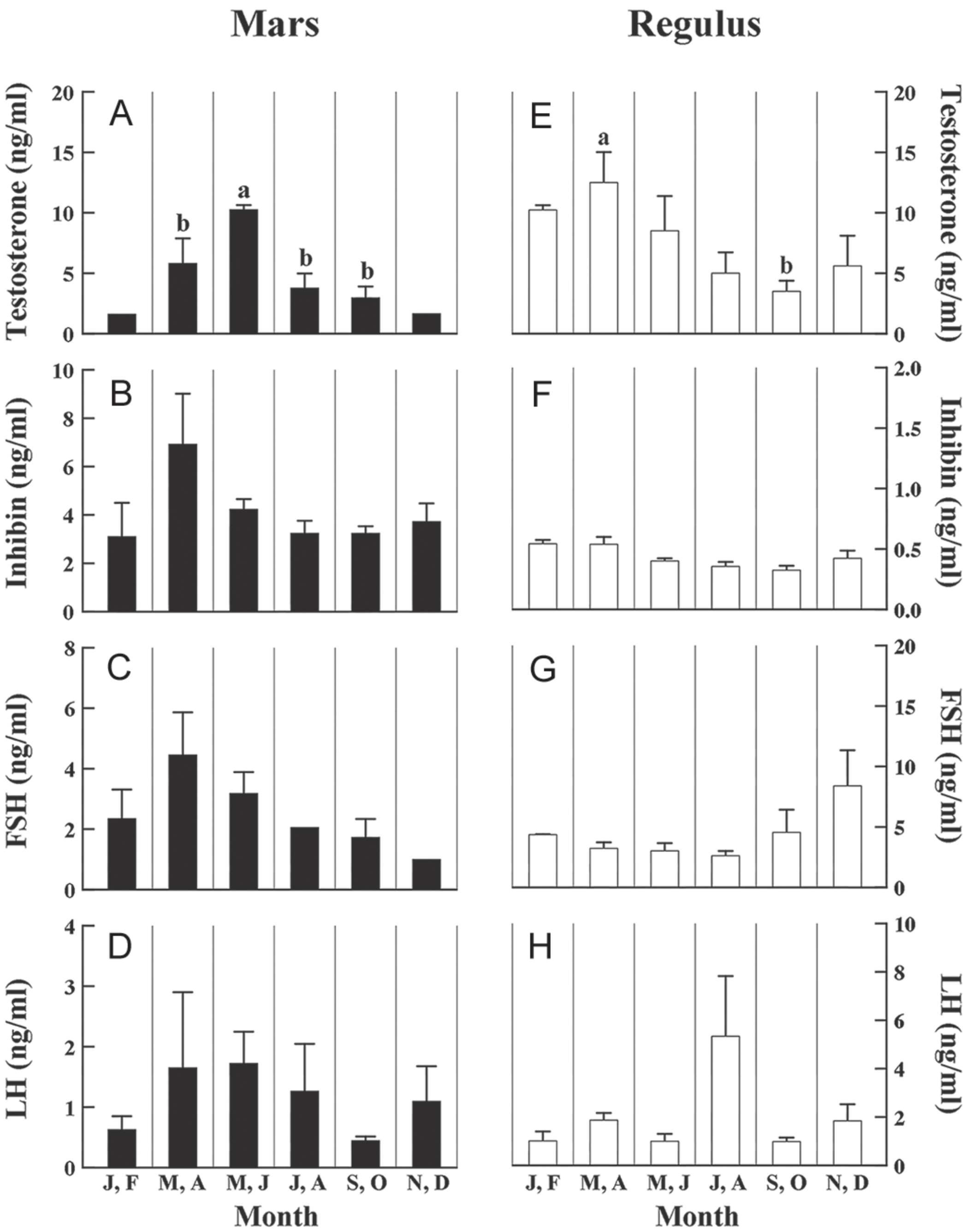

Fig. 4. Annual changes of serum concentrations of testosterone, inhibin, FSH, and LH during the mature stage of two bottlenose dolphins, Mars and Regulus. In Mars, data for after maturation were used. Values are expressed as the mean ± SEM for 2–5 observations in Mars; a vs b: $P<0.05$ and for 8–10 observations in Regulus; a vs b: $P<0.05$.

stopped at around 20 years of age [11]. The sexual maturity age of Mars was slightly delayed compared to wild male bottlenose dolphins living in Japanese coastal waters. Mars's first successful mating behavior that resulted in conception occurred at 19 years of age. This might have been due to the influence of his health condition, despite the well-known fact that animals under captivity breed at a younger age than wild animals do. Mars's mating behavior at a younger age (15 years of age) with a sexually mature female was observed; however, the female did not conceive. The circulating testosterone level was low during this time, i.e., around 1 *ng*/m*l*, and it was predicted that the spermatogenic function was immature. Conversely, Regulus was already considered to be sexually mature when he was transported to Kamogawa Sea World, which was assumed from his body length nearly reaching 300 cm, the typical size of mature males of this species, as well as serum testosterone concentrations being high from the day of relocation to Kamogawa Sea World from the wild population. For Mars, after reaching sexual maturity, circulating testosterone levels peaked during May through to June. Although this was two months later than Regulus, the tendency for the concentration to be high in spring and low in autumn was similar. The seasonal change in testicular functions of male bottlenose dolphins, as noted above, was similar to the results of studies by Katsumata *et al*. [12] and Schroeder [25]. A study by Schroeder [25], which conducted semen collection and circulating testosterone level measurements to reveal seasonal changes in sperm concentration, reported that sperm concentration was highest in October and circulating testosterone level was lowest. This indicated that there is no seasonality in spermatogenesis of male bottlenose dolphins and breeding is possible throughout the year. Similar testicular function has been reported previously in stallions [19].

In previous studies on circulating inhibin, FSH, LH and testosterone levels in relation to age in bull calves [7–9, 18], pigs [10, 22] and horses [2, 3] it was reported that circulating inhibin concentration is higher in the sexually immature stage than the mature stage. Similarly, it was reported that the circulating inhibin level is remarkably high in calves, until they reach sexual maturation [17]. Also, a study that measured circulating inhibin A and inhibin B levels separately in male chimpanzees [15], revealed that the inhibin B level is higher after than before sexual maturation and correlates with circulating testosterone levels, while no correlation could be observed between circulating inhibin A and testosterone levels.

In previous studies the present study, circulating inhibin levels of Regulus, a sexually mature male, was low: below 1 *n*g/m*l* and seasonal changes were not observed. As for Mars, a statistically significant difference was observed in circulating inhibin levels before and after sexual maturation. The result of the present study, showing that circulating inhibin levels are higher in sexually immature individuals than in mature individuals, was similar to the reports in bulls. However, when comparing data from Mars after sexual maturation and Regulus, a sexually mature individual, the data from the former was 9.5 times higher on average than that of the latter. A possible cause for such a difference in inhibin levels between the two mature individuals is yet unknown. While inhibin is a glycoprotein hormone secreted from Sertoli cells in testes, it is also reported that inhibin is secreted from the Leydig cells in studies that utilized horses [19], golden hamsters [6, 14], and African elephants [16]. Therefore, further consideration is needed regarding the relationships between spermatogenesis function and inhibin in dolphins.

Schneyer *et al.* [24] utilized commercially available assay kits for humans to analyze FSH and LH levels in bottlenose dolphins. Although this was the first research that analyzed the FSH and LH serum concentration for wild and captive male bottlenose dolphins, the values were only provided as averages. The present study has further revealed the long-term changes of FSH, LH, testosterone and inhibin levels during the growth period, and conducted a comparative study between pre- and post-sexual maturation in male bottlenose dolphins. In addition, an evident seasonal change was not observed in LH levels. Analyzing the relationship between inhibin and FSH concentrations of Mars, inhibin levels were high in the sexually immature stage and low in the mature stage, while FSH levels were low in the sexually immature stage and high in the mature period, showing a negative correlation. This result indicates that inhibin is functioning as a FSH secretion suppressor in male bottlenose dolphins during the immature stage.

The present study is the first report that reveals circulating inhibin concentrations in bottlenose dolphins, using blood samples taken from two male bottlenose dolphins over a long period.

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