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High-resolution monitoring from birth to sexual maturity of a male reef manta ray, *Mobula alfredi*, held in captivity for 7 years: changes in external morphology, behavior, and steroid hormones levels

Ryo Nozu[1,2*] , Kiyomi Murakumo[2], Rui Matsumoto[1,2], Yosuke Matsumoto[2], Nagisa Yano[2], Masaru Nakamura[1], Makio Yanagisawa[1,2], Keiichi Ueda[1,2] and Keiichi Sato[1,2]

Abstract

Background: In this study, we report the first case of a male reef manta ray, *Mobula alfredi*, becoming sexually mature in captivity and present its reproductive characteristics.

Methods: We investigated changes in external morphology, behavior, and levels of steroid hormones in a male *M. alfredi* during its sexual maturation process.

Results: At 2 years and 6 months of age, the male exceeded 300 cm in disc width. Then, at around 3 years of age, the male started to chase a female in the tank and exhibited androgen levels similar to that of another matured male, indicating that the study specimen had begun sexual maturation endocrinologically. Its first copulation event was observed at 5 years and 4 months and appeared behaviorally similar to field observations. Seven months after copulation, we performed a biopsy to collect its semen, including any motile sperm.

Conclusion: Taken together, these results indicate that the captive male *M. alfredi* individual shows signs of sexual maturation (size of disc width, testosterone levels, reproductive behavior) already with the age of 2.5 to 3 years. As a first copulation was observed with 5 years 4 months of age and the presence of sperm were confirmed at the age of 5 years 11 months of age, the studied animal reached its full sexual maturity at the latest at the age of around 5 years.

Keywords: Reef manta ray, *Mobula Alfredi*, Male, Sexual maturation, In-captivity, Mating behaviors, Sex steroid hormones

Background

Manta rays comprise two species that occur in tropical, sub-tropical, and temperate waters around the world. As fully-grown adults, they are among the largest ray species. Due to their tendency to aggregate predictably in relatively shallow waters around islands and along coastlines, they have become an attraction for recreational divers in many countries. Manta ray-based ecotourism potential can be important for coastal communities, particularly in developing countries, where it can generate great economic benefits [1, 2].

Until recently, the genus *Manta* was considered mono-specific, but was redescribed [3] in 2009 as comprising the reef manta ray, *Manta alfredi* (Krefft 1868) and the giant manta ray *M. birostris* (Walbaum 1792). In a recent study, the mitochondrial genome analysis showed that both *Manta* species are nested within the other *Mobula* species and sister to *M. mobular* [4]; the authors noted that *Manta* is an invalid generic name.

* Correspondence: r-nozu@okichura.jp
[1] Okinawa Churashima Research Center, Okinawa Churashima Foundation, 888 Ishikawa, Motobu, Okinawa 905-0206, Japan
[2] Okinawa Churaumi Aquarium, 424 Ishikawa, Motobu, Okinawa 905-0206, Japan

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Therefore, in the present paper, the genus "*Mobula*" was adopted. Both species were listed as globally vulnerable on the IUCN Red List [5, 6].

Previous reports have provided some ecological information about manta ray species, such as distribution, movement patterns, courtship, mating behavior, gestation period, and reproductive periodicity [7–9]. However, there is little information on their reproductive biology. Observations of captive specimens in aquariums enable us to obtain accurate information of the same individual over time. At the Okinawa Churaumi Aquarium (OCA), *M. alfredi* individuals thrive since 1988; much information on the biology of this species has been generated in this aquarium. For example, the OCA recorded the first captive reproduction of a *M. alfredi* [10, 11]. On June 8, 2006, a male mated with a female and on June 16, 2007, the female gave birth to a female pup (193 cm disc width and 68.5 kg body weight). On June 17, 2008, the same pair gave birth to a male pup that was used in the present study. In addition, the copulating behavior was similar to that reported for Ogasawara wild mantas [12] and the duration of clasper insertion into the cloaca was approximately 11 to 18 s. Based on the observation, the authors concluded that the duration from copulation to parturition was approximately 1 year, which were consistent with field observations [7]. On the other hand, it is thought that *M. alfredi* usually takes a resting interval of at least 1 year between pregnancies [7]. The captive female in the OCA, however, mated with a male in the tank immediately after giving birth; the female manta ray became pregnant again and gave birth the next year [10]. This suggests that breaks in pregnancy in the wild are not due to the physical conditions, however, only environmental cues may be essential. Additionally, aspects of the embryonic respiratory system of *M. alfredi* were revealed by an ultrasonographic experiment on the pregnant individual [13]. This study provides the first direct evidence of the respiratory behavior of a *M. alfredi* embryo by demonstrating that the embryo acquires oxygen from the uterine fluid using gill ventilation. Thus, observations on *M. alfredi* in captivity can not only strengthen previous studies but also provide new biological information.

To achieve efficient captive breeding of *M. alfredi* in other facilities, it is essential to accumulate more information on its reproductive ecology and physiology. However, only few studies on these aspects exist because of the difficulty in closely following *M. alfredi* over its complete life-span and the high effort involved. Here, we confirm that a male born in the OCA reached sexual maturity in captivity. Moreover, we provide behavioral and physiological information on its captive reproduction obtained from detailed and continuous time series observations.

Methods

An observed individual and rearing conditions

A male (identification number: No10–2) was born in the "Kuroshio" tank (a 10 m deep, rectangular aquarium (35 × 27 m)) of the OCA on June 17, 2008, and was observed continuously (Fig. 1a). After birth, the neonate was immediately moved to the sea pen (diameter: 30 m, depth: 12–15 m) and kept there for approximately 10 months to naturalize feeding and allow the development of swimming ability without any influence from other individuals. The pup was then moved to the main tank again. Our study period ended at the end of November 2015 as the animal has been moved and been kept solo since then. The circulation rate of water in the tank was 16 turnovers per day (recycled seawater 12 turnovers and fresh seawater 4 turnovers) and water temperature was not under thermal control. The daily feed dosage was 0.5–0.8% of its body weight. The body mass was calculated using the allometry equation (Body weight (kg) = 0.000013563* disc width (cm) $^{2.889}$ (R^2 = 0.973)) that was derived from actual measurement values of 18 *M. alfredi* individuals (Matsumoto et al., personal communication). Feeding was performed twice a day, and the feed included *Euphausia superba*, *E. pacifica*, *Sergestes lucens*, and *Engraulis japonicus*.

Estimation of disc width

Disc width (DW) of specimen No10–2 was measured twice: at his birth (182 cm) and at 10 months of age (261 cm). Thereafter, its outer interorbital distance (IOD) was measured in June and/or December of each year. Estimated DW was calculated from IOD using the growth equation (LogDW (cm) = 0.84841*LogIOD (cm) + 0.88452, R^2 = 0.94) that derived from actual measurement values of 18 *M. alfredi* individuals (Matsumoto et al., personal communication).

Behavior observation and test for stiffness of claspers

During the day, aquarium staff visually monitored the individual but not continuously. When the individual exhibited specific behaviors, we recorded the behaviors through photographs and/or videos from inside and/or outside the tank. To confirm the claspers status, we hand-tested them for stiffness at the birth (June 2008), 5 year and 4 months (Oct 2013) and 5 years 11 months of age (May 2014) while No10–2 swam.

Semen collection

To confirm whether or not the individual produced mature sperm, we obtained semen from No10–2 via a biopsy while swam (Additional file 1: Movie S1). At 5 years and 11 months of age (on May 30, 2014), a plastic tube (All Silicone Foley Balloon Catheters, Product No. 001027 0120, Create Medic Co., Ltd., Kanagawa)
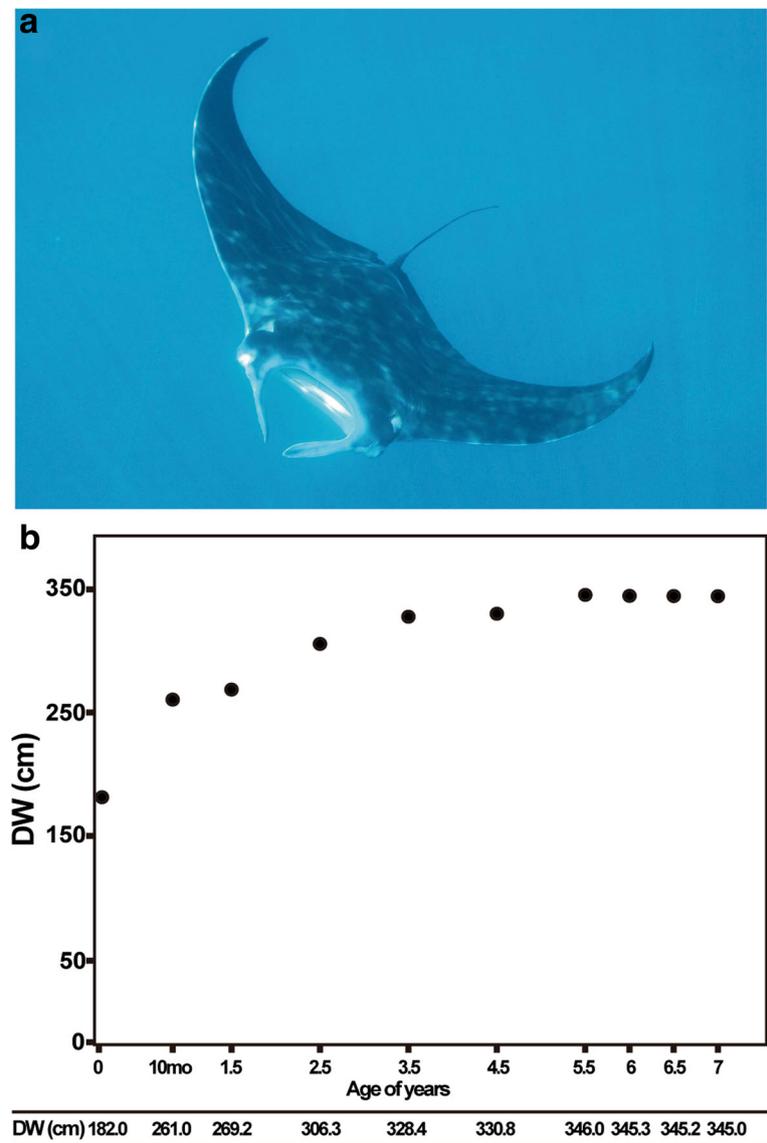
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Fig. 1 Photograph of No10–2 soon after his birth and a chart of his growth of disc width (DW). **a** No10–2 swimming in the ocean sea pen. **b** Actual DW measurements were taken at birth and at 10 months of age. After that, estimated DW values were calculated by interorbital distance. mo; months

connected with syringes (5 ml for ballooning, 50 ml for collecting semen, Terumo Corporation, Tokyo) was slipped into his clasper and semen was obtained. The semen was observed under light microscopy and video-recorded; it was also observed under a light microscope after Giemsa staining. We estimated that the specimen did not suffer severe stress due to the biopsy because feed consumption was unaffected after the semen collection.

Blood collection and measurement of plasma steroid hormones levels

No10–2 had received husbandry training since it was approximately 1 year old, which enabled us to collect blood samples regularly after 3 years and 3 months of age, from its left or right pectoral fin vein or artery, using a syringe (Terumo Corporation, Tokyo) with a 20 gauge needle (Terumo Corporation, Tokyo) while No10–2 swam. The time of blood collection was fixed in the morning. However, during certain months, we could not collect blood samples. The collected blood was placed into a heparinized tube and plasma was obtained by centrifugation (1400×*g* for 20 min. at room temperature). Obtained plasma was stored at –30 °C until analysis. To compare mature and immature male characteristics, the preserved plasma obtained from other males (i.e., No6 mature, 318-cm DW; No26

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immature, 215-cm DW) was also analyzed. Both male individuals were caught from the wild. No6 was regarded as sexually mature because previous observations showed that No6 mated with a female in the OCA [10]. In addition, No26 was regarded as immature based on the previous report that DW at sexual maturity in wild was 270–300 cm [8].

Extraction of plasma steroid was performed according to a previous procedure [14]. Briefly, plasma steroids were extracted three times using 2.5 mL diethyl ether. The extracts were evaporated and the residue was reconstituted with 2 times its volume of assay buffer (0.05 M borate buffer, pH 7.8, containing 0.5% bovine serum albumin). Estradiol 17 beta (E2), testosterone (T), and dihydrotestosterone (DHT) were determined by ELISA, following the methods of Asahina et al. (1995) [15]. Additionally, progesterone (P4) was determined using the Progesterone ELISA kit (Item No. 582601, Cayman Chemical Company, MI) according to the manufacturer's instructions. We made it a top priority to measure testosterone levels based on the volume of preserved plasma sample. Samples and standards were applied in duplicate to each plate. Based on the information from the supplier (Cosmo Bio Co., Ltd., Tokyo), the major cross-reaction of the E2 antibody for estrone was 0.8%, and 0.5% for estriol. The cross-reaction of the T antibody for DHT and 11-ketotestosterone (11KT) was 7.0% and 0%, respectively; the cross-reaction of DHT antibody for T and 11KT was 48% and 0%, respectively; the cross reaction of the P4 antibody for E2 and T was 7.2% and <0.05%, respectively.

Results

Increase in disc width and elongation of claspers
DW at birth of the individual was 182 cm. Growth data of DW are shown in Fig. 1b: DW increased from the birth to 5 years and 6 months of age, after which it remained at ca. 345 cm until the end of the study. At birth, its claspers were shorter than its pelvic fins (Fig. 2a). The organ elongated during development. At an examination at 5 years and 4 months of age, its length exceeded the pelvic fins already (Fig. 2b).

However, we could not confirm when exactly the clasper started to increase in size and exceeded the pelvic fins during our observation period. Furthermore its stiffness at this examination was similar to that of the mature individual No6.

Appearance of reproductive behavior
At 3 years of age (in June 2011; * in Fig. 5), we observed that No10–2 slowly chased females; this behavior is known as a "mating train" (Additional file 2: Movie S2). This behavior was observed several times over several days. At 5 years and 4 months of age (on October 6, 2013; ** in Fig. 5), No10–2 copulated with a female for the first time (Fig. 3; Additional file 3: Movie S3). The male chased the female actively (Fig. 3a), which attempted to position immediately over the dorsal surface of the female and cause the female to rear up, after then bit the tip of the female left pectoral fin, and then inserted its clasper into the female cloaca (Fig. 3b). During copulation, both sank to the bottom of the tank; once they reached the bottom, they swam away separately. The duration from biting the female left pectoral fin to the separation was 40–50 s. After the copulation, vigorous chasing of No10–2 continued for several days and then quieted. Two months after copulation, we confirmed that the female was not pregnant, using ultrasonographic imaging. At 5 years and 11 months of age (May 2014; *** in Fig. 5), the male chased the female actively for several days, but no mating was observed. When the individual reached 6 years and 11 months (May 2015; *** in Fig. 5), we observed active chasing without copulation for several days. And then, we again observed active chasing at 7 years and 2 months of age (August 2015) for a couple of weeks, followed by copulation at 7 years and 3 months of age (on September 2, 2015; ** in Fig. 5). Accordingly, we confirmed that the female was not pregnant.

Production of motile spermatozoa
At 5 years and 11 months of age, mobility of spermatozoa in the semen collected from No10–2 was confirmed (Fig. 4a, b; Additional file 4: Movie S4). The sperm had a
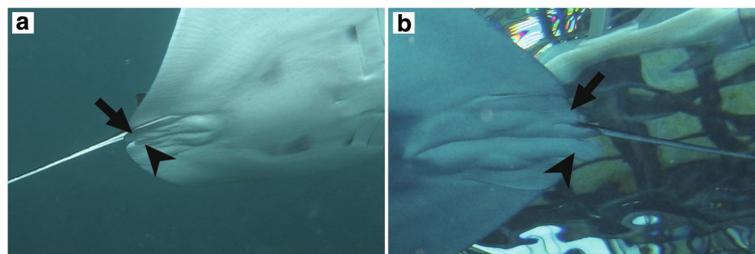

Fig. 2 Morphological changes in claspers. a Claspers at 4 months old (in October 2008). b Claspers exceeded the distal end of the pelvic fins at 5 years and 4 months of age (in October 2013). Arrowheads indicate claspers; arrows indicate pelvic fin
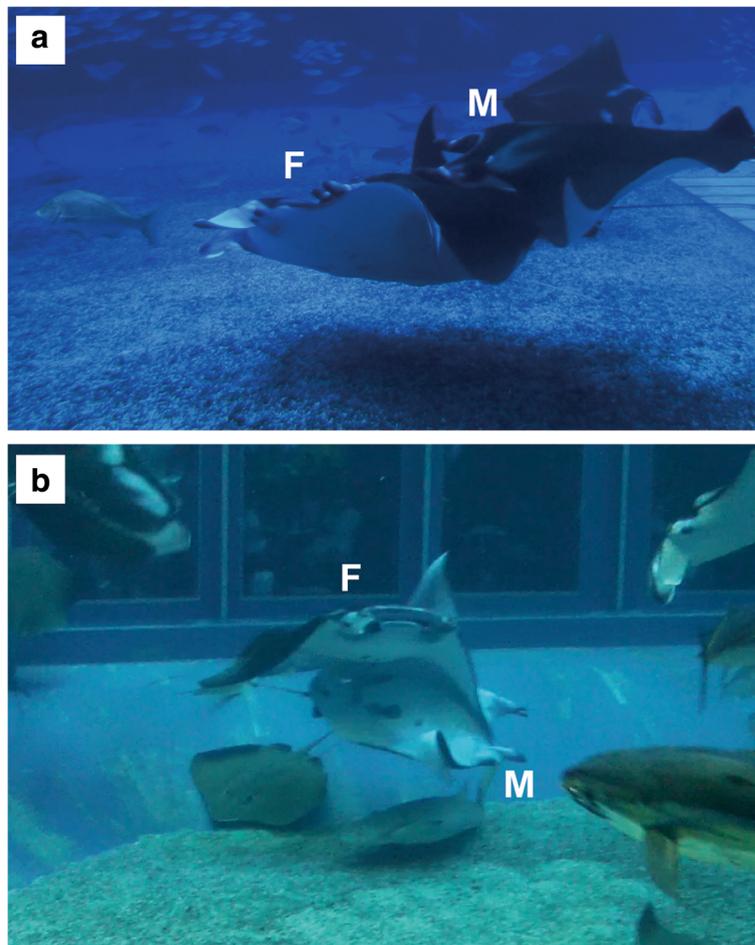
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Fig. 3 Snapshots of mating behaviors in No10–2. **a** Active chasing behavior observed in October 2013. **b** Copulation observed in October 2013

typical chondrichthian spiral head structure and rotated along the long axis (Fig. 4c).

Changes in the levels of steroid hormones

Changes in the concentrations of steroid hormones of No10–2 are shown in Fig. 5 and Additional file 5: Table S1. Androgens levels were stable from 3 years of age.

Irregular peaks of testosterone levels were detected from age 6 onwards. Variation in dihydrotestosterone showed a pattern similar to that of testosterone. Estrogen showed basal level and did not range dramatically throughout the observation period. Progesterone level increased at 6 years of age, from February to June, then decreased. At 3 years of age, androgen levels of the
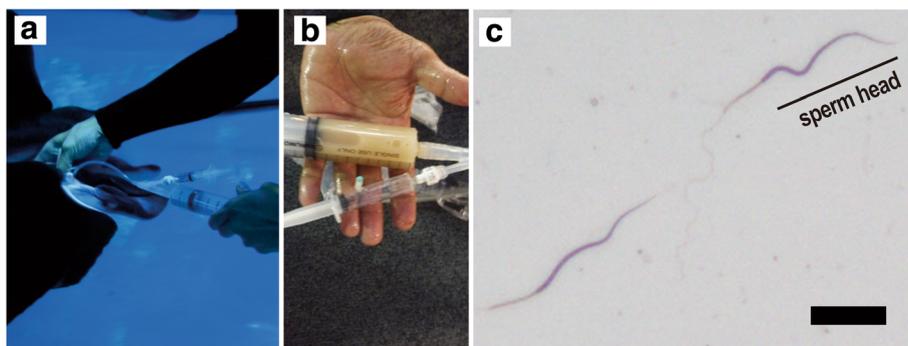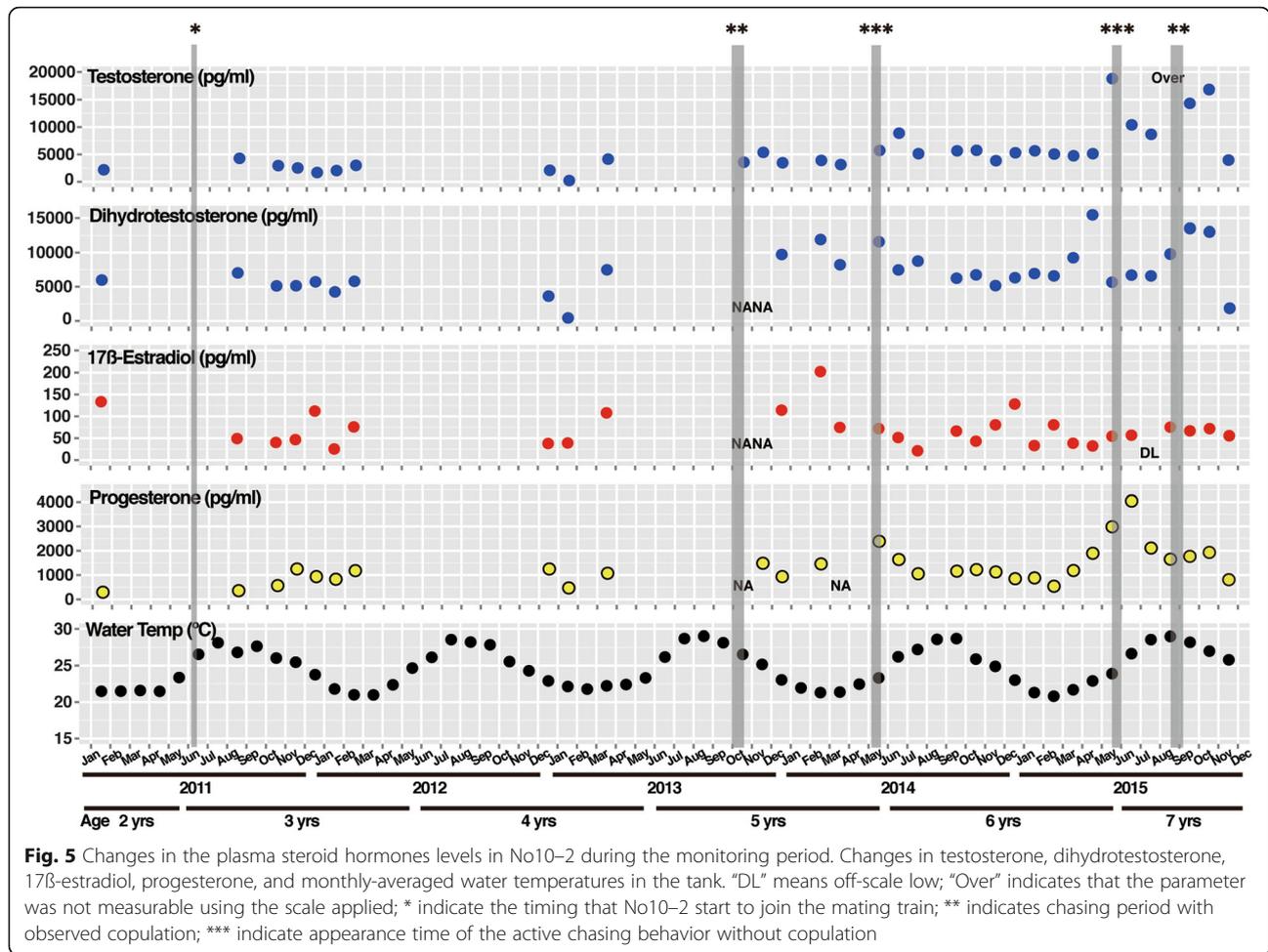

Fig. 4 Semen from No10–2. **a** Plastic tube inserted into the clasper. **b** Collected semen. **c** Giemsa-stained sperm. Scale bars = 20 μm
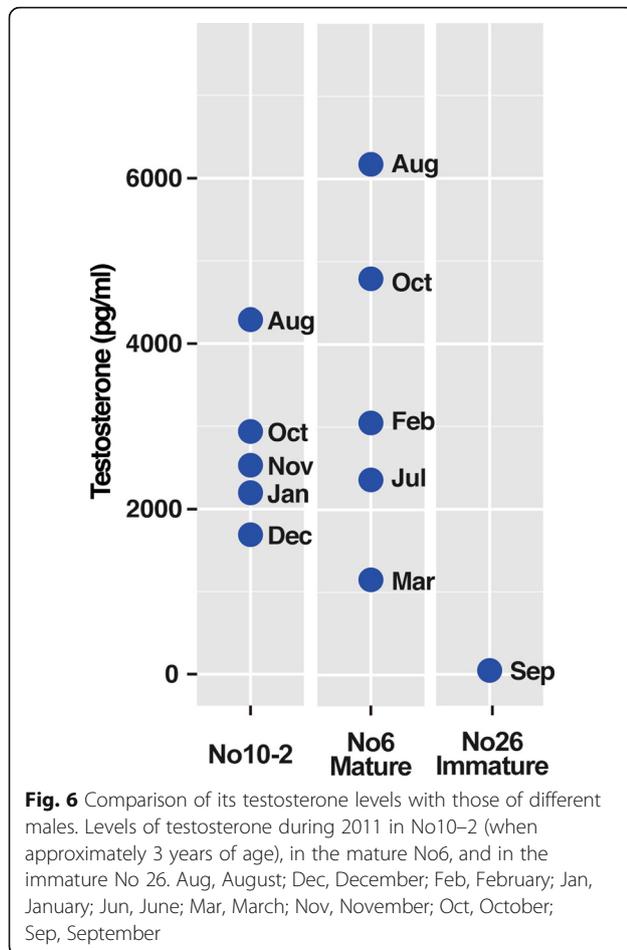
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Fig. 5 Changes in the plasma steroid hormones levels in No10–2 during the monitoring period. Changes in testosterone, dihydrotestosterone, 17ß-estradiol, progesterone, and monthly-averaged water temperatures in the tank. "DL" means off-scale low; "Over" indicates that the parameter was not measurable using the scale applied; * indicate the timing that No10–2 start to join the mating train; ** indicates chasing period with observed copulation; *** indicate appearance time of the active chasing behavior without copulation

studied specimen was within the range of androgen levels of the matured male (No6; Fig. 6, Additional file 6: Table S2), which in turn was approximately 25–135 times higher than that of the immature male (No26; Fig. 6, Additional file 7: Table S3). Immediately prior to the chasing behaviors seen in May and August 2015, increases in testosterone levels were observed, especially for T levels in August that exceeded the upper threshold of the standard curve (Fig. 5).

Discussion

It has been previously reported that the age and DW at maturity in males was 3–6 years and 270–300 cm, respectively [8, 16]. Our observations of reproductive age in captivity are consistent with these field observations [8]. On the other hand, regarding DW, when No10–2 was only 2 years and 6 months old — that is, 6 months younger the minimum age for maturity than in a wild male — No10–2 already had exceeded the DW size at maturity (300 cm) of wild males. However, although the testosterone level is already higher than in an immature animal, the animal did not show chasing or

mating behavior at this time. These results suggest that age might be a more important factor than attaining sexual maturity size in *M. alfredi*. In contrast, it has been reported that the timing of pubertal development in male bonnethead sharks, *Sphyrna tiburo*, may be more associated with its size than its age [17]. On the basis of these disparities, it is likely that the limiting factor of sexual maturation in elasmobranchs depends on the particular species and/or its environment. Moreover, the results of the present study implied that that growth rates and possibly size at maturity might be quite plastic in *M. alfredi*, and very dependent on the individual's experience.

Clasper length and calcification are often-used criteria of male sexual maturity in elasmobranchs, in that a rapid increase in clasper length marks the onset of sexual maturity [18]. Under these criteria, the length of the claspers of No10–2 had already exceeded that of the pelvic fins, and its stiffness at 5 years and 4 months of age was similar to that of matured No6, this suggested that No10–2 had completed sexual maturation by this age. However, we could not confirm when exactly the clasper started to increase in size and exceeded the

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Fig. 6 Comparison of its testosterone levels with those of different males. Levels of testosterone during 2011 in No10–2 (when approximately 3 years of age), in the mature No6, and in the immature No 26. Aug, August; Dec, December; Feb, February; Jan, January; Jun, June; Mar, March; Nov, November; Oct, October; Sep, September

pelvic fins during our observation period. Therefore, in order to determine whether the timing of clasper elongation is consistent with other characteristics of sexual maturation (especially mating behavior and/or steroid hormone levels), further detailed observations are needed if a new male is born.

In this study, we observed that a *M. alfredi* born in captivity could exhibit mating behavior in the tank. Mating behavior of the giant manta ray, *M. birostris*, was observed in detail at Ogasawara Islands, Japan [12]; in this study, the serial mating behavior was classified into five steps: chasing, biting, copulating, post-copulating, and separating. In the case of *M. alfredi*, mating behavior observed in Mozambique also followed these five steps [7]. The present observations revealed that the captive male manta ray is able to engage in serial mating behavior typical of that seen in the field.

The first copulation of No10–2 was observed at 5 years and 4 months of age (in October 2013), a second one at 7 years and 3 months of age (in September 2015). Because this behavior was observed during visual monitoring in the day, we were unable to eliminate the

possibility that any other occasion may have been overlooked. However, in here, we discussed based on the copulations we observed actually. Most of the copulations in *M. alfredi* at the OCA were observed from May to July, indicating that their breading season in captivity lasted for several months [10]. However, copulation in 2015 occurred in September, which differed from that in the past. In Hawaii and the Maldives, *M. alfredi* exhibits two mating seasons in a given year [7, 19]. For Hawaii, it was suggested that females that are unsuccessful in getting pregnant during the first mating season may mate again during the second season [19]. In the present study, copulation occurred in September, which was an unusual time at the OCA, presumably because of the failure to copulate successfully during the ordinary breeding season (May to July).

The results of the present study showed the changes in steroid hormones over time in a male *M. alfredi* born in captivity. In particular, it is reported that the increase in androgens could be associated with puberty in male elasmobranch. In male bonnethead sharks, *S. tiburo*, androgen increased during puberty [17]. Additionally, in a male whale shark kept for over 20 years in the OCA, its testosterone levels definitely increased during the dramatic extension of his claspers (Matsumoto et al., personal communication), indicating that testosterone levels increased during sexual maturation in male whale sharks as well. On the other hand, in the present study, the androgen level of No10–2 did not exhibit such an upward trend during monitoring. Furthermore, testosterone levels in another mature male (No6) were the same range as those of No10–2 at 3 years of age (in 2011), whereas testosterone levels in the immature male (No26) were extremely lower than those in No10–2. On the basis of these facts, it is highly likely that No10–2 had already started the sexual maturation process endocrinologically before 3 years of age.

Conclusion

Although born in captivity, a male *M. alfredi* reached sexual maturity at the age of 5 years. This fact raises the possibility of complete breeding of *M. alfredi* in captivity. Importantly, the present study is the first to provide endocrinological data on a male *M. alfredi* in captivity. Since there have been limited published reports about captive breeding or copulation of *M. alfredi*, it is important that the present information obtained at the OCA be shared to achieve breeding of *M. alfredi* at other institutions. We hope to contribute to a better understanding of the reproductive biology of *M. alfredi* through long-term observations in captivity.

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Additional files

Abbreviations
DHT: Dihydrotestosterone; DW: Disc width; E2: Estradiol 17 beta; IOD: Interorbital distance; P4: Progesterone; T: Testosterone; The OCA: Okinawa Churaumi Aquarium

Availability of data and materials
Measurement value of steroid hormones supporting the conclusions of this article is included within an Additional file 5.

Authors' contributions
RN, MN and KS conceived of the study, and participated in its design, and wrote the manuscript. RN performed steroid hormones assay. KM, RM, NY, YM, MY and KU collected blood samples, carried out the morphometry and observed behaviors. KM and RM carried out the semen collection and observation. All authors read and approved the final manuscript.

Ethics approval and consent to participate
Animal handling and sample collections were conducted by veterinary staffs in accordance with the Husbandry Guidelines (in Japanese) approved by the Ethics and Welfare Committee of Japanese Association of Zoos and Aquariums.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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