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RESEARCH ARTICLE

# Ultrasound Examination and Behavior Scoring of Captive Broadnose Sevengill Sharks, *Notorynchus cepedianus* (Peron, 1807)

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Serial ultrasound examination of four mature female sevengill sharks (*Notorynchus cepedianus*) was carried out over 18 months. Monitoring the reproductive cycle and development of follicles and fetuses in sharks in a noninvasive manner using this technique has not been reported previously. Sharks were caught out of the "Oceanarium" tank by divers using a specially made catch-out bag, and brought to a holding area for examination. A behavior scoring system was used to monitor the impact of regular handling on the well-being of the animals. Ultrasound showed the growth and regression of follicles in sevengill ovaries, and allowed an approximation of the reproductive stage of these sharks. Monitoring behavior at five time points during the procedure showed that regular handling of sharks for clinical studies could be done with minimal impact on animal welfare. The ability to follow reproductive events in elasmobranches using ultrasonography is an important step in the application of assisted reproductive technology in these species. Assisted reproductive technology , such as monitoring female reproductive cycles and artificial insemination, could potentially be used to

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maintain genetic diversity and compliment aquaria-based breeding programs for endangered species such as the gray nurse shark (*Carcharias taurus*). Zoo Biol 26:1–13, 2007. 

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## INTRODUCTION

Captive breeding programs for large sharks are becoming increasingly important, especially for existing display species that are threatened in their natural habitat. The low fecundity of large sharks in captivity [Kirby, unpublished data] indicates a need for aquaria to take an active role in shark reproductive research. Research into shark reproduction in aquaria involves repeated serial examinations over time that requires careful management to minimize stress to the animals.

Ultrasonography is commonly used to assess internal organs, soft tissue damage, and pregnancy in human and veterinary medicine [Andrew, 1964; Miller et al., 1982; Pipers, 1982; Bondestam et al., 1983; Pipers et al., 1984]. Walsh et al. [1992] tested the use of ultrasonography in 11 sharks from three species (*Ginglymostoma cirratum*, *Negaprion brevirostris*, and *Sphyrna tiburo*) and showed for the first time that this technique could be successfully applied to sharks. This was further expanded by Carrier et al. [2003] who used mating observations and ultrasound in the field to select five pregnant *G. cirratum*. These sharks were then housed in captivity, and ultrasound was carried out monthly throughout pregnancy, enabling the investigators to observe fetuses in utero and determine the length of gestation.

The use of ultrasonography in sharks housed in aquaria has so far been limited to individuals whose reproductive status is already known [Carrier et al., 2003]. This is often difficult to determine when dealing with long-term captive populations of large viviparous sharks, especially if mating is sporadic owing to a range of environmental factors. Ultrasonography has the potential to determine and monitor stages of the reproductive cycle, assess maturity of female sharks, and provide valuable knowledge required for assisted breeding programs in a noninvasive manner. This technique could be used with or without blood collection and steroid hormone analysis, that has been used in the past to determine elasmobranch reproductive cycles in an experimental setting [Callard et al., 1988; Callard and Koob, 1993; Ramussen and Gruber, 1993; Hamlett and Koob, 1999; Koob and Callard, 1999]. Difficulties of hormone assay development and validation, and of serial sampling limit the use of hormone analysis by aquaria.

Carrier et al. reported that the stress of repeated handling, and the effect that this can have on embryonic survival, could be a limiting factor on the regular use of ultrasonography in sharks. If ultrasound is to be applied serially to a small population in a captive setting, it is important to be able to assess the effect of regular handling, and to recognize the signs of excessive stress during examination so that an individual can be released before damage occurs.

Our study was based on clinical observations in the broadnose sevengill shark, *Notorynchus cepedianus*, that is a common species in the temperate waters off the southern and eastern coasts of Australia [Last and Stevens, 1994]. Their reproductive mode is classified as lecithotrophic viviparity [Hamlett, 2005] so developing fetuses gain nutrients solely from an external yolk sac. Follicles in the paired ovaries can

grow to over 7.0 cm in diameter at ovulation [Ebert, 1989, 1996], and females have been reported with up to 82 young per litter [Ebert, 1989]. All female *N. cepedianus* used in this study were considered sexually mature, based on their size and estimated ages, but there has been no successful mating or pregnancy within the population so their reproductive status was unknown. The objectives of this work were to test the effectiveness of regular ultrasound examinations in monitoring reproductive cycles in female sharks, and to determine if reproductive cycles were occurring in the female *N. cepedianus* population at Melbourne Aquarium. A further aim was to monitor behavior subjectively throughout the study to assess the degree of stress being imposed by the examinations, and to determine whether these responses changed over time.

## METHODS

### Animals

The population of *N. cepedianus* used in this study has been housed at Melbourne Aquarium since January 2000 (with the exception of one animal collected from the wild in July 2004). All sharks were wild-caught for aquarium display purposes. Sharks were housed in the "Oceanarium" tank on display at Melbourne Aquarium, containing 2.2 million liters of oxygenated seawater. The aquarium operates as a closed system, and Oceanarium water is treated by mechanical, chemical, and biological filtration at a rate of 440,000 L/hr. Water temperature over the study period ranged from 17 to 22.5°C to coincide with local seasonal variation.

Each shark was identified by differences in fin or body shape and individual markings (e.g. skin pigments, spots, scars) and referred to by name (Storm, Dusky, Bella, Lonnie). Female *N. cepedianus* are reported to mature at around 220 cm total length (TL) [Last and Stevens, 1994]. All the four sharks used in this study were of 240–265 cm TL (Storm 242 cm, Dusky 265 cm, Bella 249 cm, Lonnie 260 cm), so were considered mature.

### Capture and Handling

*N. cepedianus* were caught out of the Oceanarium by four aquarium divers in self-contained underwater breathing apparatus (SCUBA) equipment, using a 300 cm long "catch-out bag" made of flexible clear vinyl. The entrance to the bag was 150 cm in diameter and tapered to 50 cm in diameter at the closed end, giving the bag a conical shape. A Velcro (Velcro Australia; Hallan, Victoria, Australia) seal along the top surface allowed access to the shark. The narrow, closed, end of the bag was covered with a nylon mesh to enable water flow to be directed toward the face of the shark. Three divers operated the bag, one at the closed end and two at the entrance. A fourth diver guided the shark into the bag, that was closed behind it and sealed with Velcro straps. The bag and shark were then brought into the lock (Fig. 1), that is a holding area connected to the Oceanarium, containing approximately 17,500 L of water at a depth of 100 cm. Oxygen was supplied via a hose and air stone, placed near the sharks' head, connected to a D-class cylinder containing medical grade oxygen. Water flow from a 5-cm diameter hose connected to a recirculation pump was directed toward the face and mouth of the shark, to ensure adequate flow across the gills. The shark remained in the catch-out bag during the examination, and dissolved oxygen concentration near the head and behind the gills was monitored

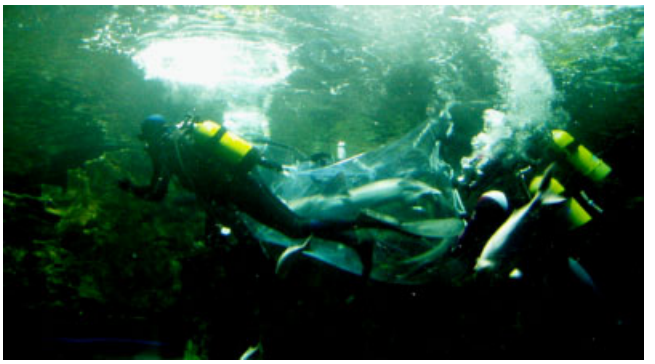

Fig. 1. Capture of *Notorynchus cepedianus* from the Oceanarium, showing divers directing the bag with the shark inside toward the examination area.

throughout the procedure with a dissolved oxygen meter (YSI 550, YSI Environmental, Yellow Springs, OH).

## Ultrasound Examination

The shark was accessed by the Velcro-lined opening along the top of the catch-out bag, placed in dorsal recumbency, and supported against the wall of the lock at the water surface. Ultrasonography was carried out using an Aloka SSD500 machine with a 3.5 MHz convex sector/linear scanner (Aloka, Tokyo, Japan). A plastic bag containing a small amount of ultrasound coupling gel was wrapped around the probe to protect it from damage by the shark's skin and seawater. The probe was placed in a transverse plane on the skin surface and moved slowly from anterior to posterior, beginning anterior to the pectoral girdle and finishing at the pelvic girdle (Fig. 2). A sagittal orientation was also used to assist in the identification of various organs. Images were recorded using a videocassette recorder (VCR) and television connected to the ultrasound machine. The largest diameter of eight to ten randomly selected follicles was measured using the distance tool on the ultrasound machine and the results recorded. Sharks were released after a maximum of 10 min in the lock and their recovery was observed by divers who were on hand to provide assistance if necessary.

Individual sharks were examined at intervals of 6–8 weeks over a total period of 18 months, and each examination lasted no more than 10 min. Curatorial staff observed the animals daily to assess general health and well-being of all the sharks.

## Behavior Scoring

For each catch-out, the reaction of the shark to the catching and examination process was scored for each of five time points during the procedure (Table 1).

Scores at each time point were added together to give an overall score for the examination, and these overall scores were used for subsequent statistical analyses. The same observer was responsible for assessing behavior scores for all the examinations.

## Statistical Analyses

Serial follicle diameter measurements from each shark were analyzed using the Repeated Measures General Linear Model within SPSS (Version 12.0.1, SPSS Inc.,

Fig. 2. The ultrasound procedure, showing the shark in dorsal recumbency and ultrasound being carried out.

Chicago, IL), followed by Tukey's Honestly Significant Difference post-test. Results for Lonnie in June/July 2005 were not included in statistical analyses as the ovoid structures imaged were not located in the ovary. Behavior scores were analyzed using the one-way analysis of variance and paired *t*-test functions within SPSS (Version 12.0.1, SPSS Inc., USA).

## RESULTS

### Ovarian Cycles

Ultrasound allowed easy visualization of ovarian follicles. Follicles seemed as distinct, ovoid structures, of low sonographic contrast with a clearly defined periphery (Figs. 3 and 4). They were most commonly located near the ventral midline in the anterior portion of the body cavity. The exception was in the June and July (2005) examinations of Lonnie, where ovoid structures were observed in the posterior part of the abdomen.

Two sharks, Lonnie (Fig. 5a) and Dusky (Fig. 5b), showed a significant increase in mean and maximum follicle diameter over the study period ($P<0.05$; 3.62–5.50 and 1.58–3.61 cm diameter, respectively). One shark, Storm (Fig. 5c), showed a significant decline in mean and maximum follicle size over the study period ($P<0.05$). The fourth shark, Bella, displayed no discernable trend (Fig. 5d), with no significant difference in mean follicle diameter between the first examination

**TABLE 1. Scoring system for behavior of sharks at five time points (i–v) during capture, examination by ultrasound, and release**

| Time point/score | Criteria |
|---|---|
| **(i)** Before capture | |
| 1 | Swimming normally with normal respiration |
| 2 | Abnormal swimming pattern and/or respiration, mild |
| 3 | Abnormal swimming pattern and/or respiration, moderate |
| 4 | Severe abnormal swimming pattern and/or respiration leading to abandonment of catch-out |
| 5 | Very severe abnormal swimming pattern and/or respiration leading to abandonment of catch-out |
| **(ii)** During capture, from divers guiding sharks toward the catch-out bag to when the shark in the bag arrived at examination area | |
| 1 | Minimal attempt at avoiding the bag or trying to escape |
| 2 | Slow avoidance behavior, weak attempts at turning in the bag and escaping |
| 3 | Moderate avoidance behavior, strong attempts at turning in the bag and escaping |
| 4 | Rapid movement away from the bag and vigorous attempts at escaping |
| 5 | Rapid and vigorous escape movements ending in abandonment of the attempted catch-out |
| **(iii)** During handling, restraint and turning to dorsal recumbency | |
| 1 | Minimal active twisting of the body resulting in easy and quick turning of the shark |
| 2 | Weak resistance with short-lived body movements, easily overcome to turn the shark |
| 3 | Moderate prolonged resistance (to 15 sec) and moderately strong muscular contractions resulting in moderate effort needed by the operators to restrain and turn the animal |
| 4 | Active and prolonged resistance ($>15$ sec) with strong twisting and tail thrashing, animal turned and restrained with some difficulty |
| 5 | Active and prolonged resistance ($>15$ sec) with strong twisting and tail thrashing leading to abandonment of the procedure |
| **(iv)** During examination while in dorsal recumbency | |
| 1 | Shark quiet throughout with no resistance |
| 2 | Weak body twisting movements, short lived, requiring mild pressure of hands to restrain |
| 3 | Moderate body twisting movements restrained with moderate effort but not interfering with ultrasound examinations |
| 4 | Moderate to strong twisting movements interrupting the ultrasound examination and requiring moderate effort to restrain |
| 5 | Strong twisting movements and efforts to escape requiring the examination to be terminated |
| **(v)** After release | |
| 1 | Swimming normally with normal respiration |
| 2 | Abnormal swimming pattern and/or respiration, mild |
| 3 | Abnormal swimming pattern and/or respiration, moderate |
| 4 | Severe abnormal swimming pattern and/or respiration requiring diver assistance |
| 5 | Very severe abnormal swimming pattern and/or respiration requiring diver assistance |

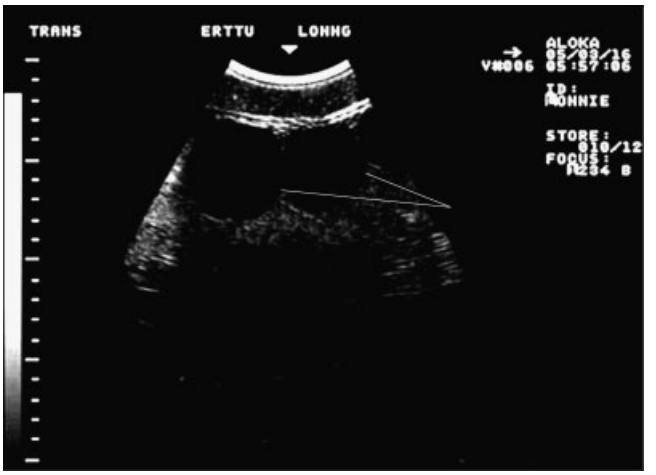


Fig. 3. Ultrasound image of Lonnie showing large follicles with clear structure.

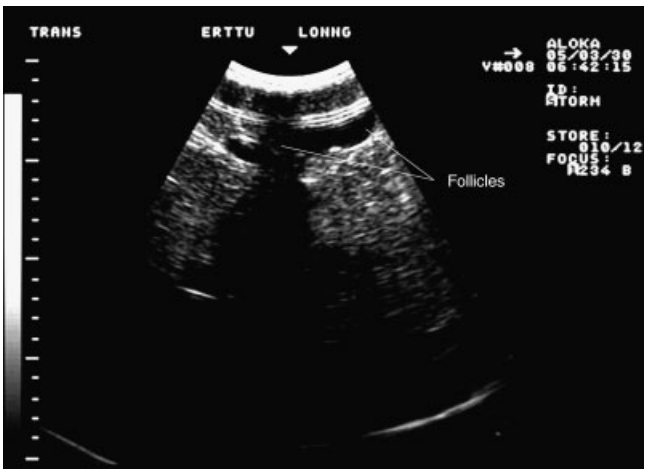


Fig. 4. Ultrasound image of Storm showing follicles with uneven shape and size.

($2.63 \pm 0.55$ cm) and the last ($2.59 \pm 0.22$ cm) ($P = 1.00$). Large numbers of ovarian follicles ($>40$) were observed in Lonnie (Fig. 3), who was wild-caught in July 2004. Serial measurements of ovarian follicles in this shark showed an increase in mean follicle diameter over the study period, starting at $3.62(\pm 0.46)$ cm in August 2004 and leveling out at a maximum of $5.50(\pm 0.34)$–$5.60(\pm 0.38)$ cm from March to June 2005 (Fig. 5a). Ovarian follicles in Storm, who had been in captivity since 2000, seemed much smaller and had a less distinct, irregular, structure and shape (Fig. 4). The mean follicle diameter was observed to decrease by 3.07 cm over 12 months of the study period, from $4.25 \pm 0.07$ cm in June 2004 to $1.18 \pm 0.13$ cm diameter in June 2005 (Fig. 5c).

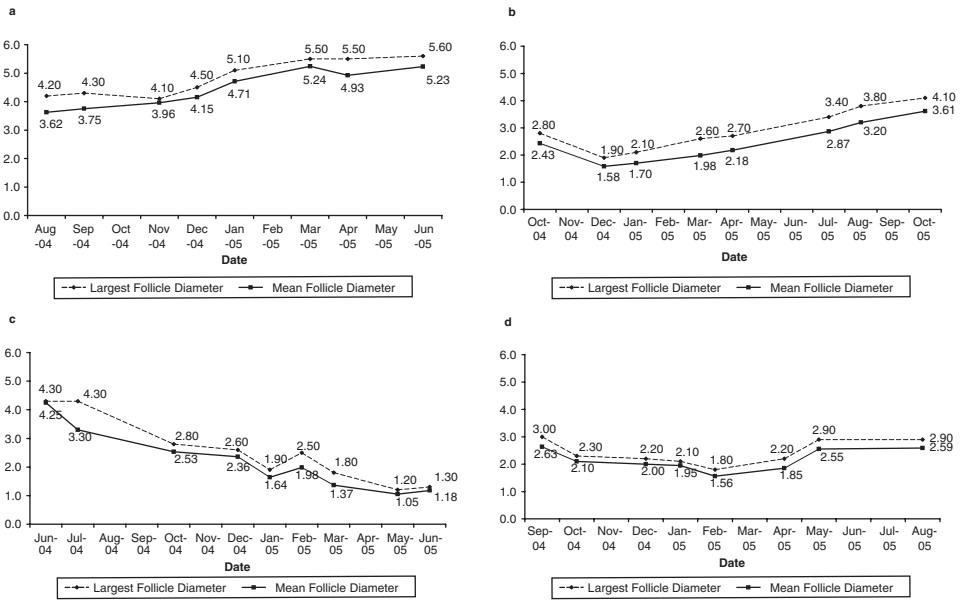


Fig. 5. Changes in follicle size observed over the study period for Lonnie (a), Dusky (b), Storm (c), and Bella (d).

## Behavior Scoring

Scores of 1–3 were given at all five time points in most examinations (81% of 41 examinations), but scores of 4 were recorded in seven examinations (17% of 41 examinations) (Fig. 6). The scores of 4 were spread evenly across the four sharks, and the single score of 5 (Dusky) was in response to an unexplained continued resistance to handling that prompted immediate release of the shark. Mean behavior scores for all sharks were lowest before capture ($1.11 \pm 0.16$) but increased during capture ($2.44 \pm 0.15$). Highest scores were observed during handling and restraint ($2.70 \pm 0.38$), whereas scores during examination ($2.24 \pm 0.18$) and after release ($2.11 \pm 0.16$) were relatively low. Standard deviations in Figure 6 indicate that relatively low scores of 2–3 can be expected in most examinations.

Individual sharks responded differently to capture and handling, but only two sharks were significantly different from each other in this response (Storm and Dusky, $P < 0.05$). Storm remained reasonably calm throughout the procedure, as indicated by minimal change in mean scores over the five time points (Fig. 7). Dusky showed a strong initial response to capture and handling but then settled rapidly once placed in dorsal recumbency, remaining calm throughout the actual ultrasound procedure. This is shown by the relatively high mean scores during handling and restraint, and the low mean score during examination (Fig. 8). Dusky also consistently displayed avoidance behavior at the beginning of catch-out, moving to the other side of the Oceanarium when the divers entered with the catch-out bag. The response of individual sharks to the examination procedure did not change significantly during the course of the study (Storm, $P = 0.099$; Dusky, $P = 0.374$; Bella, $P = 0.374$; Lonnie, $P = 0.374$). Similarly, there was no change in the response

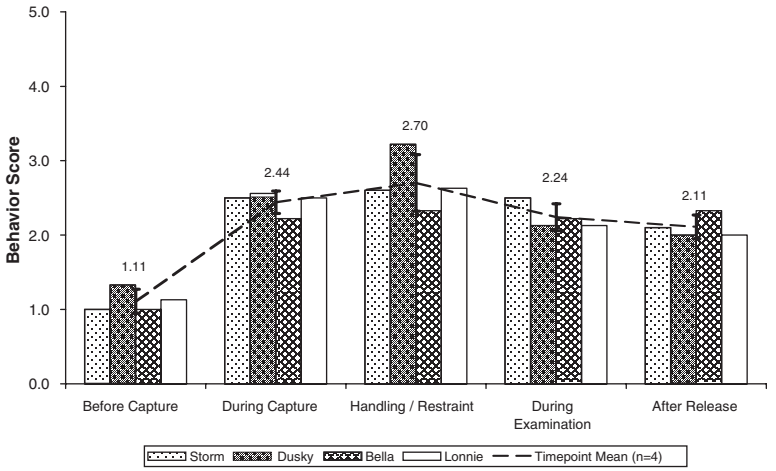


Fig. 6. Mean individual behavior scores and mean group scores for the four sharks at five time points. Error bars show $\pm 1$ SD, $n = 8$ observations for each shark at each time point.

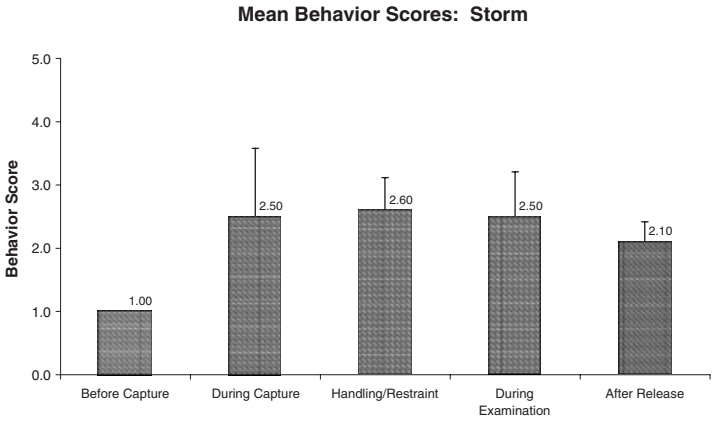


Fig. 7. Individual mean behavior scores for Storm.

to the examination procedure when the population was considered as a whole ($P = 0.139$). Curatorial staff observed no adverse effects on behavior and well-being of the sharks over the study period.

## DISCUSSION

This is the first report of ultrasound being used successfully to assess the female reproductive status in a long-term captive population of elasmobranches. The increase in mean and maximum follicle diameter observed in Lonnie and Dusky over the sample period indicates that they have been in the follicular growth stage of the reproductive cycle. Ebert [1989, 1996] found that follicles could reach over 7.0 cm in diameter in the ovaries of wild sevengills caught off the coasts of South Africa and

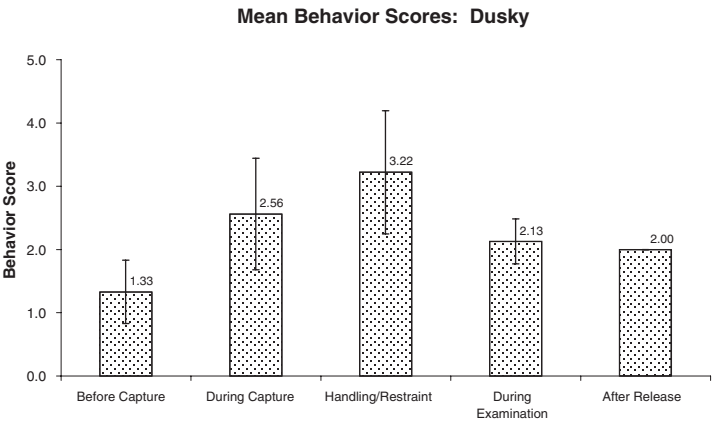


Fig. 8. Individual mean behavior scores for Dusky.

northern California, and proposed that ovulation and fertilization occurred at around 7.5 cm in diameter. Follicle diameters measured in Dusky between December 2004 ($1.58 \pm 0.26$ cm) and October 2005 ($3.61 \pm 0.26$ cm) indicate that she was in the early stages of yolk accumulation. One month after capture from the wild, Lonnie's mean follicle diameter was $3.62 \pm 0.46$ cm (August 2004), and over the past year has increased by 1.62–$5.24 \pm 0.34$ cm (March 2005). The mean follicle diameter reached a plateau at this size, with the largest follicle diameter remaining at 5.50–5.60 cm from March to July 2005. From the data presented by Ebert [1996], there is a correlation between the TL of the female, oviducal gland width, and maximum follicle diameter. His data indicate that larger, and presumably older, females have a higher fecundity and larger follicle diameter than smaller females that may have had fewer reproductive cycles. At around 260 cm of TL Lonnie is not yet fully grown, so it is possible that her follicles reached ovulatory size at 5.50–5.60 cm diameter rather than 7.00–7.50 cm reported for large females in the wild [Ebert, 1989, 1996].

This is supported by the observation (on June 1, 2005) that ovoid structures thought to be ova were present for the first time in the posterior portion of the body cavity, all the way to the pelvic girdle. This suggests strongly that ovulation occurred and ova were now in the uterus. The *N. cepedianus* population at Melbourne Aquarium includes a mature male, so copulation in captivity is possible. Mating bites were observed on Lonnie's abdomen and pectoral fins at the time of capture, suggesting that she had recently mated in the wild. Fresh mating bites were also observed in captivity in January 2005, so it is possible that sperm storage and multiple paternity will be involved if pregnancy occurs.

Ebert [1989, 1996] proposed that accumulation of yolk and growth of follicles occurred over a 12-month period, from small ($< 1.8$ cm diameter), white, oocytes in the ovaries to the final ovulation of large yolky ova. The evidence gathered so far (Fig. 3a and b) indicates that yolk accumulation may occur over a much longer period in captivity, possibly up to 2 years. Lonnie and Dusky showed an increase in follicle diameter of 0.23 and 0.18 cm per month, each over a 7-month period. If this rate (0.2 cm/month) is consistent until ovulation, a diameter increase from 1.8 to 7.0 cm [Ebert, 1989, 1996] would take 2 years approximately. An 18–24-month

period of follicular growth would be consistent with other lecithotrophic viviparous species, that often require well over 12 months to accumulate yolk [Hamlett and Koob, 1999]. Although the rate of follicular growth was similar between Lonnie (recently wild-caught female) and Dusky (long-term captive female), more data are needed to determine whether the increased vitellogenic period observed indicates a longer vitellogenic period for the Australian population of this species, or if it is an artifact of the captive environment.

The decrease in follicular diameter and change in shape observed in Storm indicate that atresia and yolk resorption was occurring. Follicle atresia has been observed in many species of shark [Hamlett and Koob, 1999], and is the expected fate of anovular follicles in a normal reproductive cycle. On the basis of reviews of elasmobranch reproductive behavior, vitellogenesis occurs either concurrent with pregnancy, or after a quiescent period after parturition, and is followed by mating and ovulation [Hamlett and Koob, 1999; Carrier et al., 2004]. Atresia of follicles in Storm is unexplained but could be part of a natural cycle or influenced by captivity. As yolk resorption and follicle atresia have not been monitored in shark species in the past, it is unknown how long the atretic phase will last.

The lack of any discernable trend in Bella's follicle data is unexplained, and is different from the other sharks used in this study. Ovarian acyclicity can occur in animals for many reasons. Bella sustained a muscular injury after a weighing attempt before the study period, and it is possible that this affected her reproductive cycle. The adverse effects of stress on the reproductive cycle of nonmammalian vertebrates have been well established [Contreras-Sanchez et al., 1998; Pankhurst and Van Der Kraak, 2000; Ganesh and Yajurvedi, 2002]. Importantly, the process of vitellogenesis can be affected by stress in the rainbow trout (*Oncorhynchus mykiss*) [Contreras-Sanchez et al., 1998] and the lizard *Mabuya carinata* [Ganesh and Yajurvedi, 2002]. In the latter study, seasonal resumption of ovarian activity was impaired by stress. The authors concluded that this was due to impairment of vitellogenesis by an apparent decrease in the serum follicle-stimulating hormone and estrogen. The stress caused by a muscular injury may have had a similar effect on Bella's reproductive cycle, resulting in the asynchronous pattern of follicular development observed during the study. Bella's behavioral reaction to examination was not significantly different from sharks that showed follicular activity (i.e. growth or regression). Hence, there was no reason to link her acyclicity to the examination process. Continued monitoring and blood hormone assays could help to distinguish the abnormal from normal function in this case.

The behavior scoring system implemented during this study has allowed assessment of the effects of capture and restraint on the well-being of the sharks. In any captive study on a wild species, it is necessary to have an understanding of the effect that the study has on the animals. Analysis of corticosteroid concentrations in elasmobranch blood serum has been done in the past [Ramussen and Crow, 1993; Ramussen and Gruber, 1993], but is limited by the time delay associated with analysis of results and the fact that handling and blood sampling cause stress. This makes it difficult to get baseline levels for comparison and is more appropriate for chronic rather than acute stress. Although behavioral observations are subjective, they allow an instant assessment of the condition of the shark as well as displaying trends over time. As a result, clinical examinations can be adapted for different situations and animal temperaments, and impact on the shark can be minimized.

Scores for the four sharks have shown a behavioral trend that occurs across the catch-out process, from first diver interaction to release back into the Oceanarium. Agitated behavior generally increases from the time of capture through handling and restraint in the lock, culminating in the divers turning the shark onto its back. Once the shark is in dorsal recumbency, agitated behavior and resistance to restraint decreases and ultrasound can usually be carried out without difficulty. High scores were quite uncommon, as shown by the relatively low mean scores for each shark across all time points. There was no discernable increase in scores over the study period, indicating that there has not been an effect on the long-term stress levels of the sharks resulting from the work. These data indicate that the sharks neither developed an aversion, nor became acclimated, to the capture and examination procedure. Similarly, curatorial staff reported no adverse effects on the behavior and well-being over the study period.

## CONCLUSIONS

1. Ultrasonography can be effectively used as a noninvasive means of assessing and monitoring reproductive status of large sharks from long-term populations in aquaria.
2. Yolk accumulation and follicular growth in captive *N. cepedianus* ovaries is longer than that reported for this species in the wild, and could take up to 18–24 months.
3. Long-term clinical studies, involving regular capture and handling, can be carried out on captive shark populations with minimal effect on their well-being. Agonistic behavior during ultrasound examination was minimal.
4. The ability to follow reproductive events in elasmobranches using ultrasonography is an important step toward the long-term goal to apply assisted reproductive technology to these species. Assisted reproductive technology could be used to maintain genetic diversity and compliment aquaria-based breeding programs for endangered species such as the gray nurse shark (*Carcharias taurus*).

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