Chapter 4

Clinical studies in captive elasmobranchs

4. Clinical studies in captive elasmobranchs

4.1. Introduction

There are an estimated 140 species of elasmobranch held in aquaria worldwide (Firchau et al. 2004) for display, conservation, and education purposes. With the exception of species that are endangered in their natural habitat, elasmobranch populations held in aquaria are usually caught from the wild as required. The difficulties associated with maintaining both elasmobranchs and the environment in which they are housed, has meant that captive breeding programs for these species are not as extensive or developed as those for terrestrial species held in zoological parks (Choromanski 2004). Aquarium breeding programs for elasmobranchs traditionally rely on the occurrence of natural mating. This approach has been successful in breeding many species of elasmobranch in aquaria (Henningsen et al. 2004), but success rates vary greatly between species. Some species regularly reproduce in captivity (e.g. smooth stingray, *Dasyatis brevicaudata*), while others reproduce sporadically (e.g. grey nurse shark, *Carcharias taurus*), or not at all (e.g. broadnose sevengill shark, *Notorynchus cepedianus*).

Captive breeding programs for elasmobranchs are becoming increasingly important, especially for display species that are threatened in their natural habitat. This is the case with *C. taurus*, which is a popular aquarium species with a long history of aquarium display. The New York aquarium, one of the first public aquariums in the world, displayed *C. taurus* as early as 1896. *C. taurus* are held in aquaria all over the world, including five out of six major public aquaria in Australia. Despite this long history as an aquarium species, *C. taurus* have traditionally been wild caught for display with very few individuals captive born. In the wild, *C. taurus* are classified by the International Union for Conservation of Nature (IUCN) as threatened worldwide. In Australia, the east coast population is classified by the federal government as critically endangered (*Commonwealth Environment Protection and Biodiversity Conservation Act 1999*), with estimates of only 300 individuals remaining.

A range of factors, including sub-optimal population structure and environmental factors created by the need to cater for multiple species in a single tank

or system, can limit the success of breeding programs for large sharks in aquaria The relatively small elasmobranch populations held by aquaria (compared to those in the wild) can also result in a loss of genetic diversity even when successful breeding is achieved. The use of reproductive technologies such as ultrasound, sperm collection, and artificial insemination (AI) has the potential to help overcome some of the issues around captive breeding of large sharks in aquaria. These technologies provide a greater control over reproduction by reducing the reliance on natural mating and allowing selective breeding to maximise the genetic diversity of offspring.

Reproduction in viviparous elasmobranchs is usually seasonal, with the breeding season occurring during spring/summer (Hamlett and Koob 1999). In most species, vitellogenesis and follicular growth occur over a period of months leading up to the breeding season (Hamlett and Koob 1999). Similarly in male elasmobranchs, spermatogenesis in most species occurs seasonally leading up to the breeding season (Parsons and Grier 1992). In the wild, many elasmobranch species undertake seasonal migration for breeding purposes. Environmental cues such as temperature are known to influence reproductive seasonality in many animal species, but little is known about the effect of these factors on elasmobranch reproductive cycles (Hamlett and Koob 1999). Elasmobranchs in captivity are not exposed to many of the environmental cues that they would normally receive in the wild, but aquarium water temperature is often varied seasonally. While open-system aquaria follow a similar temperature cycle to the environment from which seawater is drawn, closed-system aquaria often manually vary temperature in order to mimic a seasonal cycle.

Research to develop and apply assisted reproductive technologies to elasmobranchs held at Melbourne Aquarium (MAQ) in Melbourne, Victoria, was conducted between 2004 and 2008. Three species were used in this research: *Carcharias taurus*, *Notorynchus cepedianus*, and *Dasyatis brevicaudata*. *N. cepedianus* and *D. brevicaudata* are both temperate species common in the waters of southeastern Australia. *C. taurus* is a temperate species that undertakes seasonal migration along the coast of New South Wales and Queensland. The aims of the research were to develop methods for collection of semen from male elasmobranchs, to follow female *N. cepedianus* ovarian cycles using ultrasound, and investigate the timing for artificial insemination in *N. cepedianus* and *C. taurus*. Initial work to develop the techniques

was conducted mainly in *N. cepedianus*. Work in *D. brevicaudata* was aimed at expanding and developing sperm collection techniques, and gaining an understanding of male reproductive cycles in this species.

4.2 Methods

4.2.1 Animals

Mixed sex populations of *C. taurus* (one male, two females), *N. cepedianus* (one male, four females), and *D. brevicaudata* (six males and five females) were already established at Melbourne Aquarium when the research began. Male and female *C. taurus* were between 250 and 300 cm total length. Male *N. cepedianus* were 160 to 180 cm total length, and female *N. cepedianus* 230 to 270 cm. Disc widths of male *D. brevicaudata* were 110 to 120 cm. *C. taurus* were originally transported from Underwater World (UWW) in Mooloolaba, Queensland. The two females were captive born, and the male was wild caught. Two mature male *C. taurus* at UWW were also examined during the course of the research. *N. cepedianus* were all wild caught in Victorian waters. During the course of the research, one male *N. cepedianus* was added to the population, and four female *N. cepedianus* were brought in to replace animals that died due to illness or were released as part of the management program. A maximum of two mature males and four mature females were present in the Oceanarium at any one time. Female *D. brevicaudata* were all wild-caught in Victorian waters, while two males were wild-caught and four were the offspring of the females. All animals were housed in the 2.2 million litre "Oceanarium" on display at Melbourne Aquarium The aquarium operates as a closed system, and seawater is treated by mechanical, chemical, and biological filtration. Water temperature followed a controlled annual cycle, ranging from 17–22.5°C to coincide with local seasonal variation. Animal care and use was approved by Monash Medical Centre Animal Ethics Committee A (MMCA 2004/12).

4.2.2 Capture and handling

Procedures for capture and handling of sharks and stingrays were developed early to enable regular examination during the course of the research. This involved two main aspects, the first being to quickly and safely capture the animal and bring it to the

examination area, and the second to ensure adequate restraint of the animal to reduce the risk of injury to the animal or people involved. Elasmobranchs can be restrained for examination by holding them in dorsal recumbency (Daly et al. 2007). In this position, the animal remains relatively relaxed to enable examination and minor procedures (e.g. ultrasound, blood collection, catheterisation of the reproductive tract) to be conducted. In the case of stingrays, sedation or anaesthesia may be required for restraint. Stingrays have a venomous barb on the tail, so conducting the examination under sedation or anaesthesia reduces the risk of injury to the animal and people involved.

C. taurus, N. cepedianus, and *D. brevicaudata* were caught out of the Oceanarium by aquarium divers in SCUBA equipment using a 300 cm long 'catch-out bag' made of flexible clear vinyl. The entrance to the bag was 150 cm diameter and tapered to 50 cm diameter at the closed end, giving the bag a conical shape. A Velcro® seal along the top surface allowed access to the shark, and the narrow, closed, end of the bag was covered with nylon mesh to enable water flow to be directed towards the mouth of the shark and across the gills.

For capture of *C. taurus* and *N. cepedianus*, the bag was operated by three divers (one at the closed end and two at the entrance) and a fourth diver guided the shark into the bag (Fig. 1). The bag and shark were then brought into the "lock", which is a holding area connected to the Oceanarium containing approximately 17,500 liters of water at a depth of 100 cm (Fig. 2). 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 towards 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 (DO) concentration near the head and behind the gills was monitored throughout the procedure with a DO meter (YSI 550, YSI Environmental, USA).

For capture of *D. brevicaudata*, two divers operated the catch-out bag (both at the entrance) and a third diver directed the stingray into the bag. The stingray was then brought up and released into the lock prior to anaesthesia. For anaesthesia, stingrays were transferred to a large plastic tub containing 1000 litres of seawater and 30 ppm Aqui-S® (AQUI-S New Zealand Ltd., New Zealand). Water in the tub was oxygenated

Figure 1. Capture of *N. cepedianus* by divers using the catch-out bag.

Figure 2. The examination area ("lock") where procedures were conducted. The water surface of the Oceanarium can be seen in the background.

via a hose and air stone connected to D-class cylinder containing medical grade oxygen and DO was monitored throughout the procedure. A veterinarian visited MAQ one to two times a week throughout the study and was present during examinations.

4.2.3 Ultrasound of female *N. cepedianus*

Once in the lock, the shark was placed in dorsal recumbency and allowed to settle. Ultrasonography was performed using an Aloka SSD500 machine with a 3.5 MHz convex sector/linear scanner (Aloka, Japan) (Fig. 3). 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. Images were recorded using a VCR and television connected to the ultrasound machine. The largest diameter of 8-10 randomly selected follicles was measured using the distance tool on the ultrasound machine and the results recorded. Sharks were released after a maximum of ten minutes in the lock

Figure 3. The ultrasound procedure, showing the shark in dorsal recumbency and ultrasound being performed.

and their recovery was observed by divers who were on hand to provide assistance if necessary. Individual sharks were examined at intervals of at least six to eight weeks, and each examination lasted no more than ten minutes. Curatorial staff observed the animals daily to assess general health and wellbeing of all sharks.

4.2.4 Semen collection

4.2.4.1 Notorynchus cepedianus and *Carcharias taurus*

Sharks were caught out of the Oceanarium and placed in dorsal recumbency as described. The posterior portion of the body was supported out of the water, and the cloacal area was wiped with paper towel. Ampullae of the vasa deferentia, the site of semen storage in male elasmobranchs prior to ejaculation, are located posteriorly within the body cavity immediately ventral to the kidneys. Semen was collected by either massage (Fig. 4a) or catheterisation (Fig. 4b) of the ampullae. During the breeding season, the ampullae can be felt through the wall of the colon, which lies ventral to the ampullae. Semen was obtained by applying firm but gentle downward pressure on the ampullae with one finger, moving slowly towards the cloaca. Semen was collected directly from the urogenital papilla using a 10 ml syringe. Catheterisation was performed using a 2.0 or 2.4 mm diameter dog urinary tract catheter, with a stainless steel stylet inserted to provide rigidity, attached to a 10 ml syringe. The catheter was passed approximately 10 cm into the ampulla of the vas deferens via the urogenital papilla and urogenital sinus, and semen was gently withdrawn with the syringe. This procedure was quite difficult to perform successfully, so massage was used where possible. Semen was collected from *N. cepedianus* in most months of the year between 2006 and 2007. Examination of *C. taurus* was restricted by curatorial concern for these valuable endangered animals, so only five semen collection attempts were made. After semen collection, sharks were released back into the Oceanarium and their recovery monitored by divers.

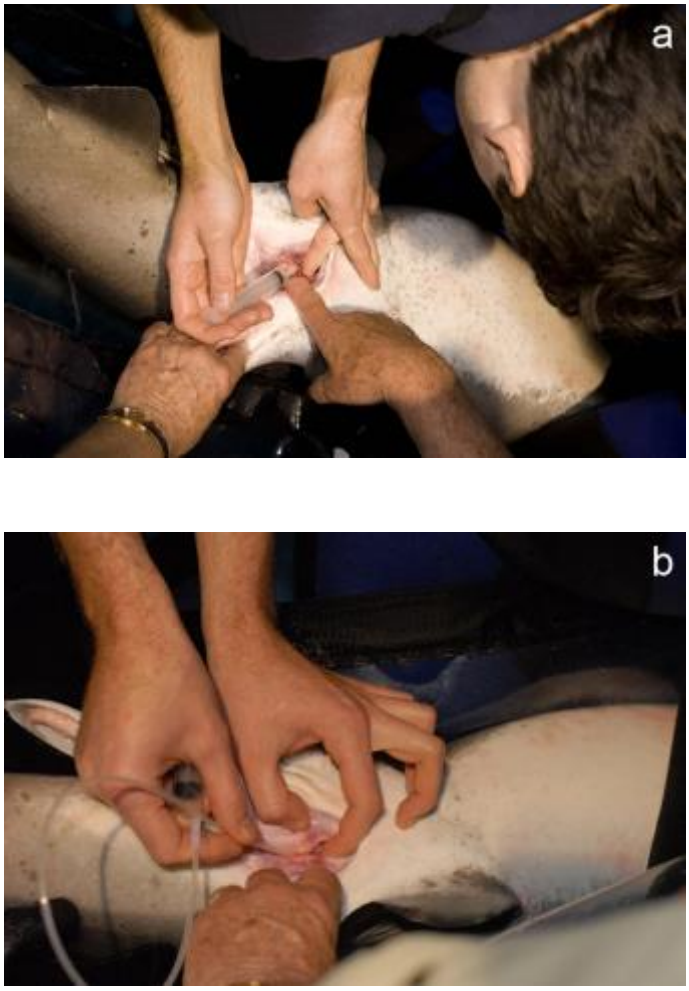

Figure 4. Semen collection by massage (a) and catheterisation (b). The sharks pictured are *N. cepedianus*, the same procedures were used for *C. taurus*.

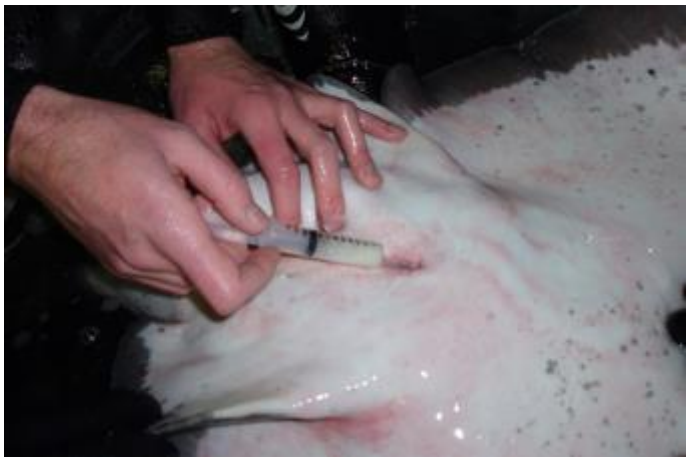

Figure 5. Collection of semen from *D. brevicaudata*.

4.2.4.2 Dasyatis brevicaudata

After catch out and anaesthesia as described, the stingray was placed in dorsal recumbency to allow access to the cloaca. The posterior portion of the body was supported out of the water and the cloacal area was wiped with paper towel. This species differs from the two shark species examined in that there are separate openings in the urogenital papilla for urinary and reproductive tracts. A 2.4 mm diameter catheter with a 10 ml syringe attached was inserted approximately 10 cm into the ampulla of the vas deferens via the urogenital papilla, and semen was gently withdrawn with the syringe (Fig. 5). After semen collection, stingrays were returned to the lock to recover from the anaesthesia and then released back into the Oceanarium. Semen was collected from *D. brevicaudata* every two months over a twelve-month period, between April 2006 and March 2007.

4.2.5 Assessment of semen quality

4.2.5.1 Visual assessment of motility

Collected semen was transferred to a 15-ml Falcon tube and kept at room temperature. Semen samples were immediately assessed visually under light microscope at 400 × magnification by placing a small drop (1-2 µl) onto a glass slide and adding 10 µl of elasmobranch ringer solution (see Chapter 5 for composition). A coverslip was applied and motility was estimated independently by two observers and the average percentage moving actively forward was recorded. The remaining semen sample was cooled to 4°C and transported to Monash Institute of Medical Research on ice for further assessment.

4.2.5.2 Fluorescent staining and flow cytometry

Sperm membrane integrity of semen collected from *D. brevicaudata* was assessed using a live/dead fluorescent staining protocol (SYBR-14/Propidium Iodide [PI], Molecular Probes, Eugene OR) as follows. Samples were diluted 1:9 with

elasmobranch ringer solution. SYBR-14 was diluted 1:24 with DMSO and 2 µl added to the samples. After incubation at room temperature for five minutes, 2 µl PI (diluted 1:1 with Milli-Q water) was added and the samples incubated for a further five minutes. Samples were centrifuged at 1000 × *g* for 1 minute and the supernatant removed. The pellet was resuspended in 500 µl ER and the procedure repeated. Finally, sperm were resuspended in 500 µl ER solution. Flow cytometry was performed using a BDFACS Canto™II flow cytometer (Becton Dickinson, USA) with 488nm laser excitation. SYBR-14 was detected with a 530/30 nm band pass filter and Propidium Iodide with a 670 nm long pass filter. Cell populations stained with SYBR-14 alone (membrane intact) were considered alive. Those stained with both SYBR-14 and PI or PI alone were considered dead (membrane compromised).

4.2.6 Reproductive behaviour

Water temperature and behaviour of *C. taurus* were recorded by curatorial staff at both Melbourne Aquarium and Underwater World between 2001 and 2007. Behavioural observations were made continuously throughout the day and often overnight at both aquaria. *C. taurus* at UWW were housed in the 2 million litre main display tank, which operates as a flow through system. Water in the tank is taken directly from the ocean near Mooloolaba, with chemical and mechanical filtration of seawater prior to entry into the display. Water temperature cycles in the display closely follow those in the local environment. *C. taurus* at MAQ were housed as described in section 4.2.1. Data from the two facilities were collated to observe and compare trends in temperature and behaviour. Observations included pre-copulatory behaviours such as "fast swimming" (males swimming rapidly near the water surface), "tailing" and "nosing" (the male following the female or swimming with his nose in close proximity to the females' cloaca), and "biting" (the male biting the female along the flank or pectoral fin). Copulation ("mating") was also recorded.

3.2.6 Artificial insemination

The artificial insemination technique was developed in *N. cepedianus* at MAQ. The male shark was caught out of the Oceanarium and semen collected by massage or catheterisation as described. The male was released back into the Oceanarium and the

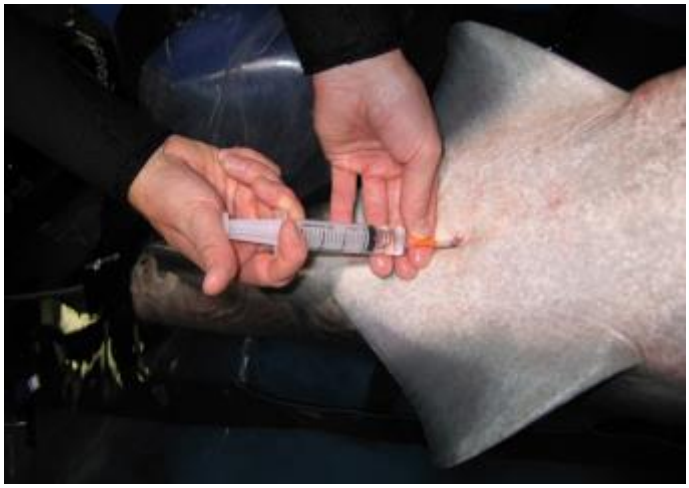

Figure 6. Artificial insemination procedure in *N. cepedianus*. The same technique was used for artificial insemination of *C. taurus*.

semen was assessed under light microscope. If motility in the sample was good (>50%), semen was diluted 1:4 with elasmobranch ringer solution and kept at room temperature until needed. The female shark was caught out of the Oceanarium as described and brought to the examination area. Diluted semen was drawn into a 20 ml syringe with a 2.4 mm diameter, 50 cm long catheter attached. The catheter was inserted approximately 40 cm into the uterine horn on one side via the cloaca, and half the diluted semen deposited (Fig. 6). The procedure was repeated in the second uterine horn and the shark released back into the Oceanarium. Artificial insemination was performed twice each on two female *N. cepedianus* at MAQ, and once on a female *C. taurus* at MAQ using semen collected from a male at UWW.

4.3 Results

4.3.1 Ovarian cycle in female *N. cepedianus*

Observations from the early part of this research (2004 to 2005) have been published in Daly et al. (2007) (Chapter 2). Additional data obtained during 2006 – 2008 are combined in this chapter with the published material, together with general observations not reported previously. Ovarian follicles were observed by ultrasound in seven of eight female *N. cepedianus* examined during the research. Of these, three females showed no sign of ovarian development on ultrasound when first introduced

into the aquarium and were considered immature at that time. Subsequent follicular development in these animals was observed after a period of nine months (two sharks) to two years (one shark) in the aquarium. One female (in the aquarium for two years) was immature and showed no signs of ovarian development during the course of the research. Follicles in maturing females were first detected at 15 – 20 mm diameter. The largest follicle diameter observed during the study period was 57 mm.

Combined data from the seven female *N. cepedianus* in which follicles were observed showed two distinct ovarian patterns (Fig. 7). Follicles were observed to increase in size from 15 – 20 mm diameter to 30 – 35 mm diameter over a period of approximately nine months. At this point, follicles either continued to grow in size (three sharks) or began to regress (three sharks). Follicles in one female *N. cepedianus* wild-caught in 2005 with fresh mating bites continued to grow past 30 – 35 mm diameter, while follicles in resident females that had not been mated regressed in size. The two other female *N. cepedianus* in which follicles continued to grow past this point were artificially inseminated prior to follicle size reaching 30 – 35 mm diameter. Follicles undergoing regression were identified by a ruffled, less clearly defined periphery on ultrasound and a decline in size over time. It was hypothesized that these follicles were atretic. The period of regression lasted approximately nine months, during which follicles reduced in size to around 15 – 20 mm diameter. At this point, a new cohort of follicles began the growth phase. Growth and regression of follicles were not observed concurrently in the same female at any stage of the research.

4.3.2. Observations on male urogenital papilla morphology

Distinct differences were observed in urogenital papilla morphology between the three species studied (Fig. 8). In *C. taurus*, a single large pore was present at the tip of the urogenital papilla leading to the urogenital sinus. Inside the urogenital sinus were two small, dorsolateral, papillae that lead to the two sides of the reproductive tract. In *N. cepedianus*, the urogenital papilla has two separate pores that sit alongside one another at the tip of the papilla. Each pore leads to distinct halves of the urogenital sinus, which appear to be separate from one another. At the anterior end of each side of the urogenital sinus is a narrow elongate papilla (7 mm in length) that leads to the

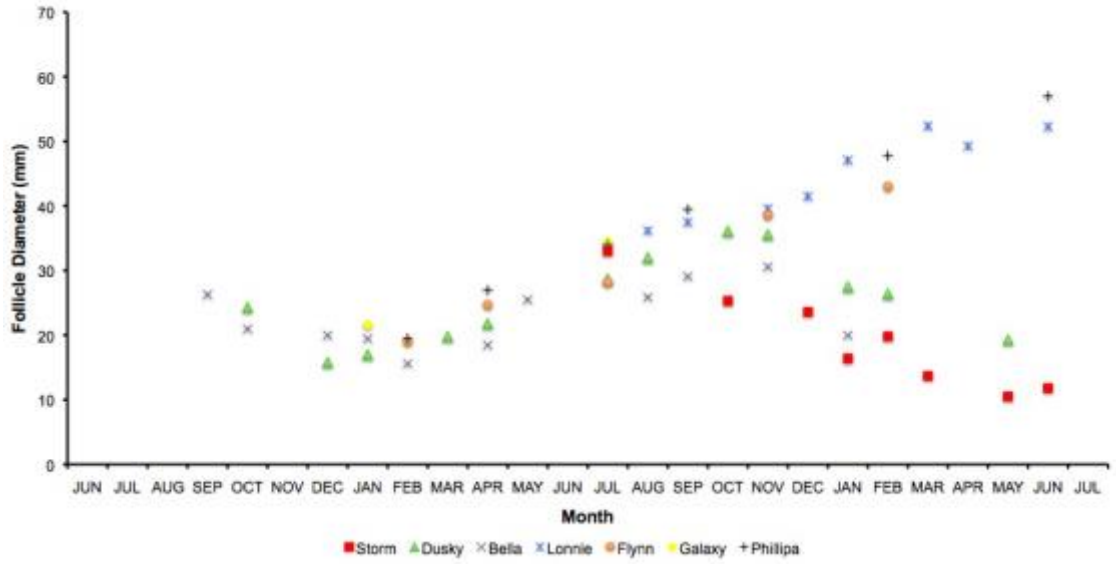

Figure 7. Follicle diameter data from seven female *N. cepedianus*. Data have been combined and plotted over a two-year period to show seasonal patterns.

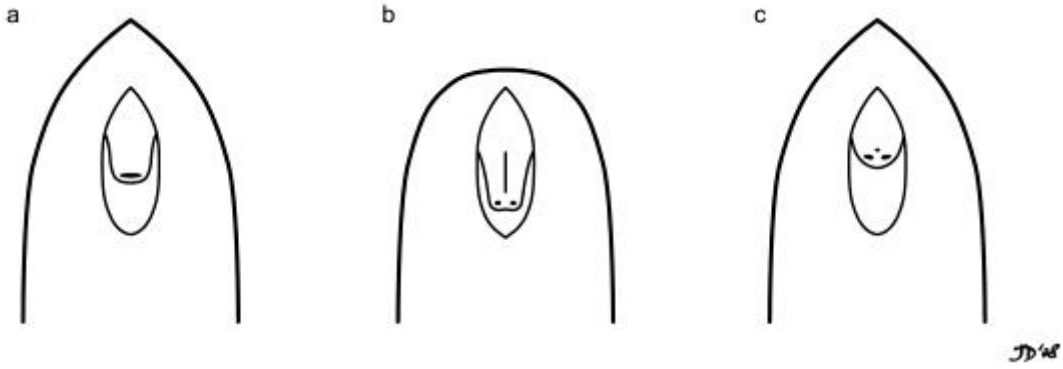

Figure 8. Urogenital papillae in *C. taurus* (a), *N. cepedianus* (b), and *D. brevicaudata* (c) showing species differences in external morphology. C. taurus has a single large pore at the tip of the urogenital papilla. N. cepedianus has two pores that sit alongside one another and lead to separate halves of the reproductive tract. D. brevicaudata has three pores, two sit alongside one another and lead to separate halves of the reproductive tract and the third (central) pore leads to the urinary tract.

reproductive tract on that side. In *D. brevicaudata*, the tip of the urogenital papilla has three separate pores. Two pores, located alongside each other, lead to separate halves of the reproductive tract. The third, located above and medial to the first two, leads to the urinary tract.

4.3.3 Seasonal changes in semen quality

4.3.3.1 Notorynchus cepedianus

Semen quality followed a marked seasonal cycle in this species (Fig. 9). Sperm concentration and motility increased concurrently in late winter to early spring and declined in autumn. Sperm motility was lowest (20%) during April to June, and sperm concentration at this time was very low. Samples collected during this time often contained a high proportion of abnormal sperm and cellular debris. Sperm concentration and motility began increasing in July and August. Highest sperm motility was observed in February (65%), which corresponded with the time when male-female interactions were highest. The period of high semen quality lasted from September to February. Semen collected during this time was highly viscous, and was sometimes observed oozing from the urogenital papilla. Individual sperm were present in all semen samples collected from *N. cepedianus*, and sperm bundles were not observed.

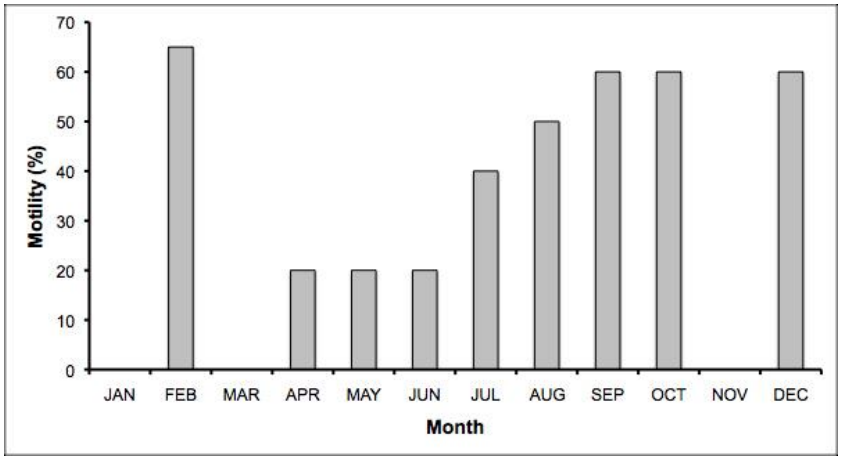

Figure 9. Seasonal changes in motility of *N. cepedianus* (n = 2) sperm during the year. Data is combined from examinations conducted between 2006 and 2008.

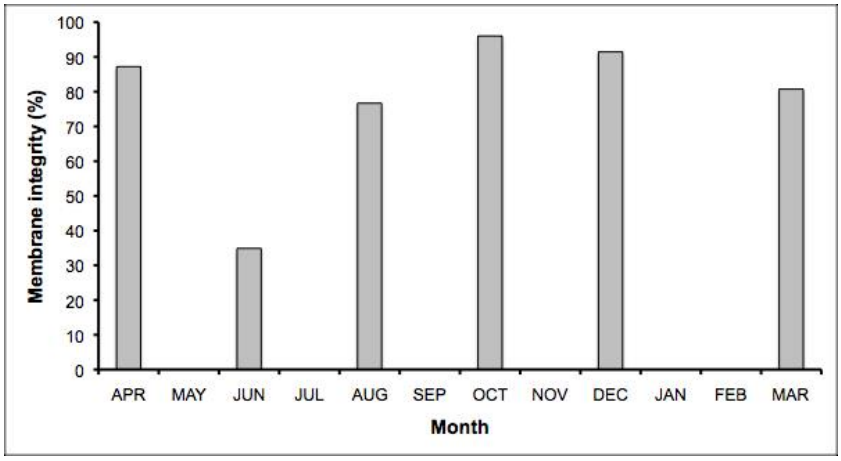

Figure 10. Seasonal changes in membrane integrity of *D. brevicaudata* (n = 5) sperm between April 2006 and March 2007.

4.3.3.2 Dasyatis brevicaudata

A marked seasonal cycle in sperm membrane integrity and semen quality was observed in this species (Fig. 10). Lowest mean sperm membrane integrity was observed in June 2006 (34.9 ± 12.1%), and the highest in October 2006 (96.0 ± 1.8%). During the period when sperm membrane integrity was high (October 2006 to April 2007), sperm were highly motile (>90%) and were observed in tightly packed bundles that moved as a single unit. Individual sperm were also observed during this time and these were also highly motile. In contrast, mainly individual sperm were present in semen collected when membrane integrity was lowest (June 2006), and motility varied greatly in these samples. Several females of this species were mated naturally during December and January, and subsequently gave birth to live young following a gestation of approximately twelve months.

4.3.4 Reproductive behaviour in relation to water temperature

Mean water temperatures at both MAQ (manually controlled) and UWW (natural cycle) followed a similar seasonal pattern. Lowest mean temperatures recorded were 17.0°C and 20.7°C at MAQ and UWW respectively in July. The highest mean water temperature recorded at MAQ was 21.0°C in January, and the highest at UWW was

27.9°C in February. In both aquaria *C. taurus* pre-copulatory behaviour was associated with rising water temperature in the spring and early summer (Fig. 10a-b). Male fast swimming began in early spring at UWW, and in early summer at MAQ. Fast swimming preceded the other behaviours and was the first sign that the breeding season was approaching. Most male-female interactions were observed in October at UWW (Fig. 11a) and in January at MAQ (Fig. 11b). The majority of the observed interactions involved tailing and nosing. Male biting of the female was usually associated with mating or mating attempts. The majority of recorded matings at UWW occurred in October and early November, with water temperatures ranging between 23.0°C and 24.5°C (Table 1). One mating event was recorded at MAQ in January when water temperature was 20.5°C (Table 1). *C. taurus* breeding areas under natural conditions in the Southern Hemisphere are in the temperate zone, with surface water temperatures during the year ranging from around 19 to 25°C. In Queensland where the temperature is higher it is confined to the three spring months (Fig. 11a). In Melbourne where the temperature reaches only the lower limits of the preferred natural range the onset of sexual behaviour is slower and it lasts until March (Fig. 11b).

Reproductive behaviour similar to that in *C. taurus* was also observed in *N. cepedianus* and *D. brevicaudata* at MAQ in December and January, when water temperatures were 20 – 21°C. In *N. cepedianus*, reproductive behaviour was minimal and consisted mainly of male biting of the female along the flank and pectoral fins, and this was associated with a mating attempt on one occasion. For the rest of the year there was very little male-female interaction. Reproductive behaviour in *D. brevicaudata* during this time was much more pronounced. Females were observed with extensive bite marks on the pelvic fins and were often followed closely by up to three males. Mating occurred annually, and sometimes within weeks of the female giving birth.

4.4 Discussion

Reproductive biology and behaviour in the three species studied followed a seasonal pattern. Water temperature and behaviour records from MAQ and UWW indicate that there is an association between reproductive behaviour and water temperature in male *C. taurus*. Reproductive behaviour is restricted to a distinct breeding season from early to late spring at UWW and early summer to early autumn at

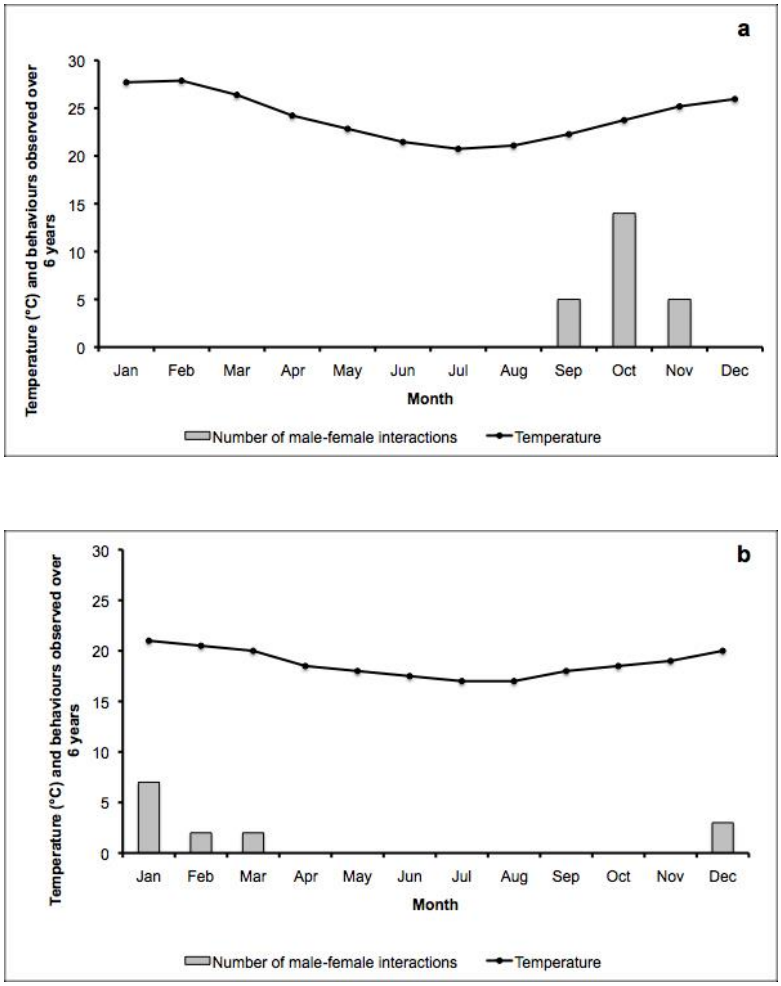

Figure 11. Temperature variations and *C. taurus* reproductive behaviour at UWW with 3 males and 3 females (a) and MAQ with one male and two females (b). Behaviours included pre-copulatory following, nosing, and biting, as well as mating.

Table 1. Dates of recorded matings of *C. taurus* and water temperature from 2001 – 2007.

Male *C. taurus*	Date	Water Temp (°C)	Aquarium
Joker	16/10/2001	24	UWW QLD
	31/10/2001	24.5	UWW QLD
Uncle Ray	29/10/2003	23.5	UWW QLD
	16/11/2004	24	UWW QLD
	29/10/2006	23	UWW QLD
	02/11/2006	23.5	UWW QLD
Romeo	30/10/2006	23.5	UWW QLD
Mitch	19/12/2007	20.5	MAQ VIC

MAQ. In both aquaria seasonal increase in temperature is followed by sexual activity, beginning with male fast swimming. At UWW pre-copulatory behaviour begins almost as soon as water temperature starts to rise, and mating has been consistently recorded at 23.0 – 24.5°C. Pregnancies have occurred in *C. taurus* at UWW but reproductive efficiency has been low, with four pups born and nine stillborn between 1992 and 2008. At MAQ pre-copulatory behaviour does not begin until four months after temperature begins to rise, when the water is around 20°C. Water temperature at MAQ does not reach the range at which mating occurs at UWW, and reproductive behaviour has only been recorded at the upper end of the seasonal temperature range (20.0 – 21.0°C). At MAQ only one mating has been recorded between the one male and two females and this at a lower temperature and later in the year than in Queensland. No other matings have been recorded in Melbourne and the outcome of the one mating observed will not be known for some time. These records suggest that *C. taurus* in captivity respond more quickly to increasing water temperature at ranges above 20°C, and that a temperature of 20°C or above is required for sexual behaviour and mating. This observation agrees with information from other Australian aquaria where sexual behaviour has been observed in *C. taurus*. Gordon (1993) reported that pre-copulatory behaviour in *C. taurus* held at Manly Oceanworld, New South Wales, occurred between November and January. Water temperature was reported to range between 15 and 24°C throughout the year, with sexual behaviour and mating beginning at 20°C. A recent study reported male-male and male-female sexual conflict in C. taurus held at the National Aquarium in Baltimore and Sea World Orlando, in the U.S.A. (Henningsen et al. 2008). These authors reported seasonal changes in steroid hormones and sexual conflict at water temperatures of 22.2 – 24.4°C. Interestingly, these changes were also observed in *C. taurus* held at a constant 24.4°C, although in this case photoperiod was varied seasonally. It appears that a water temperature of at least 20°C is required for sexual behaviour of *C. taurus* in captivity, but more information is required to test the hypothesis that actual mating occurs when a threshold temperature is reached.

A similar association can be made between increasing water temperature and sperm production in *N. cepedianus* and *D. brevicaudata* at MAQ. In both species, sperm production followed the same seasonal cycle as water temperature, and it is possible that rising water temperature acts as an environmental cue for the resumption of spermatogenesis. This agrees with observations made by Dobson and Dodd (1977c)

who suggested that temperature was the main environmental cue for the seasonal reproductive cycle in the male small spotted catshark (*Scyliorhinus canicula*). These authors found that increasing water temperature stimulated mitosis in late stage spermatogonia in *S. canicula* that had undergone partial hypophysectomy (Dobson and Dodd 1977a). They suggested that the seasonal decline in spermatogenesis that occurs naturally was likely caused by a concurrent decline in pituitary hormones, and that these changes were temperature dependent (Dobson and Dodd 1977a). The recent study by Henningsen et al. (2008) found that a seasonal rise in male steroid hormone levels was correlated with increased sexual conflicts in *C. taurus*. The effects of temperature on these hormone cycles are not clear, as water temperature was maintained at a constant 24.4°C for part of the study. It seems possible that seasonal changes in semen quality and behaviour in the present study are triggered by changes in water temperature. Further studies are required to determine if changes in pituitary or steroid hormone levels in response to temperature changes mediate this effect. The low number of animals and limited chances to collect blood samples from *C. taurus* restricted the use of hormone studies in the present research.

Seasonal cycles in reproductive behaviour and spermatogenesis are well documented in many elasmobranch species, and four basic patterns have been defined (Wourms 1977; Parsons and Grier 1992). Both *N. cepedianus* and *D. brevicaudata* fit into the group having a defined seasonal cycle in testicular function and a defined mating season (Parsons and Grier 1992). There are conflicting reports on male *N. cepedianus* reproductive biology in the wild. Lucifora *et al.* (2005) reported that male gonadosomatic index (GSI) varied seasonally in males from a population in Argentina, with a minimum in April and maximum in October – November. This fits with the pattern of sperm production observed in the present study. Ebert (1996) reported that males from a population in southern Africa also showed seasonal changes in GSI. Interestingly, in the latter study, as well as in a previous study by the same author (Ebert 1989), "viable" sperm was found to be present throughout the year. It is unknown whether captivity could have an effect on seasonality of sperm production, although it is worth noting that a male *N. cepedianus* introduced to MAQ late in the study showed the same seasonal variation in semen quality as the male that had been in captivity for five years.

Data from the present study indicate that the follicular cycles of female *N. cepedianus* in captivity are long, with vitellogenesis potentially taking up to two years to complete. In the present study, the number of follicles in the ovary was difficult to count by ultrasound, but was estimated from images of successive transverse planes through the ovary at well over fifty in some females. In these sharks, follicles were detected all the way from the pectoral region to three-quarters of the way along the body. Follicles were detected in the same image as the spiral valve on a number of occasions. Two females with growing follicles that died during the study were dissected, and in both the ovary extended almost the full length of the body and took up a large proportion of the body cavity. Studies on wild female *N. cepedianus* have found large numbers of eggs in the ovary of mature females. Lucifora et al. (2005) found 59, 94, and 107 follicles in the ovaries of three female *N. cepedianus* from a population in Argentina, with the largest measured at 69 mm diameter. Similarly, Ebert (1996) reported 67 to 104 follicles in the ovary of 19 *N. cepedianus* from southern Africa, and suggested that follicles were up to 70 mm diameter at the time of ovulation. In the latter study, vitellogenesis was thought to occur over a twelve-month period. Data from the present study indicate that vitellogenesis in *N. cepedianus* in captivity occurs over a period closer to two years. The largest follicles observed by ultrasound took sixteen months to grow from 20 mm to 57 mm, and will require several more months to attain the suggested 70 mm ovulation size. It is not clear whether this long period of follicle growth is an artefact of captivity or if this represents the natural vitellogenic period for this species.

The two patterns of follicular development and long reproductive cycle observed in female *N. cepedianus* in the present study pose difficulties for timing of artificial insemination in this species. Follicle growth data indicates that artificial insemination should take place when follicles are around 30 – 35 mm diameter, since follicle regression was observed to begin at around this point in females examined in the early part of the study (2004 – 2006). It was hypothesised that mating and/or sperm storage in the female oviducal gland may influence whether follicles continue to grow or become atretic. This was based on the observation that follicles in a female *N. cepedianus*, wild-caught in 2005 with fresh mating bites, continued to grow past 30 – 35 mm diameter, while follicles in resident females that had not been mated became atretic. Since then, two female N. cepedianus have been artificially inseminated using

sperm collected from males in the display. Both of these females, showed continued follicular growth beyond 30 –35 mm diameter. Unfortunately one of these females has since died due to unknown causes, but at the time of death the ovary contained a very large number of follicles at 40 – 45 mm diameter. The remaining artificially inseminated female also showed continued follicle growth, last measured at 57 mm in June 2008. The idea that part of the female reproductive cycle is induced by mating in some elasmobranchs is not new. Pratt and Carrier (2005) suggested that the pre-copulatory biting that occurs in some species during courtship and copulation may stimulate ovulation or some other physiological response in the female. Maruska et al. (1996) suggested that mating may actually stimulate the beginning of ovarian development in *D. sabina*. They found that mating in this species occurred up to seven months before ovulation and fertilization, but shared a close temporal relationship with the beginning of ovarian development. Although an attractive idea, there is not enough information to determine whether mating and/or insemination stimulate follicular growth beyond 30 – 35 mm in *N. cepedianus*. Continued observations of female follicular growth and regression patterns may help to elucidate this point.

Natural mating is always the first choice for breeding elasmobranchs in aquaria, but there are many factors that can affect the success of such programs. Data in the present study indicate that seasonal temperature cycles play an important role in breeding of some species, but many other factors may contribute to the build up of sexual activity and ultimate mating. These include population composition, light regimens, feeding, and stress from the captive situation. There are also limits on the degree of selective breeding that is possible in aquaria. The tank size required to house large sharks means that aquaria do not always have space to isolate individual sharks (for example a dominant male) for long periods of time to allow other animals to contribute to breeding. Reproductive technologies have the potential to overcome some of these issues, and a large portion of the work conducted during this study was on the development of reproductive technologies for application in elasmobranch species. The approach taken (i.e. ultrasound, semen collection, and artificial insemination) was seen as the simplest way to monitor male and female reproductive cycles and assist breeding in the two large shark species examined. The outcome of the artificial insemination of a female *C. taurus* at MAQ using semen collected from a male at UWW is unknown at this stage. Ultrasound examination of this shark, and the artificially inseminated *N.*

cepedianus, in the 2008/09 breeding season will help determine the success of this procedure. This work represents the first steps toward general application of these techniques in breeding programs for endangered elasmobranchs in aquaria. The ultimate goal is to be able to selectively breed endangered aquarium species such as *C. taurus* to maintain existing stock and maximise genetic diversity. Maintenance of captive populations by supplementing them from the wild, even if the wild population is relatively healthy, is becoming less acceptable than it has been in the past. Aquaria need to strive towards self-sustaining populations that only require minimal input from wild stocks to bolster genetic variability. In this way, aquarium populations can maintain their place as valuable insurance populations for endangered species such as *C. taurus*, and help to prevent other species from going down a similar path.