The use of reproductive technologies in breeding programs for elasmobranchs in aquaria

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Abstract: Despite the use of reproductive technologies in terrestrial and aquatic species, very little work has been done with elasmobranchs. Reproductive technologies such as sperm collection and quality assessment, sperm cryopreservation, artificial insemination, and monitoring female reproductive condition and gestation could potentially be used to complement existing breeding programs for elasmobranchs in aquaria. As a greater emphasis is placed on self-sustaining aquarium populations, reproductive technologies will become an increasingly important component of breeding programs for elasmobranchs in aquaria. Ongoing research at SEALIFE Melbourne Aquarium, Australia, since 2004 aims to create a basis for future use of reproductive technologies in elasmobranchs in aquaria worldwide.

INTRODUCTION

Elasmobranch breeding programs in aquaria

It has been estimated that over 210 species of elasmobranch are held in major aquaria worldwide (American Elasmobranch Society) for display, conservation, and education purposes. With the exception of species that are endangered in their natural habitat, elasmobranch populations held in aquaria are traditionally 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. Aquarium breeding programs for elasmobranchs traditionally rely on the occurrence of natural mating. While this approach has been successful in breeding many species of elasmobranch in aquaria (Henningsen et al. 2004), there are still many species that have never reproduced in captivity (e.g., broadnose sevengill sharks, *Notorynchus cepedianus*, Peron, 1807) while other species reproduce sporadically (e.g., sand tiger sharks, *Carcharias taurus*, Rafinesque, 1810) (personal observation). 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 aquarium breeding programs for elasmobranchs and, in particular, large sharks.

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 has been held in aquaria for over one hundred years (Koob, 2004) and remains a popular aquarium species today. Despite its long history as an aquarium species, *C. taurus* has traditionally been wild caught for display with relatively few individuals born in captivity. *Carcharias taurus* is classified by the International Union for Conservation of Nature (IUCN) as threatened worldwide, and the Australian east coast population is classified by the Australian federal government as critically endangered. Captive breeding programs for *C. taurus* are likely to be crucial to its future as an aquarium species.

One way to assist current breeding programs for elasmobranchs in aquaria is with the use of reproductive technologies. The ability to monitor reproductive cycles,

collect semen, and artificially inseminate elasmobranchs would provide greater control over reproduction in aquaria. The use of sperm cryopreservation to store the genetics of individual males would also be of benefit, enabling males to contribute to the gene pool of a population beyond their normal reproductive lifespan, and allowing transfer of genetic material among aquaria for selective breeding to ensure genetic diversity within captive populations. Although well established in many mammalian and teleost species, reproductive technologies have had relatively little application in elasmobranchs.

Definition of reproductive technologies

Reproductive technologies are a broad group of techniques that can be used to assist reproduction in animals. A distinction should be made between the reproductive technologies that are currently used in elasmobranchs, and the more complex and well established assisted reproductive technologies (ARTs) used in humans. The World Health Organization (WHO) defines ARTs as "all treatments or procedures that include the *in vitro* handling of both human oocytes and sperm, or embryos, for the purpose of establishing a pregnancy" (Zegers-Hochschild et al., 2009). The definition of ARTs provided by the WHO specifically excludes artificial insemination, as it does not involve manipulation of both male and female gametes *in vitro*. This chapter will therefore refer to the use of "reproductive technologies", which is a more appropriate term in the context of elasmobranchs. For the purposes

of this chapter, reproductive technologies will be defined as techniques used to monitor or manage reproduction in elasmobranchs, and include monitoring of reproductive cycles with ultrasound or hormone analysis, hormone treatments to alter reproduction, semen collection, sperm cryopreservation, artificial insemination, and artificial rearing of embryos of viviparous elasmobranchs. These techniques have all been used to varying degrees in elasmobranchs, and offer great potential for the reproductive and genetic management of elasmobranch populations in aquaria.

MONITORING REPRODUCTION

Reproduction in elasmobranchs is usually seasonal, with the breeding season occurring during spring/summer (Hamlett and Koob, 1999). Vitellogenesis and follicular growth generally occur over a period of months leading up to the breeding season, although there is variation among species, notably some oviparous species that lay eggs year round (Hamlett and Koob, 1999). Similarly in male elasmobranchs, spermatogenesis in most species occurs seasonally leading up to the breeding season (Parsons and Grier, 1992). While there are some species that reproduce regularly in aquaria and for which reproductive cycles of individuals can be easily inferred, the reproductive conditions of many species in aquaria are not so easily apparent and require routine monitoring in order to define. This may be due to reproductive asynchrony of individuals within the population, single sex populations

where reproductive activity cannot be observed, or simply a lack of reproductive activity within a population. In these situations, techniques that can be used to assess and monitor reproductive condition in male and female elasmobranchs such as ultrasound, hormone analyses, and semen assessment can be useful to help manage the reproductive health of a population. While all three are useful techniques, all are somewhat limited in the amount of information that they can provide from a single examination. If the reproductive state of the elasmobranch is unknown a number of regular examinations may be required to build a pattern of reproductive activity. In the absence of regular examinations it is often difficult to determine where an individual examination fits within the overall reproductive cycle of an individual or species. For this reason, reproductive monitoring for the purposes of managing reproduction should be performed on a regular basis as part of routine health examinations.

Ultrasound

Ultrasound is a non-invasive means of directly visualizing reproductive structures, and has been used to monitor ovarian changes and pregnancy in several species of elasmobranch. It has also been used to visualize embryos developing inside the egg case of the oviparous Port Jackson shark (*Heterodontus portusjacksoni*, Meyer, 1793) and horn shark (*Heterodontus fransisci*, Girard, 1855) (Pereira, personal communication), both of which produce thick, opaque egg cases that are not

conducive to 'candling'. One of the earliest published reports on the use of ultrasound in elasmobranchs was from Walsh et al. (1993). These authors assessed the potential diagnostic applications of ultrasound in nurse sharks (*Ginglymostoma cirratum*, Bonnaterre, 1788), lemon sharks (*Negaprion brevirostris*, Poey, 1868), and bonnethead sharks (*Sphyrna tiburo,* Linnaeus, 1758), and were able to obtain images of fetal spines in a pregnant *S. tiburo*. The utility of reproductive ultrasound in elasmobranchs was further demonstrated by Carrier et al. (2003), who captured female *G. cirratum* that were observed to mate in the wild and followed their progress in captivity throughout gestation. The use of ultrasound specifically on elasmobranchs in aquaria has since been reported for reproductive purposes including following folliculogenesis in *N. cepedianus* (Daly et al., 2007) and detection of pregnancy in white-tip reef sharks (*Triaenodon obesus*, Ruppell, 1837) (Schaller, 2006).

Although there is only a handful of published reports on the use of ultrasound in elasmobranchs, it is the most commonly used reproductive technology among aquaria and has been used for reproductive assessment in at least 39 species of elasmobranch (Table 1). Ultrasound has become a regular part of routine examinations in many aquaria, and is typically used to monitor reproductive cycles in female elasmobranchs to enable better management of individuals within a population. By measuring the diameter or volume of follicles in the ovary over a period of time it is possible to follow folliculogenesis in individual females (Figure 1), and by monitoring the growth of fetuses with ultrasound it is possible to estimate

delivery dates (e.g. bluespotted ribbontail stingray, *Taeniura lymma*, Forsskal, 1775), Pereira, personal communication) and to transfer pregnant females to nursery tanks prior to parturition so that neonates have a better chance of survival (e.g. cownose rays, *Rhinoptera bonasus*, Mitchill, 1815) (George, personal communication).

Table 1. Species for which reproductive ultrasound has been reported or observed.

Species	**Common name**	**Reference**
Aetobatus narinari (Euphrasen, 1790)	spotted eagle ray	George (pers. com.), Mylniczenko (pers. com.)
Carcharhinus plumbeus (Nardo, 1827)	sandbar shark	Mylniczenko (pers. com.), Hadfield (pers. com.)
Carcharhinus limbatus (Muller & Henle, 1839)	blacktip shark	Mylniczenko (pers. com.)
Carcharias taurus	sand tiger shark	Mylniczenko (pers. com.), Daly & Jones (personal observation)
Chiloscyllium punctatum (Muller & Henle, 1839)	brownbanded bamboo shark	Mylniczenko (pers. com.), Daly & Jones (personal observation)
Dasyatis americana (Hildebrand & Schroeder, 1928)	southern stingray	Mylniczenko (pers. com.), Hadfield (pers.com.), Pereira (pers. com.)
Dasyatis sabina (Lesueuer, 1824)	Atlantic stingray	Hadfield (pers.com.)
Ginglymostoma cirratum	nurse shark	Carrier et al. 2003, Hadfield (pers. com.)

Gymnura altavela (Linnaeus, 1758)	spiny butterfly ray	Hadfield (pers. com.)
Hemiscyllium ocellatum (Bonnaterre, 1788)	epaulette shark	Daly & Jones (personal observation)
Heterodontus francisci	horn shark	Lécu & Herbert (pers. com.)
Heterodontus portusjacksoni	Port Jackson shark	Daly & Jones (personal observation)
Himantura uarnak (Gmelin, 1789)	reticulated whip ray	Hadfield (pers. com.)
Himantura dalyensis (Last & Manjaji-Matsumoto, 2008)	Australian freshwater whip ray	Hadfield (pers. com.)
Manta alfredi (Krefft, 1868)	reef manta ray	Tomita et al. 2012
Mobula hypostoma (Bancroft, 1831)	lesser devil ray	Mylniczenko (pers. com.)
Myliobatis aquila Linnaeus, 1758	common eagle ray	Pereira (pers. com.)
Myliobatis freminvillii (Lesueur, 1824)	bullnose eagle ray	Hadfield (pers. com.)
Notorynchus cepedianus	broadnose sevengill shark	Daly et al. 2007, Grassman (pers. com.)
Orectolobus ornatus (De Vis, 1883)	ornate wobbegong	Otway & Ellis 2011
Platyrhinoidis triserata (Jordan & Gilbert, 1880)	thornback guitarfish	Hadfield (pers. com.)
Potamotrygon motoro (Muller & Henle, 1841)	ocellate river stingray	Daly & Jones (personal observation)

Pteroplatytrygon violacea (Bonaparte, 1832)	pelagic stingray	Hadfield (pers. com.)
Raja clavata (Linnaeus, 1758)	thornback ray	Whittamore et al. 2010
Raja eglanteria (Bosc, 1800)	clearnose skate	Hadfield (pers. com.)
Rhinobatos cemiculus (Geoffrey Saint Hilaire, 1817)	blackchin guitarfish	Lécu & Herbert (pers. com.)
Rhinoptera bonasus	cownose ray	George (pers. com.), Mylniczenko (pers. com.), Hadfield (pers.com.)
Rhynchobatus laevis (Bloch & Schneider, 1801)	smoothnose wedgefish	Hadfield (pers. com.)
Scyliorhinus canicula (Linnaeus, 1758)	smallspotted cat shark	Whittamore et al. 2010
Sphyrna tiburo	bonnethead shark	Walsh et al. 1993, Hadfield (pers. com.)
Stegostoma fasciatum (Hermann, 1783)	zebra shark	Hadfield (pers. com.), Daly & Jones (personal observation)
Taeniura grabata (Geoffrey Saint-Hilaire, 1817)	round fantail stingray	Pereira (pers. com.)
Taeniura lymma (Forsskal, 1775)	bluespotted ribbontail stingray	Pereira (pers. com.)
Taeniura meyeni (Muller & Henle, 1841)	blotched fantail ray	Hadfield (pers. com.)

Triaenodon obesus	whitetip reef shark	Schaller et al. 2003, Daly & Jones (personal observation)
Triakis megalopterus (Smith, 1839)	sharptooth houndshark	Smale 2003
Triakis semifasciata (Girard, 1855)	leopard shark	Stetter 2004, Lécu & Herbert (pers. com.)
Urobatis halleri (Cooper, 1863)	round stingray	Jirik & Lowe 2012
Urobatis jamaicensis (Cuvier, 1816)	yellow stingray	Mylniczenko (pers. com.), Hadfield (pers. com.)

Sharks are usually placed in dorsal recumbence for ultrasound examination as this induces tonic immobility, and imaging of internal organs is usually easiest via the ventral surface. Tonic immobility (Henningsen, 1994) enables examination without the use of anesthetic agents, although sedation may still be preferred for longer examinations (Mylniczenko, personal communication). In the case of rays, examination can be conducted either ventrally or via the dorsal surface, particularly in the case of large rays that are difficult to place in dorsal recumbence. Sedation or anesthesia is often necessary for ray species, large or small, to minimize the risk of the animal causing injury to itself or the person conducting the examination (Daly & Jones, personal observation), and in some cases, where regular examination is required, the barb is removed either partially by clipping or completely by excising the barb and germinal plate to prevent re-growth (George, personal communication; Mylniczenko, personal communication).

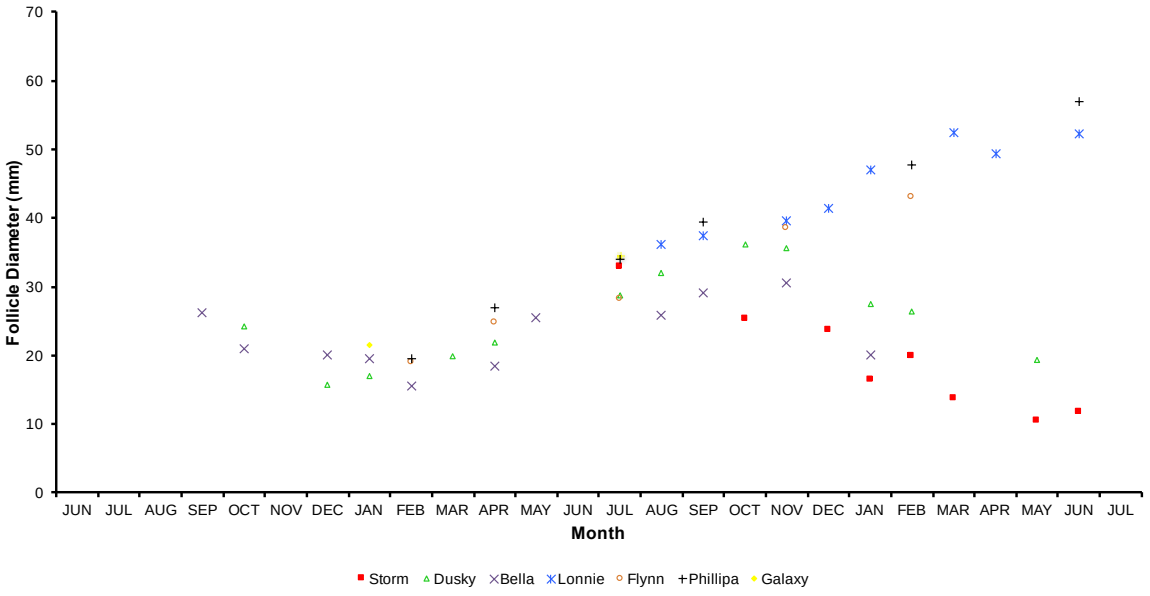

Figure 1. Changes in ovarian follicle diameter in broadnose sevengill sharks (*Notorynchus cepedianus*) measured by ultrasound.

Hormone analysis

Measurement of circulating reproductive hormones in serum or plasma by radioimmunoassay (RIA) provides an accurate means of determining the reproductive status of an individual animal. Several reproductive hormones are present in the blood of elasmobranchs, and these can be associated with reproductive parameters in live sharks. The main hormones associated with reproductive cycles in elasmobranchs are estradiol, progesterone, and testosterone in females, and testosterone in males (Hamlett, 1999; Hamlett and Koob, 1999). Early work on developing RIA for use in elasmobranchs involved collecting blood

from sharks maintained for a short period in captivity, and comparing the concentrations of reproductive hormones with the condition of reproductive organs following dissection (e.g. Koob et al., 1986). Since then, RIA has been used to compare circulating reproductive hormone levels with ovarian and testicular cycles in several elasmobranch species from wild populations, either with or without dissection for assessment of reproductive organs (Rasmussen and Gruber, 1993; Tricas, 2000; Awruch et al., 2008).

Analysis of reproductive hormone levels has so far had limited application in elasmobranch populations in aquaria. RIA has been used to monitor circulating hormone levels in captive elasmobranchs to assess the reproductive status of individual *C. plumbeus*, bull sharks (*Carcharhinus leucas*, Muller & Henle, 1839), and juvenile *N. brevirostris* (Rasmussen and Murru, 1992), *R. eglanteria* (Rasmussen et al., 1999), *C. taurus* (Henningsen et al., 2008), and *S. tiburo* (Gelsleichter et al., 2002). More recently, reproductive hormone analysis has been used to monitor the reproductive cycle in bowmouth guitarfish (*Rhina ancylostoma*, Bloch & Schneider, 1801) (Hanna, personal communication), and blackchin guitarfish (*Rhinobatos cemiculus*, Geoffrey Saint-Hilaire, 1817) (Lécu & Herbert, personal communication) in aquaria, and has been used to assist in the diagnosis of reproductive disease in *D. americana* (Mylniczenko and Penfold, 2012).

Although there are still many gaps in the knowledge of elasmobranch reproductive endocrinology (Awruch, 2013), hormone analyses have a lot to offer to reproductive management of elasmobranchs in aquaria. Monitoring reproductive hormones has

already been used to assist in the management of reproductive populations (Henningsen et al., 2008), and could be used either in place of or in conjunction with ultrasound to monitor female reproductive cycles. Hormone assays could also be used to monitor reproductive cycles in male elasmobranchs, for which ultrasound monitoring is of limited use. It may also be possible to predict the timing of ovulation in female elasmobranchs, which would be of great benefit to artificial insemination studies. At present the ranges of estradiol, progesterone, and testosterone during the reproductive cycle in male and female elasmobranchs are yet to be established for most species. In most cases, regular assay of reproductive hormones over at least a one to two-year period will be required for the establishment of 'normal' ranges, which will be essential to the utility of hormone analyses in breeding programs for elasmobranchs in aquaria.

Semen collection and sperm quality assessment

Traditionally aquaria have focussed on the female reproductive cycle and in particular the detection and monitoring of pregnancy. As a result, there is relatively little information available on monitoring reproductive cycles in male elasmobranchs compared to females. In many elasmobranch species the testes undergo annual seasonal changes in spermatogenesis, which are often accompanied by changes in gonadosomatic index (GSI) (Parsons and Grier, 1992; Maruska et al., 1996; Lucifora et al., 2005). In some species, seasonal changes in GSI correspond with the annual

breeding season, while others breed all year round irrespective of GSI, or have a defined mating season but no change in GSI (Parsons and Grier, 1992). As a result of this, and the relatively minor changes in size that the testes undergo throughout the reproductive cycle compared to ovaries in the female, ultrasound is of limited use in the monitoring of reproductive cycles in male elasmobranchs. Indeed, there are no published reports on the use of ultrasound to monitor reproductive cycles in male elasmobranchs.

Semen collection and sperm quality assessment have been used as a means of monitoring male reproductive function in aquaria (Daly, 2008). Semen can be collected from male elasmobranchs during the breeding season either by exerting gentle pressure on the distal reproductive tract proximal to the cloaca (e.g. Masuda et al., 2003; Masuda et al., 2005), or by passing a thin tube or catheter into the ampulla of the ductus deferens via the urogenital papilla (e.g. Minamikawa and Morisawa, 1996). Both methods have been used successfully to collect semen from a number of elasmobranch species in aquaria (Table 2). Semen collection and sperm quality assessments have been used to monitor seasonal reproductive cycles in male *N. cepedianus* and smooth stingrays (*Dasyatis brevicaudata*, Hutton, 1875) (Daly, 2008), and preliminary observations in other species including *C. punctatum*, *P. motoro*, and southern eagle rays (*Myliobatis australis, Macleay, 1881*), indicate that this method will be useful for following reproductive activity in a number of elasmobranch species held in aquaria (Daly & Jones personal observations).

The simplest way to assess the quality of a semen sample is by observing motility under light microscope, however there is very little information available in the literature on the handling conditions required for observing activity of elasmobranch sperm. The high osmotic pressure of elasmobranch bodily fluids means that sperm should be assessed in solutions with an osmolality of approximately 900 – 1100 $mOsmkg^{-1}$ in order for them to be maximally active. Most studies to date have utilized ionic solutions based on elasmobranch biological fluids. Jones et al. (1984) reported that sperm from *H. portusjacksoni* were active in a phosphate-buffered elasmobranch ringer solution that was based on the ionic composition of elasmobranch blood, and similar solutions have been used to observe the activity of elasmobranch sperm from a range of species (Daly and Jones, personal observations). Work by Minamikawa and Morisawa (1996) showed that sperm from the banded houndshark (*Triakis scyllium*, Muller & Henle, 1839) could maintain activity in solutions that replicate blood or uterine conditions. These investigators also suggested that the presence of hexoses such as glucose are important for sperm activity (Minamikawa and Morisawa, 1996).

A slightly different approach was used successfully by Luer et al. (2007), who reported that sperm from *R. eglanteria*, maintained high motility in a poultry semen extender that had been modified for elasmobranch conditions by the addition of urea, trimethylamine oxide, and NaCl to raise the osmotic pressure. These authors also found that sperm were highly active in a mixture of seminal fluid and fluid from the alkaline gland, suggesting that secretions from male secondary sexual organs may

also be important for sperm activity. From the limited information available it appears that the osmotic pressure of the solutions in which the sperm are held is more important than the exact ionic composition of those solutions, however it is important to note that most of the solutions used to date for dilution and handling of shark semen have contained high amounts of NaCl and urea to mimic the conditions naturally found in elasmobranch biological fluids.

Another method that has been used to assess elasmobranch sperm quality is "viability" staining. This method involves the use of a dye that is membrane impermeant, meaning that it is excluded by normal, "viable", cells but is able to stain the nucleii of "non-viable" cells with compromised plasma membranes. The fluorescent dyes SYBR® 14 and propidium iodide (LIVE/DEAD® Sperm Viability Kit, Molecular Probes, Eugene OR, USA) have been used in conjunction with flow cytometry to assess the viability of sperm from *Urolophus. paucimaculatus* (Dixon, 1969) and *M. australis* (Daly and Jones, personal observation), and to assess seasonal changes in semen quality in *D. brevicaudata* (Daly, 2008). The disadvantage of assays that utilize fluorescent dyes is that they require access to specialized equipment (i.e. a flow cytometer or fluorescent microscope) for analysis, which limits their application in aquaria for routine semen assessment or situations where immediate sperm quality analyses are required.

An alternative to the use of fluorescent stains is the use of viability stains that can be viewed under light microscope. One such viability stain is Nigrosin-Eosin, which is commonly used to assess mammalian and avian sperm but to date has had limited

application in sperm from elasmobranchs. The simplicity of this staining method makes it a suitable means of assessing sperm quality in the aquarium setting as a complement to motility assessments. It is important to note that motility assessment and viability staining evaluate two different aspects of sperm function, so wherever possible these methods should be used in combination to maximize the accuracy of sperm quality assessments.

Table 2. Species for which semen collection has been reported or observed.

Species	**Common name**	**Reference**
Chiloscyllium plagiosum (Bennett, 1830)	whitespotted bamboo shark	Masuda et al. 2005, Adams (pers. com.)
Scyliorhinus torazame (Tanaka, 1908)	cloudy cat shark	Masuda et al. 2003
Hemiscyllium ocellatum	epaulette shark	Janse (pers. com.), Daly & Jones (personal observation)
Carcharias taurus	sand tiger shark	Daly & Jones (personal observation)
Urolophus paucimaculatus	sparsely spotted stingaree	Daly & Jones (personal observation)
Urolophus gigas (Scott, 1954)	spotted stingaree	Daly & Jones (personal observation)
Chiloscyllium punctatum	brownbanded bamboo shark	Daly & Jones (personal observation)
Notorynchus cepedianus	broadnose sevengill shark	Daly & Jones (personal observation)
Dasyatis brevicaudata	smooth stingray	Daly & Jones (personal observation)
Potamotrygon motoro	ocellate river stingray	Daly & Jones (personal observation)

Triaenodon obesus	whitetip reef shark	Daly & Jones (personal observation)
Myliobatis australis	southern eagle ray	Daly & Jones (personal observation)
Triakis scyllium	banded houndshark	Minamikawa & Morisawa 1996
Stegostoma fasciatum	zebra shark	Janse (pers. com.)
Raja eglanteria	clearnose skate	Luer et al. 2007

METHODS FOR CONTROLLING REPRODUCTION

Artificial insemination

An area of research that is gaining increasing attention among aquaria is the use of artificial insemination to achieve pregnancy in elasmobranchs (Adams, personal communication; Janse, personal communication; Daly and Jones, unpublished observation). Although still in the early stages of development, results achieved so far indicate that this technique will be an important component of future breeding programs for elasmobranchs in aquaria. Research on artificial insemination procedures is ongoing, but in general, artificial insemination of elasmobranchs involves depositing sperm in the female reproductive tract with a tube or catheter attached to a syringe or pipette.

There are only three published accounts of artificial insemination in elasmobranchs (Masuda et al., 2003; Masuda et al., 2005; Luer et al., 2007), although there have been other unpublished attempts conducted by aquaria around the world. The first published account of artificial insemination in an elasmobranch was by Masuda et

al. (2003), who achieved a fertilization rate of 76.9% in *S. torazame)* when sperm were deposited in the cloaca of the female. A follow-up study by the same investigators in *C. plagiosum* reported a fertilization rate of 23.3% when sperm were deposited in the uterus of the female (Masuda et al., 2005). An unpublished study in *C. plagiosum* found that oviduct insemination resulted in a fertilization rate of 100%, but that insemination of a second female by depositing semen in the cloaca did not result in fertile eggs (Adams, personal communication). After re-insemination using the oviduct method, this second female began to produce fertile eggs. A study by Luer et al. (2007) tested different insemination methods in *R. eglanteria* and found that fertilization occurred when sperm were deposited in the cloaca or right uterine horn of the female, but did not occur when sperm were deposited in the left uterine horn. When compared to insemination of the right uterine horn, cloacal insemination resulted in a slightly higher fertilization rate during the first six weeks following insemination (100% compared to 77%), but a slightly lower fertilization rate from six to ten weeks after insemination (10% compared to 33%). There have also been unsuccessful attempts to artificially inseminate *N. cepedianus* (Daly and Jones, unpublished observation), *C. taurus* (Daly and Jones, unpublished observation), and *S. fasciatum* (Janse, personal communication). It appears from these studies that the site at which the sperm are deposited during the artificial insemination procedure has an effect on fertilization success, and outcomes may be affected by species differences, the timing of the insemination, or by the person performing the procedure.

Artificial insemination has also been attempted using semen that has been stored for a period of hours to days prior to insemination (Adams, personal communication). The motility and fertilizing ability of semen collected from *C. plagiosum* and stored at room temperature (21°C) or refrigerated (4°C) was assessed at 24, 48, and 72 h storage. Fertilization was achieved using sperm stored for 24 h at 4°C, but not using semen that was stored at 4°C for 48 h or semen that was stored at room temperature. Successful artificial insemination of a female *C. punctatum* at SEALIFE Melbourne Aquarium (Melbourne, Australia) was achieved using sperm collected at Underwater World SEALIFE Aquarium (Mooloolaba, Queensland) and stored at 4°C for 8 hours during transport (Daly and Jones, unpublished observation). Attempts have also been made to artificially inseminate a female *C. punctatum* using cryopreserved semen, but these have not resulted in successful fertilization (Daly and Jones, unpublished observation).

Artificial rearing of embryos from viviparous species

Although the incubation of eggs from oviparous species is relatively common among aquaria, there is only one account of an attempt to artificially rear developing fetuses from a viviparous species (Otway and Ellis, 2011). In this study, investigators removed late term fetuses with no external yolk sac from a pregnant female *O. ornatus* and transferred them to an artificial uterus for the final 18 days of gestation. All fetuses survived until “birth”, and followed similar growth patterns to *O. ornatus*

pups born naturally. Artificial rearing of embryos or fetuses from earlier developmental stages is likely to be restricted by difficulties in accurately replicating the elasmobranch uterine environment and embryo nutrition in species with matrotrophy. With further research it is possible that artificial uteri could have an application in aquarium breeding programs for elasmobranchs, however this is unlikely to be in the foreseeable future.

Decreasing reproduction and associated issues

In aquaria it is sometimes necessary to reduce the reproductive capacity of an individual or population. This may be for population management, to mitigate interactions among conspecifics, or to improve the health of an animal. In elasmobranch species such as *C. taurus*, seasonal changes in water temperature are important for reproductive behavior and mating (Henningsen et al., 2004; Henningsen et al., 2008). For these species, interactions among conspecifics and reproductive activity can be reduced by maintaining a constant water temperature (Henningsen et al., 2008). For other species, the simplest and most common way to reduce reproduction in a species is by housing single sex populations, however this can have health consequences in some species. Female *D. americana* housed in single sex populations are known to develop reproductive disease characterized by increased estrogen levels, ovarian pathologies, and an overabundance of histotroph in the uterus (Mylniczenko and Penfold, 2012; George, personal communication;

Mylniczenko, personal communication). Depo-Provera® (medroxyprogesterone acetate, 400 mg/mL, Pfizer) has been used successfully to reduce the incidence of reproductive disease in single sex populations of *D. americana* (Mylniczenko, personal communication), and ovariectomy has been used in sub-adult females of this species as a more permanent means of eliminating reproductive function and preventing the development of reproductive disease (George, personal communication), although the long term effects of this procedure are as yet unknown.

Hormonal implants containing deslorelin, a GnRH agonist, have also been tested as a means of reducing reproduction in elasmobranch species with mixed success. Suprelorin™ implants (deslorelin , 4.7 mg, Peptech Animal Health) have been tested in male smooth stingrays but were unsuccessful in reducing reproductive function (Daly and Jones, unpublished observation), and have also been trialed in a male *S. fasciatum* (Mylniczenko, personal communication). In female vermiculate river stingrays (*Potamotrygon falkneri*, Castex & Maciel, 1963) Suprelorin™ implants (deslorelin, 4.7 mg) produced mixed results, with one of the two females treated producing pups while on the treatment (Hanna, personal communication). The reasons for the varied success of hormonal implants in elasmobranchs are unknown, but may be related to variation in the physiological response to the hormonal preparations or differences in the application of the implants. The utility of hormonal implants to control reproduction should therefore be assessed for each species as required.

Sperm cryopreservation

In general, sperm cryopreservation involves the dilution of semen in an extender solution containing cryoprotectants that help protect the sperm from freezing injury, followed by packaging of samples in straws or vials, freezing at a controlled cooling rate, and storage of samples in liquid nitrogen at -196°C. Despite the common use of sperm cryopreservation in breeding programs for a wide range of aquatic animals (Tiersch, 2011), there has to date been very little work done in elasmobranchs. The only published account of cryopreservation of sperm from an elasmobranch is in *U. paucimaculatus* (Daly et al., 2011). This study reported post-thaw membrane integrity of 23.4±0.4% and an initial motility of 37.5±3.4% when sperm were frozen in an extender containing elasmobranch ringer solution (257 mM NaCl, 7 mM Na_2SO_4, 2.5 mM $NaHCO_3$, 4 mM KCl, 2 mM $CaCl_2$, 3 mM $MgSO_4$, 70 mM trimethylamine N-oxide, 0.27 mM Na_2HPO_4, 0.01 mM NaH_2PO_4, 400 mM urea, 30 mM glucose) with 20% v/v egg yolk and 10% v/v glycerol. Sperm frozen using a tris-sucrose-potassium solution (30 mM Tris, 25 mM sucrose, 600 mM KCl) with 20% v/v egg yolk and 10% v/v glycerol or dimethyl sulfoxide (DMSO) had slightly lower post-thaw membrane integrity (21.7±0.5% and 18.4±0.4% respectively) and motility (35.8±4.0% and 30.8±3.3% respectively) but a longer duration of motility than other treatments.

Recent experiments on cryopreservation of sperm from *M. australis* have achieved a post-thaw motility of 30% (50% of pre-freeze motility) and membrane integrity of 57.5% using elasmobranch ringer solution containing 10% v/v glycerol (Daly and Jones, unpublished observation). The higher post-thaw motility and membrane integrity in this recent study is possibly due to the faster cooling rate used (>10°C/min) compared to the earlier study in *U. paucimaculatus* (3°C/min), but may also be due to species differences in sperm freezing tolerance. It is possible that there will be species variation in sperm cryopreservation requirements among elasmobranchs. Recent experiments on cryopreservation of sperm from *C. punctatum* have found that glycerol is toxic to sperm from this species, despite having protective effects in sperm from *U. paucimaculatus* and *M. australis* (Daly and Jones, unpublished observation). Although some components of the sperm cryopreservation protocol will remain the same for all elasmobranch species, some aspects, including cryoprotectant type, will need to be tested for each species.

FUTURE DIRECTIONS

Reproductive technologies are a relatively new addition to aquarium breeding programs for elasmobranchs, but they have already contributed greatly to the management of these species. While technologies such as ultrasound have gained relatively widespread use, others such as sperm cryopreservation and artificial insemination, are still in the early stages of development. Sperm cryopreservation

protocols have so far been tested in only three species with mixed results. There are many factors that affect sperm cryopreservation including base medium composition, cooling rate, and cryoprotectant type, concentration, and equilibration time. These factors will need to be optimized for each species or species group in order for sperm cryopreservation to be a useful technique for aquarium breeding programs and for gene banking of elasmobranch sperm.

Preliminary studies on artificial insemination have given an indication of the future potential of this technology, but there is still a lot of research required before it will become common practice among aquaria. It is worth noting that so far all successful attempts at artificial insemination have been in oviparous species, in which ovulation can be easily visualized. Further research will be needed to adapt this technique for viviparous species and to identify the optimal time for insemination. Although sperm storage has been reported for many elasmobranch species, it is possible that the receptivity of the female reproductive tract to sperm storage may be hormonally modulated and vary throughout the reproductive cycle. For species with a well-defined annual or biennial cycle it may be possible to predict the timing of artificial insemination based on behavioral cues or ultrasound examination, while other species may benefit from hormonal monitoring to predict the timing of ovulation, or even the induction of ovulation through administration of exogenous hormones.

REFERENCES

American Elasmobranch Society. 2008. The 2008 AES International Captive Elasmobranch Census. http://elasmo.org/census. Accessed 06 January 2016.

Awruch, C.A. 2013. Reproductive endocrinology in chondrichthyans: The present and the future. General and Comparative Endocrinology, 192, 60–70.

Awruch, C.A., Pankhurst, N.W., Frusher, S.D. and Stevens, J.D. 2008. Endocrine and morphological correlates of reproduction in the draughtboard shark *Cephaloscyllium laticeps* (Elasmobranchii: Scyliorhinidae). Journal of Experimental Zoology Part A: Ecological Genetics and Physiology, 309A, 184–197.

Carrier, J.C., Murru, F.L., Walsh, M.T. and Pratt, H.L. 2003. Assessing reproductive potential and gestation in nurse sharks (*Ginglymostoma cirratum*) using ultrasonography and endoscopy: An example of bridging the gap between field research and captive studies. Zoo Biology, 22, 179–187.

Daly, J. 2008. Development of Assisted Reproductive Technologies in captive fish. PhD thesis. Monash University.

Daly, J., Gunn, I., Kirby, N., Jones, R. and Galloway, D. 2007. Ultrasound examination and behavior scoring of captive broadnose sevengill sharks, *Notorynchus cepedianus* (Peron, 1807). Zoo Biology, 26, 331–343.

Daly, J., Holland, M. and Galloway, D. 2011. Preliminary Investigations on Sperm Cryopreservation of a Stingray, the Sparsely Spotted Stingaree. In Cryopreservation in Aquatic Species 2nd Edition. (Eds Tiersch, T.R., Green, C.C.) Baton Rouge, Louisiana USA: World Aquaculture Society, 337–344 pp.

Gelsleichter, J., Rasmussen, L., Manire, C., Tyminski, J., Chang, B. and Lombardi-

Carlson, L. 2002. Serum steroid concentrations and development of reproductive organs during puberty in male bonnethead sharks, *Sphyrna tiburo*. Fish Physiology and Biochemistry, 26, 389–401.

Hamlett, W.C. 1999. Male reproductive system. In Sharks, Skates, and Rays. The Biology of Elasmobranch Fishes. (Ed Hamlett, W.C.) Baltimore, MD, USA: The Johns Hopkins University Press, 444–470 pp.

Hamlett, W.C. and Koob, T.J. 1999. Female reproductive system. In Sharks, Skates, and Rays. The Biology of Elasmobranch Fishes. (Ed Hamlett, W.C.) Baltimore, MD, USA: The Johns Hopkins University Press, 398–443 pp.

Henningsen, A.D. 1994. Tonic immobility in 12 elasmobranch species: Use as an aid in captive husbandry. Zoo Biology, 13, 325-332.z

Henningsen, A.D., Murru, F.L., Rasmussen, L.E.L., Whitaker, B.R. and Violetta, G.C. 2008. Serum levels of reproductive steroid hormones in captive sand tiger sharks, *Carcharias taurus* (Rafinesque), and comments on their relation to sexual conflicts. Fish Physiology and Biochemistry, D.O.I. 10.1007/s10695-008-9202-9.

Henningsen, A.D., Smale, M., Gordon, I., Garner, R., Marin-Osorno, R. and Kinnunen, N. 2004. Captive breeding and sexual conflict in elasmobranchs. In The Elasmobranch Husbandry Manual: Captive Care of Sharks, Rays, and Their Relatives. (Eds Smith, M., Warmolts, D., Thoney, D., Hueter, R.) Columbus, OH: Ohio Biological Survey, 237–248 pp.

Koob, T.J. 2004. Elasmobranchs in the public aquarium: 1860 to 1930. In The Elasmobranch Husbandry Manual: Captive Care of Sharks, Rays, and Their

Relatives (Eds Smith, M., Warmolts, D., Thoney, D., Hueter, R.) Columbus, OH: Ohio Biological Survey, 1–14 pp.

Koob, T.J., Tsang, P. and Callard, I.P. 1986. Plasma estradiol, testosterone, and progesterone levels during the ovulatory cycle of the skate (*Raja erinacea*). Biology of Reproduction, 35, 267–275.

Luer, C.A., Walsh, C.J., Bodine, A.B. and Wyffels, J.T. 2007. Normal embryonic development in the clearnose skate, *Raja eglanteria*, with experimental observations on artificial insemination. Environmental Biology of Fishes, 80, 239–255.

Masuda, M., Izawa, Y., Kametsuta, S., Ikuta, H. and Isogai, T. 2003. Artificial Insemination of the Cloudy Catshark. Journal of Japanese Association of Zoological Gardens and Aquariums, 44, 39–43.

Masuda, M., Izawa, Y., Kametsuta, S., Ikuta, H. and Isogai, T. 2005. Artifical Insemination of the White-spotted Bamboo Shark, *Chiloscyllium plagiosum*. Journal of Japanese Association of Zoological Gardens and Aquariums, 46, 91–96.

Minamikawa, S. and Morisawa, M. 1996. Acquisition, Initiation and Maintenance of Sperm Motility in the Shark, *Triakis scyllia*. Comparative Biochemistry and Physiology--Part A: Physiology, 113A, 387–392.

Mylniczenko, N. and Penfold, L.M. 2012. Ultrasound findings in southern stingrays (*Dasyatis americana*) with and without reproductive disease. 43rd Annual IAAAM Conference.

Otway, N. and Ellis, M.T. 2011. Construction and test of an artificial uterus for ex

situ development of shark embryos. Zoo Biology, 31, 197–205.

Parsons, G. and Grier, H. 1992. Seasonal changes in shark testicular structure and spermatogenesis. Journal of Experimental Zoology Part A: Ecological Genetics and Physiology, 261, 173–184.

Rasmussen, L. and Gruber, S. 1993. Serum concentrations of reproductively-related circulating steroid hormones in the free-ranging lemon shark, *Negaprion brevirostris*. Environmental Biology of Fishes, 38, 167–174.

Rasmussen, L.E.L., Hess, D.L. and Luer, C.A. 1999. Alterations in serum steroid concentrations in the clearnose skate, *Raja eglanteria*: Correlations with season and reproductive status. Journal of Experimental Zoology, 284, 575-585

Rasmussen, L. and Murru, F. 1992. Long-term studies of serum concentrations of reproductively related steroid hormones in individual captive carcharhinids. Australian Journal of Marine and Freshwater Research, 43, 273–281.

Schaller, P. 2006. Husbandry and reproduction of Whitetip reef sharks *Triaenodon obesus* at Steinhart Aquarium, San Francisco. International Zoo Yearbook, 40, 232–240.

Tiersch, T.R. and Green, C.C. (Eds). 2011. Cryopreservation in Aquatic Species 2nd Edition, 2nd ed. Baton Rouge, Louisiana USA: World Aquaculture Society.

Tricas, T. 2000. Annual Cycles of Steroid Hormone Production, Gonad Development, and Reproductive Behavior in the Atlantic Stingray. General and Comparative Endocrinology, 118, 209–225.

Walsh, M., Pipers, F., Brendemuehl, C. and Murru, F. 1993. Ultrasonography as a

diagnostic tool in shark species. Veterinary Radiology & Ultrasound, 34, 213–219.

Zegers-Hochschild, F., Adamson, G.D., de Mouzon, J., Ishihara, O., Mansour, R., Nygren, K., Sullivan, E., van der Poel, S.on behalf of ICMART and WHO 2009. The International Committee for Monitoring Assisted Reproductive Technology (ICMART) and the World Health Organization (WHO) Revised Glossary on ART Terminology, 2009. Human Reproduction, 24, 2683–2687.

PERSONAL COMMUNICATIONS

Adams, L. 2013. Aquarium of the Pacific, 100 Aquarium Way, Long Beach CA 90802, USA. Personal communication.

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