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The Value of *Ex Situ* Cetacean Populations in Understanding Reproductive Physiology and Developing Assisted Reproductive Technology for *Ex Situ* and *In Situ* Species Management and Conservation Efforts

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Wild cetacean populations have uncertain futures in the face of shifting climate conditions and the continued encroachment of their unique ecosystem by human activities. Core conservation efforts focus on habitat protection and understanding the natural ecology of a species, but such efforts are incomplete without a comprehensive understanding of a species' physiology. *Ex situ* populations of cetaceans provide a unique opportunity to collect this physiological data, and thereby serve as an important component of any conservation effort. The sustainability of captive cetacean populations is in turn dependent on a thorough understanding of reproductive physiology, and such research has facilitated the development of assisted reproductive technology (ART). ART, specifically gamete preservation for genome resource banking, artificial insemination and sperm sexing, has been used to significantly enhance the genetic, reproductive and social management of *ex situ* cetaceans. For endangered cetaceans and other marine mammals, ART will permit the establishment of permanent repositories of valuable genetic material which could be used to maximize their reproductive potential and maintain the species' genetic diversity; an approach that, when combined with *in situ* conservation efforts, may prevent their extinction.

Cetaceans have been held in aquaria for centuries, initially as novel curiosities in private collections, then for display in a zoological setting (reviewed

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by Reeves & Mead, 1999). The goals of modern zoological institutions are very different to those of their predecessors, whose sole purpose was for public display. Zoological institutions have since become cooperative operations, unified in their efforts toward global species conservation through the development of sustainable populations, and by conducting and/or supporting research and education programs both *in situ* and *ex situ*. Integration of conservation programs throughout multiple scientific disciplines (i.e., ecology, genetics, physiology, anatomy, behavioral biology) form the basis of most species management programs being implemented today. In view of the significant threats to marine environments, an understanding of a species physiology, including their reproductive physiology, is of paramount importance to their long-term conservation in the wild.

The majority of cetaceans housed in zoological settings today represent two different taxonomic families, Delphinidae and Monodontidae. The most common species of Delphinidae in aquaria is the bottlenose dolphin (*Tursiops* sp.), with the AZA managed North American region comprising 788 animals (Dudley, 2008). Well recognized but less common delphinids held worldwide in aquaria are the killer whale (*Orcinus orca*; ≈ 41 individuals, B. Andrews, personal communication), the Pacific white-sided dolphin (*Lagenorhynchus obliquidens*; ≈ 119 individuals, Robeck et al., 2009) and the false killer whale (*Pseudorca crassidens;* 23 individuals; Robeck & Arai, unpublished data). The only *ex situ* population of Monodontidae is the beluga (*Delphinapterus leucas*), with a world captive population of approximately 95 individuals (B. Andrews, personal communication). With the exception of the bottlenose dolphin, *ex situ* population sizes of cetaceans are small and animals are distributed, often disparately relative to sex and age, across numerous facilities. Cooperative research and breeding programs are therefore necessary to generate scientifically robust information and understand species-specific reproductive mechanisms. This knowledge can then be used to enhance natural breeding programs with assisted strategies such as artificial insemination (AI).

Of the predominant species of cetaceans in aquaria, the species whose wild counterparts have the most concerning status is the beluga. Though the status of this arctic and subarctic dwelling species was downgraded from vulnerable to near threatened in 2008 (Jefferson et al., 2008), the population trend remains unknown, and a subpopulation of beluga, whose habitat is the Cook Inlet in Alaska, has been classified as critically endangered in response to multifactorial impacts of habitat change and subsistence hunting (Lowry, 2006). IUCN population evaluations of killer whales are recognized as being data deficient overall (IUCN, 2008), but similar to the beluga, a subpopulation of the species, the southern resident killer whale stock in the Pacific Northwest, has been listed as endangered under the Endangered Species Act (National Marine Fisheries Service, 2008). Although the pressures on *in situ* populations remain distinctly different to that of animals in aquaria, both groups require monitoring and some degree of intervention to ensure they are healthy and sustainable. As their natural environment continues to change, the future success of *in situ* and *ex situ* populations of cetaceans are reliant on continued research across the spectra of scientific disciplines associated with their biology. This review will describe both the current status of reproductive research and assisted reproductive technology development in zoological-based cetaceans and their potential and realized impacts on *in situ* and *ex situ* species conservation.

Reproductive Research

Female and male reproductive biology

Due to the nature of their aquatic habitat, it is not possible to collect biological samples or conduct examinations on wild marine mammals at a frequency adequate to characterize a species' reproductive physiology. Observations both in the wild and in aquaria, of breeding and subsequent parturition, enabled initial estimates of reproductive seasonality, age of reproductive maturity and gestation length, for a particular species. However, the high tractability of *ex situ* cetaceans, in conjunction with operant conditioning programs and advancing monitoring technologies (as described below), has enabled scientists to garner definitive information on such reproductive characteristics as well as reproductive cycle length.

With appropriate conditioning, cetaceans can be trained for biological sample collection and ultrasonographical reproductive tract examination on a voluntary basis. Sample types such as blood (e.g., Cornell et al., 1987; Duffield, Odell, McBain, & Andrews, 1995; Yoshioka, Aida, & Hanyu, 1986), urine (e.g., Robeck et al., 2005a ; Walker et al., 1988), milk (West et al., 2000) and feces (e.g., Biancani, Da Dalt, Lacave, Romagnoli, & Gabai, 2009) have been collected and analyzed for longitudinal hormone monitoring studies. Operant conditioning of cetaceans has also facilitated reproductive research and management programs through the collection of semen (e.g., Schroeder & Keller, 1989), body temperature data (e.g., Katsumata, Furuta, Katsumata, Watanabe, & Taya, 2006a; Terasawa, Yokoyama, & Kitamure, 1999) and reproductive tract examinations using transabdominal ultrasound (e.g., Brook, 2001; Brook, Kinoshita, Brown, & Metreweli, 2000).

Early studies using post-mortem material provided information on reproductive anatomy in cetaceans (e.g., Harrison & Ridgway, 1971). The advent of endocrine monitoring using immunoassays, and the ability to perform longitudinal serial sample collections and ultrasonographical reproductive tract examinations in live animals, has since led to accurate descriptions of female (Table 1) and male (Table 2) reproductive characteristics for several species of cetaceans. Urinary hormone monitoring has provided the most information on female reproductive physiology due to the high frequency (up to multiple samples per day) and non-invasive method under which samples can be collected. When combined with ultrasound monitoring, urinary hormone assays have revolutionized our understanding of estrous cycle dynamics for several cetaceans and has revealed characteristics unique for each species. For example, Steinman, O'Brien, and Robeck (2007) provided the first evidence of facultative-induced ovulation in a cetacean species using serial urinary hormone monitoring and ovarian ultrasound.

Female and male reproductive research provides a number of benefits to captive breeding programs. Ovarian ultrasound can facilitate controlled natural breeding via peri-ovulation introduction or separation of males and females (Brook & Kinoshita, 2005). Endocrinological analyses in conjunction with ovarian ultrasound provide more definitive information than ultrasound alone on the appropriate timing of controlled breeding efforts or AIs (e.g., Robeck et al., 2009). As a result of reproductive studies, management of pregnant animals through different gestational stages can be accomplished along with fetal health monitoring and prediction of parturition timing (Katsumata et al., 2006b; Lacave et al., 2004; Robeck, Gili, Iannaccone, Steinman, & O'Brien, 2005c; Williamson, Gales, & Lister, 1990). Reproductive research is also facilitating the development of male and female contraceptive methods for cetaceans such as the bottlenose dolphin (Briggs, 2000). Contraceptive programs are crucial for managing animals in limited enclosure spaces, as is the case for many cetaceans which reproduce well in aquaria.

Information on spermatogenesis and sperm biology represents important components of a species-specific reproductive baseline database, which can be incorporated into health assessments for both captive and free-ranging cetaceans. Although numerous males have been trained for voluntary semen collection (Table 2), high quality samples, indicative of ejaculates produced by natural matings, can only be collected on a routine basis after development of suitable training and collection methods (beluga: Graack & Grovhoug, 2003; O'Brien, Steinman, Schmitt, & Robeck, 2008; bottlenose dolphin: Robeck & O'Brien, 2004; Schroeder & Keller, 1989; killer whale: Fripp, Rokeach, Robeck, & O'Brien, 2005; Pacific white-sided dolphin: Robeck et al., 2009). Training and collection methods differ slightly across species, but all techniques are based on maximizing the quality of the ejaculatory response and avoiding saltwater and urine contamination.

Seasonal effects on testosterone secretion and sperm production (Table 2) need to be considered for some species when initiating a semen collection training program for either reproductive research or reproductive health evaluations of individuals. High quality ejaculates can be collected year-round from most cetaceans studied to date (Table 2), with the exception of the Pacific white-sided dolphin, where spermic ejaculates are produced only during the Northern hemisphere's summer and early fall (Robeck et al., 2009). For species exhibiting seasonal trends in sperm production such as the beluga and the bottlenose dolphin (Table 2), the annual timing of semen collection should also be taken into account. Based on weekly collections, annual sperm production in a beluga was highest in winter and early spring (O'Brien et al., 2008), whereas three of four bottlenose dolphins (*Tursiops truncatus*) housed together in California produced ejaculates with significantly higher numbers of spermatozoa in spring and summer compared to fall and winter (16.8 ± 13.3 billion spermatozoa/ejaculate and 8.7 ± 8.6 billion spermatozoa/ejaculate, respectively, n = 382 ejaculates; Robeck & O'Brien, unpublished data).

Table 1

Female cetacean reproductive characteristics derived from research incorporating endocrinological and/or ovarian and uterine ultrasound analyses of zoological-based animals. Data are means and/or range (when available), unless indicated.

Species	Reproductive cycle length (d)	Preovulatory follicle size (maximum diameter, mm)	Timing of pregnancy diagnosis by weekly progestagens (P) or ultrasound (U) (d post-breeding or AI)	Gestation length (d post-breeding or AI, unless indicated)	Reproductive maturity (based on age at first presumptive ovulation and/or conception) (yrs)	Reproductive seasonality (based on cycling and/or parturition)*
ODONTOCETES						
Bottlenose dolphin *Tursiops truncatus*	33 (31-36)[1,3]	21 (17-31)[1]	P: 28; U: 50-60[2,3]	377 (357-399)[1,2,3,4]	4[5]	Seasonal trends[1,2]
Indo-Pacific bottlenose dolphin *Tursiops aduncus*	30 (27-33)[6]	21 (16-23)[7]	-	370 (352-384)[6]	-	Seasonal trends[6]
Indo-Pacific humpback dolphin *Sousa chinensis*	30 (28-36)[8]	20 (18–22)[8]	-	≈ 11–12 months[8]	-	Seasonal trends[8]
Pacific white-sided dolphin *Lagenorhynchus obliquidens*	31 (29-34)[4,9]	15 (13-18)[9]	P: 28; U: 40-50[4]	356 (348–367)[9]	3[10]	Highly seasonal[9]
Killer whale *Orcinus orca*	42 (36-47)[4,11]	39 (3.1-5.2)[4,11]	P: 35; U: 120-140[4]	530 (466-561)[4,11]	7-8[12]	Seasonal trends[11,12]
False killer whale *Pseudorca crassidens*	-	-	-	14 months[13]	5[14]	Seasonal trends[13,15]
MONODONTOCETES						
Beluga *Delphinapterus leucas*	40 (30-49)[16,17]	29 (2.4-4.2)[16,17,18]	P: 28; U: 40-50[4,18]	471 (450-491)[4]	6[19]	Highly seasonal[19]

*All species exhibit polyestrous activity. [1]Urian et al., 1996; [2]Robeck et al., 2005a; [3]O'Brien & Robeck 2006; [4]Robeck & O'Brien, unpublished data; [5]Dudley, 2008; [6]Brook, 1997; [7]Brook, 2001; [8]Brook et al., 2004; [9]Robeck et al., 2009; [10]Dalton et al., 2005; [11]Robeck et al., 2004b; [12]Robeck et al., 1993 and Duffield et al., 1995; [13]Robeck et al., 1994b; [14]Robeck et al., 2001; [15]Atkinson et al., 1999; [16]Steinman et al., 2007; [17]Robeck et al., 2010; [18]O'Brien et al., 2008. [19]Robeck et al., 2005b.

Table 2
Male cetacean reproductive characteristics derived from zoological-based animals trained for voluntary semen collection. Data are either means ±SD, and/or range (when available), unless indicated.

Species	Ejaculate volume (ml)	Sperm concentration (x10^{7}/ml)	Total spermatozoa per ejaculate (x10^{7})	Sperm progressive motility (%)	Sperm plasma membrane integrity (viability) (%)	Morphologically normal spermatozoa (%)	Reproductive maturity (age at first spermic ejaculate and/or conception) (yrs)	Reproductive seasonality (occurrence of spermatogenesis)
ODONTOCETES								
Bottlenose dolphin *Tursiops truncatus*	26 ± 18[1]	78 ± 44[1]	1580 ± 950[1]	87 ± 4[1]	88 ± 4[1]	96 ± 2[2]	5[3]	Non-seasonal[4] & seasonal trends[4,5,6]
Indo-Pacific bottlenose dolphin *Tursiops aduncus*	0.3-64[7]	2-142[7]	60-1320[7]	16-95[7]	75-97[7]	-	7-8[7]	Non-seasonal[7]
Pacific white-sided dolphin *Lagenorhynchus obliquidens*	10 ± 6[8] (1-35; *n* = 51)	70 ± 70[8] (0.5-273; *n* = 52)	902 ± 1157[8] (3-4518; *n* = 52)	92 ± 6[8] (78-98; *n* = 26)	94 ± 5[8] (76-99; *n* = 44)	96 ± 3[8] (87-99; *n* = 26)	-	Highly seasonal[8]
Killer whale *Orcinus orca*	7 ± 7[9] (0.5-40; *n* = 70)	71 ± 50[9] (8-240; *n* = 66)	538 ± 891[9] (17-4480; *n* = 66)	93 ± 4[9] (80-100; *n* = 60)	89 ± 8.8[9] (48-98; *n* = 48)	90 ± 7[9] (71-99; *n* = 30)	10[9]	Non-seasonal[9]
MONODONTOCETES								
Beluga *Delphinapterus leucas*	2 ± 1[10]	30 ± 16[10]	54 ± 49[10]	45 ± 5[10]	83 ± 6[10]	60 ± 6[10]	9[11]	Seasonal trends[a,10]

[a]Spermatozoa are produced year-round. [1]O'Brien & Robeck, 2006 (*n* = 3 males); [2]Robeck & O'Brien, 2004a (*n* = 4 males); [3]Dudley, 2008; [4]Robeck & O'Brien, unpublished data (*n* = 17 males); [5]Schroeder & Keller, 1989 (*n* = 1 male); [6]Montano et al., 2007 (*n* = 1 male); [7]Yuen, Brook, Kinoshita, & Ying, 2009 (*n* = 3 males); [8]Derived from Robeck et al., 2003, Robeck et al., 2009, Robeck & O'Brien, unpublished data (*n* = 2 males); [9]Derived from Robeck et al., 2004b, Robeck & Monfort, 2006, Robeck & O'Brien, unpublished data (*n* = 6 males); [10]O'Brien et al., 2008 (*n* = 1 male); [11]Robeck et al., 2005b (*n* = 7 males).

Consideration of the social grouping is also required to avoid social suppression of spermatogenesis by con-specific males. Observations of bottlenose dolphin social groups comprising two to three males showed that in some cases, the subordinate male had a tendency to produce ejaculates with reduced sperm concentration (< 100 million spermatozoa/ml). When the subordinate male was moved to a different social group and underwent a presumptive change in social rank, ejaculate sperm concentration increased substantially (> 500 million spermatozoa/ml; Robeck & O'Brien, unpublished data). These preliminary observations imply an ability of the dominant male to exert a suppressive effect on reproductive function in the subordinate animal, as has been reported in some terrestrial species (e.g., olive baboons, *Papio anubis*: Sapolsky, 1985). Further research incorporating monitoring of physiological (sperm concentration and quality, glucocorticoid and reproductive hormone concentrations, testicular size) and behavioral parameters is required to characterize mechanisms underlying socially-mediated effects on male cetacean reproduction. It should also be noted that for all species, information on ejaculate and sperm characteristics from larger numbers of males is required to keep building upon the current cetacean database.

Assisted Reproductive Technology

Implications of assisted reproductive technology to ex situ population management

The maintenance of sustainable *ex situ* populations of cetaceans depends on the ability to manage the genetic representation of individual animals within each species. Typically, management strategies designed to address the potential genetic bottlenecks that can occur in reproductively-isolated facilities often involve movement of breeding animals between facilities. While animal movement can be a successful means of genetic exchange, it is not without risk and significant expense, and for some species, is disruptive to the stability of social groups. Additionally, regulatory requirements surrounding marine mammals have proved increasingly difficult, and in some cases impossible, for allowing international movement of animals between many countries.

Recognition of the challenges faced when managing *ex situ* populations and the desire to participate in genetic and reproductive management on a global level became the basis for the development of a reproductive research program at SeaWorld USA (Robeck, Curry, McBain, & Kraemer, 1994a; Robeck & O'Brien, 2005). Three main goals became central to the program. Firstly, representation of founders that had not reproduced naturally was needed to ensure valuable genetic diversity was not lost. Assisted breeding practices were therefore required to help maximize the reproductive potential of founders and other genetically valuable individuals, and alleviate behavioral incompatibilities and the need to transport animals for breeding. Secondly, methodologies for the long-term preservation of spermatozoa, collected from trained animals or retrieved after the death of an animal, were required to further maintain genetic diversity that would otherwise be lost over time. Thirdly, development of sex predetermination technology was desired to efficiently manage the limited availability of enclosure space while still maintaining cohesive social groups of each species. To achieve these three goals it was apparent that a suite of assisted reproductive technologies (AI, estrus synchronization, semen preservation and sperm sexing) must be developed for each species of concern.

Artificial insemination and estrus synchronization

Characterization of reproductive cycles and ovarian function has been performed in several species of cetaceans as described previously (Table 1). Further research was then required to gain an understanding of the relationship between reproductive endocrinology, anatomy, behavior and physiological events such as ovulation. When combined with knowledge of basic anatomy (vaginal, cervical and uterine structure), such reproductive research has permitted the development of estrus synchronization and AI techniques, culminating in 35 calves from five species of cetaceans (Table 3).

Estrus has been synchronized in cetaceans using an oral synthetic progestagen treatment and trials have demonstrated that the proportion of females exhibiting estrus following treatment is maximized during the discrete (Pacific white-sided dolphin, beluga) or diffuse (bottlenose dolphins, killer whales) “breeding season” inherent to each species (Robeck et al., 2005a; Robeck & O’Brien, unpublished data). Monitoring of urinary hormones and ovarian activity led to detailed descriptions of the temporal relationship between ovulation and peak concentrations of hormones (estrogens and luteinizing hormone), thereby allowing optimal timing of AI (0-8 h prior to ovulation; beluga: Robeck et al., 2010; Steinman et al., 2007; bottlenose dolphin: O’Brien & Robeck, 2006; O’Brien et al., 2008; Robeck et al., 2005a; killer whale: Robeck et al., 2004, Robeck, Steinman & O’Brien, unpublished data; Pacific white-sided dolphins: Robeck et al., 2009).

Initial research demonstrated that intrauterine sperm deposition, as apposed to vaginal or cervical, was required to optimize the success of AI in cetaceans (Robeck et al., 1994a). Intrauterine inseminations have been accomplished in cetaceans using a non-surgical, endoscopic method comprising custom-made catheters and specialized endoscope preparation for use with spermatozoa. Females are either trained to permit a voluntary intrauterine endoscopy procedure (Neto et al., 2008; Robeck et al., 2004; Figure 1), or are removed from the water under mild or no sedation and placed on foam pads during the procedure’s 20 to 30 min duration, while they are kept cool and clinically monitored. No significant complications have resulted from any of the 153 cetacean AI procedures conducted by our research group (Robeck & O’Brien, unpublished data).

Table 3
Cetaceans produced from artificial insemination (AI) by the SeaWorld and Busch Gardens Reproductive Research Center and collaborators (n=35 across all species), and type of sperm processing prior to insemination.

Species	Sperm processing prior to AI	Number of calves[d]	Species totals
ODONTOCETES			
Bottlenose dolphin *Tursiops truncatus*	Fresh-chilled[b,1]	1	21
	Frozen-thawed[b,1,3]	6	
	Sexed-fresh-chilled[a,3]	3	
	Sexed-frozen-thawed[a,2,3]	10	
	Frozen-thawed-sexed-frozen-thawed[a,3]	1	
Indo-Pacific bottlenose dolphin *Tursiops aduncus*	Fresh-chilled[c],[4]	1	1
Pacific white-sided dolphin *Lagenorhynchus obliquidens*	Frozen-thawed[b,5]	5	5
Killer whale *Orcinus orca*	Fresh-chilled[c,6]	2	4
	Frozen-thawed[c,6]	2	
MONODONTOCETES			
Beluga *Delphinapterus leucas*	Fresh-chilled[a,7]	1	4
	Frozen-thawed[a,8]	3	

[a]One insemination per estrus. [b]One insemination or multiple inseminations per estrus. [c]Multiple inseminations per estrus. [d]Six calves are due from September 2010.

[1]Robeck et al., 2005a; [2]O'Brien & Robeck, 2006; [3]O'Brien et al., 2009; [4]Robeck et al., 2001; [5]Robeck et al., 2009; [6]Robeck et al., 2004; Robeck et al., unpublished; [7]O'Brien et al., 2008; [8]Robeck et al., 2010; Robeck et al., unpublished.

Figure 1. A killer whale undergoing trained, voluntary artificial insemination at SeaWorld San Diego, CA.

Conception rates following intrauterine AI are influenced by numerous factors, primarily sperm quality, sperm dose, AI timing relative to ovulation and quality of the oocyte; the latter of which is often influenced by the age of the female (e.g., Marsh & Kasuya, 1986). Using insemination methods optimized for each species, the success rate of intrauterine AI in dolphins and killer whales is 50-75% (O'Brien & Robeck, 2006; Robeck et al., 2004, 2005a, 2009; Robeck & O'Brien, unpublished data); such rates are comparable to those achieved in livestock species where estrus synchronization and AI techniques were first developed (reviewed by Foote, 2002). For the beluga, a facultative-induced ovulator usually requiring ovulation induction using exogenous GnRH (Steinman et al., 2007), intrauterine AI using frozen-thawed spermatozoa has resulted in a 20% conception rate (Robeck et al., 2010). This result is expected to parallel those of the dolphin and killer whale as more inseminations are performed using a deep bicornual insemination method (Robeck et al., 2010).

Sperm preservation and gamete rescue for genome resource banking

The development of effective sperm cryopreservation methods in cetaceans for use with AI is considered high priority. Sperm samples can either be collected voluntarily from trained captive animals, or post-mortem from wild or captive animals using a process known as gamete rescue. Collection and preservation of spermatozoa from either source represents an important conservation tool, which allows the indefinite storage of valuable genetic material (in the form of spermatozoa) that can be used to produce offspring long after a male has died through assisted reproductive technologies (Ballou, 1992; Wildt, 1992). Indeed, genome storage banks, facilitating the organized storage of gametes and other biological tissues have been developed for numerous endangered wildlife species (reviewed by Holt, Abaigar, Watson, & Wildt, 2003). However, before a genome resource bank can be established, controlled *in vitro* studies must be performed to ensure that cryopreserved samples retain adequate fertilizing capacity following thawing.

Short- and long-term sperm preservation methods have been reported for four cetaceans; the bottlenose dolphin (Robeck & O'Brien, 2004), killer whale (Robeck et al., 2004), Pacific white-sided dolphin (Robeck et al., 2009) and beluga (O'Brien & Robeck, 2010). Systematic banking of spermatozoa for long-term storage from 33 trained cetaceans has been accomplished by our group and collaborators (bottlenose dolphin: n = 21; Pacific white-sided dolphin: n = 4; killer whale: n = 6; beluga: n = 1) and from wild beluga (n = 4) in conjunction with native subsistence hunts (Robeck, Gearhardt, Suydam, & O'Brien, unpublished data). Due to species-specific aspects of ejaculate characteristics and sperm biology, the composition of diluents used to preserve spermatozoa varies considerably across the aforementioned species. In the bottlenose dolphin, use of a novel sperm cryopreservation method, directional solidification freezing ("directional freezing", Arav, 1999; Arav et al., 2002), led to a significant improvement of sex-sorted spermatozoa *in vitro* quality compared to a conventional straw freezing method (O'Brien & Robeck, 2006). For the beluga, a species whose spermatozoa exhibit a low tolerance to cryopreservation compared with other cetaceans, superior sperm quality post-thawing was also achieved using directional freezing with non-sexed spermatozoa compared to conventional straw freezing methods (O'Brien & Robeck, 2010).

Sex ratio modification using sperm sexing technology

Sex ratio management is of particular significance to species which naturally exist in female-dominated social groups. For such species (e.g., dolphins: Wells, 2000; killer whales: Baird, 2000), management of socially cohesive groups is better performed when animals are housed in groups mimicking sex ratios occurring in the wild. Since natural matings typically result in the production of equivalent numbers of males and females, male offspring are then often surplus to breeding requirements and can present social issues due to inappropriate levels of male–male competition and aggression. The situation can be exacerbated in zoological settings by transitory, naturally occurring sex ratio skews (Glatston, 1997). A potential solution to these social and reproductive management issues is to structure breeding programs to produce predominantly female offspring through the application of sperm sexing technology and associated assisted reproductive technology (sperm preservation and AI).

The preferential production of female offspring can be achieved by inseminating animals with X chromosome-bearing spermatozoa. Such "female" spermatozoa are obtained by separation of X and Y chromosome-bearing spermatozoa using a specialized flow cytometer, capable of detecting the small difference in DNA content between the two sperm populations (reviewed by Sharpe & Evans, 2009). Birth of offspring of pre-determined sex using flow cytometrically sex-sorted fresh spermatozoa was first performed in rabbits 20 years ago (Johnson, Flook, & Hawk, 1989). Since then offspring have been produced using sex-sorted spermatozoa and associated assisted reproductive technology from humans and numerous domesticated and farmed species. The first pre-sexed zoological species, the bottlenose dolphin, was born in 2005 (O'Brien & Robeck, 2006) and the technology has since been integrated into a global reproductive, genetic and social management program for the species (O'Brien et al., 2009) resulting in a total of 14 pre-sexed bottlenose dolphin calves to date. This technology may become a critical management tool for the growth of small *ex situ* cetacean populations. By combining sperm sexing technology with AI, the growth of small and/or critically endangered *ex situ* populations can be enhanced by the preferential production of female offspring (Figure 2). Through appropriate genetic management, samples enriched for female or male spermatozoa can be banked and selected for use to maximize the genetic diversity of these populations.

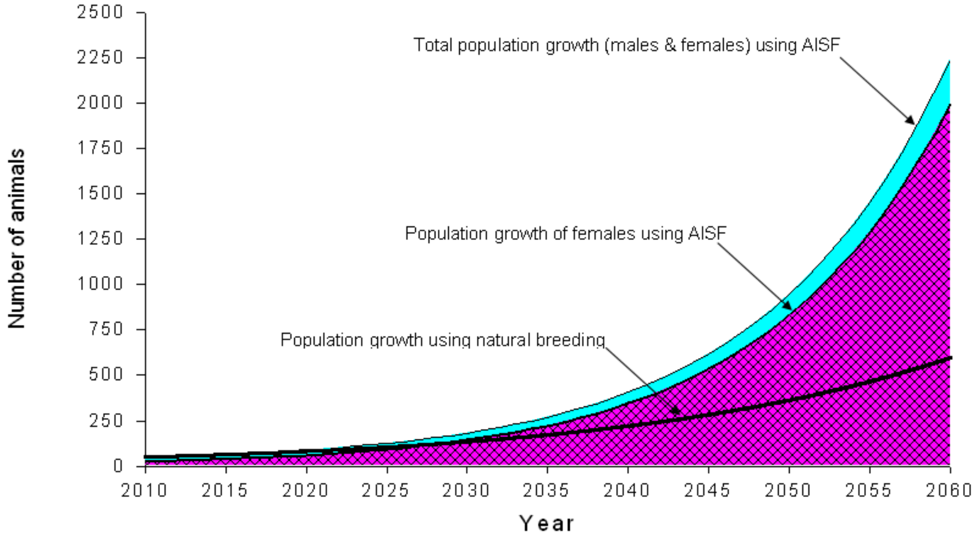

Figure 2. Simulation of the impact of artificial insemination using sex-selected (female) spermatozoa (AISF) on the growth of a normally distributed cetacean population over 50 years. The simulation includes a population size of 50 animals (25 males and 25 females), and an annual recruitment rate of 5%. The annual recruitment rate (which includes the mortality rate) was based on that of the bottlenose dolphin (*Tursiops truncatus*) population at SeaWorld (USA). The solid black line represents the total number of animals under a natural breeding program (50:50 sex ratio). The upper line of the blue region represents the total number of animals produced if all breeding is accomplished with AISF. The upper line of the overlaid hatched pink region represents the total number of females produced within the blue population when all breeding is accomplished using AISF (10:90 sex ratio). All breeding decisions are made in conjunction with appropriate genetic management.

Oocyte and embryo technologies

The development of *in vitro* maturation and fertilization techniques can provide a tool for the production of preimplantation stage embryos for developmental research or for embryo transfer. The development of viable methods for oocyte and embryo preservation would also enhance the potential use of genome banks in captive and wild species conservation.

Data collected from *in vitro* culture studies demonstrate the diverse requirements for normal gamete and embryonic development in different mammalian species (Bavister, 2000). Though no significant research has been conducted on the *in vitro* culture or preservation of delphinid oocytes, ovarian tissue or embryos, some progress has been made in several species of *mysticetes*. *In vitro* oocyte maturation, fertilization and embryo culture studies have been performed using post-mortem tissue in the common minke whale (*Balaenoptera acutorostrata*, Fukui et al., 2007), Antarctic minke whale (*B. bonaerensis*, Iwayama, Ishikawa, Ohsumi, & Fukui, 2005), sei whale (*B. borealis*, Bhuiyan et al., 2009) and Bryde's whale (*B. edeni*, Watanabe et al., 2007; Bhuiyan et al., 2009). Studies have also been extended to frozen-thawed (common minke whale: Asada, Tetsuka, Ishikawa, Ohsumi, & Fukui, 2001a) and vitrified oocytes (common minke whale: Asada et al., 2001b; Fujihira et al., 2006; Iwayama et al., 2004) but embryo preservation has not been reported.

The immediate challenge for the development of oocyte and embryo technologies in cetaceans is the lack of access to post-mortem tissue, and when available, access within a timely manner after death. Numerous studies in domestic species have shown that the developmental capacity of oocytes collected from antral follicles post-mortem is negatively impacted by increased ovary holding time and reduced storage temperature (e.g., Wongsrikeao et al., 2005). Similar studies in cetaceans are required using opportunistically collected samples to determine optimum ovary processing and oocyte collection procedures. Further, studies on immature follicle isolation and preservation are warranted in view of the potential application of such procedures toward gamete banking in threatened terrestrial species (e.g., Czarny, Harris, & Rodger, 2009). Preliminary studies in the bottlenose dolphin have demonstrated the potential of superovulation techniques as a source of oocytes or embryos for genome banking (Robeck McBain, Mathey, & Kraemer, 1998). Research on superovulation, transabdominal oocyte aspiration, uterine embryo flushing and embryo transfer techniques is required to enable the future integration of oocyte and embryo technologies into the genetic and reproductive management of cetaceans.

Benefits of Reproductive Research and Assisted Reproductive Technology for Wild Cetacean Populations

A thorough understanding of female reproductive physiology through robust scientific research offers potential benefits to the conservation of wild populations. For instance, knowledge of a species’ reproductive seasonality and parturition periods can better underpin governmental policies on habitat protection. Information on spermatogenesis and sperm biology represents important components of a species-specific reproductive baseline database, which can be incorporated into health assessments for free-ranging animals. This is particularly important in light of cetacean prey contamination with anthropogenic pollutants and the unknown effects of such pollutants on reproductive function (e.g., Fisk et al., 2005). Examination of effects of environmental toxins on reproductive characteristics such as sperm production and *in vitro* sperm quality (e.g., morphology, DNA fragmentation, metabolic potential) could be conducted in free-ranging cetaceans under anesthesia using electroejaculation. In conjunction with an appropriate anesthetic protocol, electroejaculation is considered a safe and effective technique for collection of semen from a variety of endangered wildlife (African elephant: Howard, Bush, de Vos, & Wildt, 1984; rhinoceros species: Roth et al., 2005; western lowland gorilla: Seager, Wildt, Schaffer, & Platz, 1982). Methods for electroejaculation under appropriate field anesthesia are yet to be developed for cetaceans. Captive male bottlenose dolphins would serve as an ideal model for the development of such methods for subsequent use in reproductive assessments of their free-ranging counterparts.

Formation of a genome resource bank and the organized banking of spermatozoa from males in the captive population will provide insurance against potential catastrophic losses to the species occurring in the wild. Too often, efforts toward reproductive research and the development of assisted reproductive technologies such as sperm preservation are initiated only after a species has become threatened with extinction and few individuals remain in aquaria. For example, the recent extinction of the Baiji (*Lipotes vexillifer*) could not be prevented because *ex situ* reproductive research efforts were delayed until the species was critically endangered (Turvey et al., 2007). Without an understanding of that species' physiology, and no history of successful *ex situ* breeding, little could be done to prevent their demise.

As global human populations increase, so does the concomitant anthropogenic pressure on all species. Consequently, zoological institutions are becoming increasingly responsible for preserving species close to extinction and indeed, those that have become extinct in the wild.. Even when species have been successfully re-introduced in the wild, the long-term viability of these "new" *in situ* populations often remain reliant on *ex situ* populations (e.g., populations of the California condor, *Gymnogyps californianus*; black-footed ferret, *Mustela nigripes*; Arabian oryx, *Oryx leucoryx,* IUCN, 2008).

The scimitar-horned oryx provides a good example of how conservation efforts can be enhanced through reproductive research and AI. Due to its extinction in wild, and the challenge of maintaining maximal genetic diversity within the captive population, the scimitar-horned oryx became the focus of multi-institutional efforts to study the species' reproductive biology and establish successful genome resource banking and assisted reproductive techniques. As a result, techniques for semen collection, cryopreservation and AI were developed and refined (Garland, Frazer, Sanderson, Mehren, & Kroetsch, 1992; Morrow et al., 1997; Roth et al., 1998, 1999), and 14 calves have been produced following oestrus synchronization and AI of oryx cows with frozen–thawed spermatozoa (Garland et al., 1992; Morrow et al., 2000; Morrow, Penfold, & Wolfe, 2009). A permanent store of frozen semen now exists for the species in the form of a genome storage resource bank, and captive offspring (produced from natural mating or AI) are available for re-introduction to the wild. In 2007, as part of a collaborative project led by the World Association of Zoos and Aquaria (WAZA project 05039 – Re-introduction of Sahelo-Saharan antelopes to Tunisia), captive bred oryx from Northern American and European zoos were transported to Tunisia, and underwent the first stage of re-introduction in the wild.

For cetaceans, scientific progress toward an understanding of reproductive biology and the development of ART is contributing to the sustainable *ex situ* management of several species. If required, these advancements may also be applied to the conservation of such species in the wild through re-population and re-introduction programs. However many species of cetaceans are not accessible for *ex situ* studies and consequently very little is known about their biology. These include one of the world's most critically endangered marine cetacean, the vaquita (*Phocoena sinus*), and the world's three species of solely freshwater river dolphins, whose habitat is in close contact with human activities. The status of such river dolphins is either critically endangered/functionally extinct (Yangtze river dolphin, "Baiji", *Lipotes vexillifer*), endangered (Ganges and Indus river dolphin, *Platanista gangetica*), or data deficient (Amazon river dolphin, *Inia geoffrensis*) (IUCN, 2008). The establishment of functional *ex situ* populations of these and other small cetaceans, to gain an understanding of their biology, could indeed enhance current *in situ* conservation efforts. Criticism of breeding programs by partisan conservation groups may limit the true potential of a global multi-disciplined conservation approach. Even when in aquaria, research efforts for some marine mammals become mired by such controversy. For example, although every scientist agrees that the manatee (*Trichechus* sp.) is critically endangered, efforts toward its captive breeding and reproductive research have been impeded by bureaucratic restraints The dissemination of genuine, scientifically sound data and biological discovery by zoological researchers, for the manatee and other threatened marine mammals, should be considered essential for the formulation of holistic governmental policies on species protection and management.

Concluding Remarks

Animals in the care of zoological institutions are an invaluable resource for increasing our understanding of basic reproductive biology, a pre-requisite for effective species conservation. Reproductive research has also facilitated the development and application of assisted reproductive technologies for modernized genetic, reproductive and social management of *ex situ* cetacean populations, and there exists great potential for the use of these technologies in the ongoing *in situ* conservation needs of both non-endangered and endangered cetaceans.

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