Development of breeding techniques in the crocodile industry
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Reproductive anatomy, semen collection, semen preservation and preliminary observations of artificial insemination

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Breeding practices common in livestock industries have the potential to significantly improve productivity in the crocodile industry. In current farming practices, single male crocodiles are typically penned with one to three females, due to their potential for aggressive behaviour toward other males. This means several males are required, as well as many separate pens, which have to be built and maintained. The use of artificial insemination (AI) could revolutionise the current industry, allowing a farmer to carry only a couple of proven stud males, or to buy in semen from elsewhere. This would see crocodile farming adopt practices that have become routine in many other livestock industries long ago.

Not only would AI be beneficial through reducing or eliminating the need to carry numerous males, it will also facilitate improved genetic management of valuable sires for desirable traits such as skin and meat quality. It can also be used to introduce new genetic vigour without the need to import and habituate new individuals. There is also potential benefit in the captive propagation and management of endangered crocodilians; pairing up two individuals in the hope they will find each other compatible is often not successful and sometimes one kills the other.

As described in this report the current study has made significant steps towards developing AI for the saltwater crocodile (*Crocodylus porosus*). Taking into consideration the anatomy and physiology of the male reproductive system, a successful method of semen collection was developed by means of cloacal digital manipulation of the terminal segment of the ductus deferens. This enabled characteristics of the sperm (normal and abnormal sperm) and bacteriology to be documented for the first time.

Crocodile producers, zoologists and veterinarians now have a technique that they can utilise for the safe and reliable collection and manipulation of crocodile semen. This technique will provide spermatozoa that can be assessed as part of a breeding soundness evaluation of individual males, help map reproductive seasonality, provide information pertaining to sexual maturity and develop sperm preservation protocols. Successful semen collection also represents the first step in the development of an AI protocol for the species. This study has also provided valuable baseline anatomical information for *C. porosus*, which will be of direct benefit to those involved in its commercial production. The imaging protocols developed in the study may facilitate improvements in commercial production and veterinary care.

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Executive Summary

What the report is about

This report describes successful preliminary investigations into the development of assisted breeding technology in the Australian saltwater crocodile industry, with a particular focus on semen collection and preservation. Semen collection and preservation is the first step towards the establishment of artificial insemination (AI), the successful implementation of which has the potential to revolutionise crocodile farming with respect to reproductive and genetic management. In gathering male and female reproductive anatomical information related to semen collection and AI, this study has also assembled a package of magnetic resonance and computer tomography images of the musculoskeletal system of the crocodile, which will have direct relevance to crocodile production systems.

Who is the report targeted at?

This report is targeted at crocodile farmers, veterinarians and zookeepers, to illustrate the application of semen collection and preservation to their industries. The report will provide confidence to these stakeholders with respect to the potential application and implementation of assisted breeding techniques for improving crocodile production, and facilitating the genetic management and propagation of endangered crocodilians.

Where are the relevant industries located in Australia?

There are currently six crocodile farms in the Northern Territory, six in Queensland and one in Western Australia, which vary in size from small (<2000 crocs) to large (30,000+ crocs) and supply a market of approximately 30,000 to 40,000 skins per year. The research described in this report will benefit crocodile producers, veterinarians and zookeepers charged with the responsibility of reducing production costs, improving the quality of their end product and maintaining genetic and general health of crocodile populations. Farmers that adopt these practices may benefit, potentially leading to industry growth and enhanced economic returns in export dollars.

Background

Development of a successful AI program requires:

1. A method of semen collection, evaluation and preservation;
2. A detailed understanding of the physiology and sexual behavior of the female to ascertain the most appropriate timing of insemination; and
3. An intimate knowledge of female reproductive anatomy, in order to deposit semen to allow for successful fertilization of the oocyte.

While AI has proven successful in the American alligator (*Alligator mississippiensis*), resulting in the production of 11 fertile eggs, the procedure has yet to achieve a level of success whereby it is has been implemented in the crocodile farming industry; nor has it been successfully used for overcoming infertility or facilitating genetic exchange in endangered crocodilian species.

The saltwater crocodile *Crocodylus porosus* is the largest crocodile in the world and the only farmed species in Australia; its commercial products of high-grade quality leather and meat generate in the order of $5 million per year (Goulding *et al.*, 2007). The development of artificial breeding in this species would make a significant impact on improving productivity in much the same way that it has revolutionised the cattle industry. The use of frozen-thawed semen in conjunction with AI could be used to reduce the requirement and expense of males on farm, facilitate the breeding and dissemination
of high production related genotypes, and reduce aggressive mating encounters that can lead to the death of valuable brood stock.

Aims/objectives

This project had the following objectives for developing techniques that would lead to application of assisted breeding technology in the Australian crocodile industry:

1. 3-dimensional MRI gross anatomy of sexually mature male and female crocodile
2. Reliable and safe technique for repeated collection of crocodile semen
3. Development of methods to assess the quality of crocodile semen
4. Selection of chemical media for the preservation of crocodile semen
5. Protocols for the reliable cryopreservation of crocodile semen
6. A method for crocodile artificial insemination using a speculum

Methods used

Crocodiles for this project were sourced from Koorana Crocodile Farm, at Coowonga on the central coast of Queensland. For anatomical studies, sexually mature and juvenile crocodile cadavers were supplied for traditional dissection and advanced imaging using computer tomography and magnetic resonance. Initial protocols for semen collection used electro-ejaculation on sedated crocodiles but a semen collection technique using digital massage of the ductus deferens proved to be more successful. Seminal characteristics of the 30 ejaculates were described using standard semen evaluation techniques used for domestic animals. Culture and sensitivity of the cloacal and seminal flora were described. semen samples from ten crocodiles were also examined, to establish their physiochemical tolerance to extension media, processing conditions (temperature and osmolality) and short and cryopreservation. Given the images acquired from CT and MRI scanning, a 3D musculoskeletal package of the major muscle groups involved with crocodile meat production was also produced. The cloacal anatomy of the female saltwater crocodile reproductive tract was described for the first time, and based on these findings, an artificial insemination protocol was attempted. A total of 22 sexually mature crocodiles were inseminated (ten in 2011 and 12 in 2012) without specific knowledge of their ovulatory status.

Results/key findings

1. 3-dimensional MRI gross anatomy of sexually mature male and female crocodile
   - Flare of the MRI scan associated metal fragments of the gut prevented production of specific 3D MRI images of male and female reproductive system
   - Traditional dissection and histology was therefore used to document gross anatomy of the reproductive system
   - Additionally, MRI and CT scans were used to generate a package of images of the crocodile musculoskeletal system that will be used for veterinary education and meat processing.

2. A reliable and safe technique for repeated collection of crocodile semen
   - Electro-ejaculation resulted in contamination of the ejaculate with urates and was deemed not suitable for further processing
   - Following an initial description of male reproductive anatomy, this study developed a successful method of semen collection by means of cloacal digital manipulation of the terminal segment of the ductus deferens
   - Semen was recovered from 30 of 31 collection attempts from a total of 23 sedated males ranging from 197 cm to 400 cm in body length.
3. Development of methods to assess the quality of crocodile semen

- A database of seminal characteristics was described from 30 ejaculates of 23 crocodiles. Mean (± SEM) seminal volume, pH, osmolality, sperm concentration, percentage motile sperm and the percentage of sperm with an intact membrane measured 0.91 ± 0.16 mL, 7.3 ± 0.1, 335.5 ± 9.0 mOsmKg⁻¹, 2.29 ± 0.26 x 10⁹, 50.7 ± 4.2%, and 79.9%, respectively. Sperm abnormalities included macro and microcephalic nuclei, teratoid spermatozoa, loose heads and range of abnormal flagella. Most semen samples contained spermatozoa with cytoplasmic droplets but the significance of this phenomenon, as a sperm maturational pathology, requires further validation.

- In an attempt to develop targeted antibiotics for use in semen diluents, microflora of the penile shaft, sulcus and semen of a subset of crocodiles was analysed for culture and sensitivity; a broad range of bacteria were identified and the majority were sensitive to gentamicin.

- As part of the semen collection procedure, this study also identified a male crocodile with a penile prolapse and determined that crocodile size was not a pre-determinant of semen quality – this work will form the basis of a future breeding soundness evaluation of the male crocodile.

- In addition to the work described here (not funded by RIRDC) crocodile semen was also evaluated for sperm DNA fragmentation and a technique established using the comet assay, in situ hybridisation and sperm chromatin dispersion assays (Prof Jaime Gosalvez Pers Comm).

4. Selection of chemical media for the preservation of crocodile semen

- Semen collected from ten saltwater crocodiles was used to investigate sperm in vitro manipulation and preservation. Preliminary studies revealed that:
  - Phosphate buffered saline (PBS) without Ca²⁺, Mg²⁺ and egg yolk (EY) was a suitable extender for studies of sperm physiology.
  - Spermatozoa diluted in PBS showed no change in survival [% motility (M), rate of sperm movement (R) and % plasma membrane integrity (PMI)] when diluted over a range of 1:1 to 1:16.
  - Except for a small decline in PMI, there was no change in sperm survival when semen diluted without EY was cooled rapidly to and rewarmed from 0 °C.
  - While crocodile spermatozoa exposed to a range of anisotonic media and then returned to solutions of 390 mOsmKg⁻¹ retained their M from 220 – 390 mOsmKg⁻¹, PMI remained surprisingly high in hypotonic media (25 – 280 mOsmKg⁻¹); spermatozoa also showed a significant increase in the incidence of flagella coiling (FC) with increasing hypotonic conditions. The adverse effect of anisotonic conditions on sperm M and FC recovered somewhat when spermatozoa were returned to the 390 mOsmKg⁻¹ media but not to pre-treatment levels.

5. Protocols for the reliable cryopreservation of crocodile semen

- Addition of EY (0, 5, 10 and 20% V/V) had no beneficial effect on sperm survival when incubated in PBS for 1h at 30 °C or after 24h storage at 4 °C.

- Exposure of crocodile spermatozoa to respective concentrations of 0.68M, 1.35M and 2.7M glycerol, dimethylsulphoxide (DMSO), dimethylacetamide (DMA) after 2h storage at 4°C (equilibration) resulted in a reduction in M, but no change in PMI.

- Sperm cryopreserved in the same cryoprotectant media within 0.25 mL straws at -6°C/min in a programmable freezer and thawed at 37°C for one minute, showed a major decline of M but there was moderate protection of PMI (DMA 2.7M - 17.7 ± 4.4; DMSO 2.7M – 22.7 ± 1.4 and glycerol 2.7M – 25.7± 6.4).
• Sperm thawed, and immediately washed to remove the cryoprotectant, showed a significant improvement in PMI but not M.
• Future studies of crocodile sperm preservation should explore the apparent disjunction between low levels of M and the high tolerance of the plasma membrane to anisotonic conditions and cryoprotectant toxicity.

6. Method for crocodile artificial insemination
• The results presented here are only preliminary investigations, as the major aims of this project were focussed on the male. It should also be noted that female crocodiles were inseminated without reference to their specific stage of ovulation but based on the natural mating activity of crocodiles at Koorana Crocodile Farm.
• Artificial insemination of diluted semen in 2011 of 10 crocodiles resulted in one female producing six fertile eggs, of which one animal was successfully hatched; only four crocodiles laid eggs post-insemination.
• In 2012, insemination of neat and diluted semen of 13 crocodiles resulted in one female producing one fertile egg; due to abnormally high rainfall during the 2012-2013 breeding season, only four crocodiles laid eggs post-insemination.
• For AI to be successful, there is a critical need for understanding the timing of the reproductive seasonality of the female crocodile, in developing methods for assessment of ovarian status (ultrasound and laparoscopy) and reproductive hormone secretion, and linking these to observations of behavioural oestrus.

Implications for relevant stakeholders
The use of artificial insemination in the crocodile industry has been taunted for over 30 years but the rate-limiting step has always been the reliable and safe collection of semen. This study has shown that it is possible to collect semen in quantities presumably sufficient for artificial insemination; it therefore represents the first successful step towards the implementation of assisted breeding technology in the saltwater crocodile industry. Reliable semen collection will also allow for the establishment of a database for the assessment of breeding soundness in male crocodiles, that can be used in a similar way as for domestic animals, in order to identify male infertility or the selection of males with higher levels of reproductive potential. This study has also provided a systematic evaluation of crocodile semen preservation technologies which, when used in combination with AI, will ultimately be powerful tools for future genetic management.

Although this study primarily focussed on the application of assisted breeding technology to improvements in commercial production (skin and meat) of crocodiles, the techniques also have major implications for the conservation of endangered crocodilians worldwide. Of the 24 species of crocodilian, ten are currently listed as critically endangered (n = 6), endangered (n = 1) or vulnerable (n = 3). Using the saltwater crocodile as a model species, we propose that the techniques we have developed for semen collection in this study can equally be applied to captive or wild crocodilians to assess male reproductive status, and ultimately in AI programs.

The results of this study also have implications for policy makers regarding the potential use of genetic material from wild crocodile populations. We see a future role for semen collection from selected wild males specifically caught for their phenotypic qualities or for genetic enhancement of captive animals, especially for problem animals that require relocation because of threats to human populations.

Although not the original objective of this study, we have also provided a preliminary assessment of the musculoskeletal system of the crocodile, which can be used by industry to for veterinary education and for examining current techniques of meat processing. The 3D modelling we have provided also has the potential to be used for determining the most efficient techniques of crocodile skin processing.
Recommendations

Semen collection

- Use of digital massage for safe and reliable semen collection in the saltwater crocodile
- Development of improved methods of capture and sedation to facilitate semen collection
- Implementation of male breeding soundness evaluation across the industry (using knowledge of reproductive anatomy and semen assessment), to improve reproductive potential
- Determination of reproductive seasonality and sexual maturity in the male crocodile. The success of semen collection (and semen quality) is highly likely to be related to the stage of the breeding season and age of the male; surprisingly, in this study, semen was collected from a juvenile male that measured only 195 cm in length. The use of semen from younger males has major implications regarding the acceleration of genetic improvement.

Semen evaluation and preservation

- The importance of the high incidence of cytoplasmic droplets to crocodile sperm maturation needs to be investigated further
- There was a relatively high incidence of polyploidy observed in sperm of the crocodile ejaculate. This phenomenon is related to malfunction of meiosis. It is possible that sperm abnormalities and assessment of sperm DNA fragmentation in the crocodile could serve as a biomarker of environmental conditions on the farm or in wild populations
- The disjunct between sperm motility and plasma membrane integrity is suggestive of external mechanisms controlling sperm motility, and could have relevance to sperm storage in the female. Further studies are, therefore, necessary to elucidate this phenomenon at the mitochondrial level and could involve the use of the lipophilic cationic probe $5,5'\%6,6'\%\text{-tetrachloro-1,1'\%3,3'\%}$-tetraethylbenzimidazolyl carbocyanine iodide (JC-1; Invitrogen Australia, Victoria, Australia) to examine mitochondrial membrane potential. In addition, the use of sperm media containing external treatments that increase intracellular levels of cyclic adenosine monophosphate (cAMP) should also be investigated to see whether motility can be reactivated
- Phosphate buffered saline (PBS) without Ca$^{2+}$, Mg$^{2+}$ can be used as base extender but other commercially extenders need to be tested. Semen should be buffered around neutrality and 280 mOsmKg$^{-1}$, and there is no requirement for egg yolk during storage or cooling to 4°C
- The non-toxicity of gentamicin, sulphamethoxazole/trimethroprim and amoxicillin/clavulanic and appropriate does for control of bacterial growth of contaminated semen samples requires validation
- Short-term chilled storage is the next logical step in the development of artificial insemination protocol in the crocodile. A storage period of three to five days should allow the shipment of semen nationally
- Initial studies of sperm cryopreservation of crocodile semen suggest that higher levels of cryoprotectants may be required for post-thaw survival, but the detrimental effect of DMSO, DMA and glycerol on sperm motility needs to mediated.

Artificial insemination

- Alternative cryoprotectants need to be examined, as does the extreme tolerance of the crocodile sperm plasma membrane to hypotonic environments. Determining the reproductive seasonality and sexual maturity of female crocodiles for the purposed timing of AI is essential; this includes developing relatively non-invasive techniques for establishing ovarian (follicular) and oviducal status
- The importance of sperm storage in the female crocodile and its relevance to AI and semen evaluation needs to be established.
Introduction

Crocodile farming is a relatively new agricultural practice and so has a limited information base from which to make improvements on productivity and efficiency. Based upon strong arguments pertaining to animal welfare, genetic management and economic viability, we have conducted studies designed to implement the use of artificial insemination (AI) in the Australian saltwater crocodile (*Crocodylus porosus*) industry. Similar strategies have been implemented for decades in domestic animal production systems but despite some limited success with the use AI in the American alligator (*Alligator mississippiensis*; Larsen et al., 1982), the procedure has not yet become commercially viable (Huchzermeyer, 2003), and nor has it been attempted in the saltwater crocodile.

Saltwater crocodiles kept on Queensland farms typically breed for only one month per year, while the rest of the year is associated with management of production stock (John Lever, Unpublished Observations). Many crocodile farms use a single 1:1 sire dam mating system, to reduce aggressive behavior, and ensure paternity. The cost of this production system is considerable, and includes additional general husbandry, maintenance of water quality and feeding. AI (in combination with frozen semen) has the potential to reduce the need for males on farm and dramatically reduce production costs, in a similar way to the cattle industry. Moreover, crocodile AI would greatly facilitate the transfer and delivery of selected genetics for improvements in production traits (Isberg et al., 2003; 2011; b; 2006a; b). Semen, rather than crocodiles, can be shipped from farm to farm at a reduced cost, and semen from wild animals could be used to improve genetic vigour without bringing males into captivity. AI could also be used for behaviorally incompatible or incapacitated crocodiles, thereby reducing the incidence of mating failure associated with physical trauma or a lack of sexual drive.

An important component of routine assessment of male fertility and the implementation of AI in any species is the development of a reliable and safe method of semen collection. A pioneering study by Larsen and Cardeilhac (1981) indicated that electroejaculation was not an appropriate semen collection technique for the alligator. Subsequently, successful semen collection was described in the American alligator (Cardeilhac et al., 1982; Larsen et al., 1996) and Broad-nosed caiman (*Caiman latirostris*; Larsen et al., 1992) by means of aspiration and “scraping” of the penile sulcus, but these techniques have been known to produce trauma to the penis and blood cell contamination of the ejaculate (Larsen et al., 1992). Although Larsen and colleagues successfully produced 11 fertile eggs using AI (Gainesville Sun Newspaper, Page 4B, July 24, 1981), the semen used for this procedure was recovered from the ductus deferens post-mortem. Larsen et al. (1984) also used semen dissected directly from the reproductive tract to examine extenders and test sperm preservation procedures. Following initial examination of the saltwater crocodile male reproductive tract to gain insight into semen recovery methods, this study reports on the successful and repeatable application of a non-invasive method of semen collection by means of cloacal digital massage; the seminal characteristics of 30 ejaculates from 23 males is described and documented for the first time, as is the culture and sensitivity of the microflora of the crocodile penis, sulcus and semen.

To establish appropriate sperm preservation protocols, further study is required to characterise the fundamental physiology and extension requirements of the crocodile sperm cell. Although spermatozoa have now been successfully recovered from a range of reptiles (Millar and Watson 2001; Brown et al., 2011; Hoser 2008), systematic observations of sperm preservation are rare or typically only preliminary in nature (SERPENTES - Mengden et al., 1980; Quinn et al., 1989; Fahrig et al., 2007; Mattson et al., 2007; TESTUDINES – Platz et al., 1980; Sirinarumitr et al., 2010; LACERTILIANs – Depeiges and Dacheux, 1985). In a rare study of reptilian sperm physiology, Depeiges and Dacheux (1985) noted that the motility of lizard (*Lacerta vivipara*) epididymal sperm responded to a phosphodiesterase inhibitor (caffeine) with increased velocity, indicating that motility in this species was most likely cyclic AMP-dependent. Short-term preservation of crocodilian sperm has been described in the American alligator (*Alligator mississippiensis*; Larsen et al., 1984 – up to 9 days at 5°C) and broad-nosed caiman (*Caiman latirostris*; Larsen et al., 1992 - up to 5 days at 5°C) but
cryopreservation has resulted in nil or only very low levels of motility. These same authors also found glycerol (2-10% V/V) and DMSO (5–10% V/V) to be highly detrimental to motility during the equilibration period, prior to the cryopreservation procedure. However, it should be noted that both studies only assessed sperm survival in terms of motility; no data was gathered on the integrity of the plasma membrane.

An examination of the scant literature related to sperm preservation in reptiles indicates that the majority of studies have, thus far, primarily been empirical and somewhat piecemeal. A systematic, controlled examination of the fundamental parameters governing survival and extension of the spermatozoon has yet to be conducted. The current study examined the physiological tolerance of saltwater crocodile spermatozoa to the effects of serial dilution of the seminal plasma, anisotonic osmotic stress and rapid changes in temperature. It also determined the need and importance of egg yolk for short-term preservation (extension and chilled) and evaluated preliminary cryopreservation protocols. Understanding the cellular and biochemical mechanisms that promote or limit reptile sperm physiology is vital for establishing methods of sperm quality assessment and informed selection of semen extenders and supplements for preservation and AI (Watson 1979; 1990).

The success of any artificial insemination program is ultimately measured by the production of live offspring. In this study, we describe the cloacal anatomy of the female saltwater crocodile with respect to the development of a practical insemination protocol. While we also describe preliminary attempts at artificial insemination of the crocodile, which resulted in the production of fertilised eggs and a viable offspring, the primary objective of the artificial insemination work was, in the first instance, to develop a protocol for semen delivery rather than production of offspring.

An original objective of this project was a detailed description of the male and female reproductive anatomy based on magnetic resonance imaging technology. However, the majority of the crocodiles provided for this part of the project possessed small fragments of accumulated metal in their digestive tract that, on MRI scanning, produced major flaring artefacts which obscured visualisation of the reproductive tract. Nevertheless, the data obtained from these images was still usable and, when combined with Computer Tomography and Magnetic Resonance imaging and conventional dissection, allowed a 3D description of the crocodile musculoskeletal system that we have related back to the various ‘cuts of meat’ currently used by the crocodile industry, and can equally be used for veterinary education.

Commercial production of crocodiles represents an emerging primary industry in rural Australia, generating A$9 million in 2006/7 (ABARE 2008; ABS 2008; Foster and Parker 2009). The industry was established in 1970s, with the primary focus of meeting the demands for the supply of crocodile skins to the luxury good market. Both native crocodile species, the saltwater or estuarine crocodile (Crocodylus porosus) and the freshwater crocodile (Crocodylus johnsoni) were originally farmed, however increased competition within the market place has seen production centred on C. porosus due to its higher skin quality and larger size (Miles et al., 2010). Within an increasingly competitive worldwide marketplace, meat once considered only as a by-product of farming now represents consideration in overall commercial productivity, albeit only contributing a relatively small component (< 3%) of overall product value (Foster and Parker 2009; Foster 2009).

Crocodile meat is renowned for its low fat and high protein content, and is becoming established within the niche market of ‘exotic’ meats, with an emphasis on ‘meat quality’, selling at premium prices reaching up to $30 per kilogram (Peucker 2005). Cuts are regularly prepared from the neck, back, and tail. These, along with the limbs, yield approximately five to six kilograms of meat at slaughter, on average. The domestic market accounts for the majority of meat sales (approximately 90%), with the remaining sales to export markets in SE Asia and Europe (Foster 2009). However, the recent emergence of the Chinese marketplace, where crocodile meat is used both in traditional and medicinal cuisine, presents the industry with significant opportunities to further develop its export sales (Deng et al. 2011).
With increasing emphasis being placed upon meat production, there is a need for knowledge and understanding of the musculoskeletal anatomy of *C. porosus*, and in particular the anatomical disposition of the main muscle groups relevant to commercial meat production. This is necessary to ensure effective and optimal methods for carcass preparation and meat retrieval, to aid guidance of breeding programs designed to improve meat quality and production, and in addition, to enhance the general husbandry, welfare management and veterinary care of this production animal species.

There has been great interest in crocodilian anatomical form, which has continued from the early pioneering work of Haughton (1863-66, 1865), Parker (1881) Stokes (1887), Romer (1924), who have all provided detailed accounts of various aspects of crocodilian cranial and post-cranial musculoskeletal anatomy. This information has been embellished in more recent times, primarily by the functional anatomists and palaeontologists, who have studied the crocodile from the perspective of a living archosaur, a ‘close relative’ to the dinosaurs. Despite this diverse wealth of information, a detailed summary overview of the musculoskeletal anatomy of *C. porosus* aimed primarily for those involved in commercial production of this species is lacking. Although Grigg and Gans (1993) provided much needed summary information, Huchzermeyer (2003) stated that we are still awaiting a standard text on crocodilian anatomy, a position that remains unchanged to this day.

Traditionally, the preparation of anatomical information relied upon hours of careful dissection, preparing specimens for illustration and/or photography. This was a costly and time-consuming operation, which can seldom be afforded in today’s economic climate. However, with the advent of modern imaging techniques, specifically computed tomography (CT) and magnetic resonance (MR), and the development of computer-based modelling software, it is possible to generate this essential baseline anatomical data time efficiently and cost-effectively. Both these imaging techniques rely on inherent differences in signal intensity that exist between structures of different tissue composition. Hence, by acquiring a sequential series of images, taken at different locations with the body, it is possible to inspect the anatomical disposition and inter-relationships of different organs and/or tissue types at these different anatomical locations. Furthermore, the entire sequential series of images can be used to provide a 3-D representation of scanned anatomy. This new approach to the generation of anatomical information affords the opportunity to readily deliver new insight and understanding of anatomical form.

The principle aim of this additional study was, therefore, to provide a baseline description of the musculoskeletal anatomy of *C. porosus*, with specific relevance to those muscle groups involved in the commercial production of this species, using anatomical data generated by CT and MR imaging techniques. Wherever possible, the study also aimed to link information with previously published work regarding the crocodilian muscular skeletal system, to give a comprehensive summary overview for this species.
Objectives

Original objectives of the project are 1 to 4; 5 to 7 are additional objectives that were also achieved.

1. 3-D MRI gross anatomy of sexually mature male and female crocodile
2. Reliable and safe technique for repeated collection of crocodile semen
3. Development of methods to assess the quality of crocodile semen
4. Method for crocodile artificial insemination using a speculum and otoscope
5. Selection of chemical media for the preservation of crocodile semen
6. Protocols for the reliable cryopreservation of crocodile semen
7. 3-D CT and MRI anatomical description of the saltwater crocodile musculoskeletal system.
Methodology

Animals and husbandry

For preliminary studies of male reproductive anatomy, reproductive tracts were dissected from a 280 cm long mature male and two 200 cm long juvenile male crocodile cadavers in mid-August 2011. During this same period, the anatomy of the female cloacal region from a 320 cm long sexually mature female and a 200 cm long juvenile 200 was also described. Semen collection was attempted (n = 31) from a total of 22 mature (length > 200 cm) and two juvenile (180-200 cm) salt-water crocodiles over a period that extended from mid-Spring (late September and early October 2012; n = 22) to early summer (early December 2012; n = 9), at Koorana Crocodile Farm (http://www.koorana.com.au/) located at Coowonga (Latitude: -23.2833; Longitude: 150.71666). Bacterial swabs were taken from a further six sexually mature crocodiles for culture and sensitivity of the penis and semen. Male crocodiles were kept in separate bachelor pens, or housed individually with females as part of the normal breeding season, which in the central Queensland coast extends from September to December (John Lever, Personal Observations).

Preliminary artificial insemination attempts were conducted on ten sexually mature females in 2011 to determine the practicality of the semen delivery procedure, and a further 13 (Length: 238.9 ± 9.3 cm; Range: 200 – 316 cm) were inseminated using refined semen placement methods in 2012 either with undiluted or fresh semen. A total of six cadaver crocodiles, humanely slaughtered for reasons other than this study, were obtained from Koorana Crocodile Farm to document the musculoskeletal system. The study cohort comprised four juvenile and two young adults, ranging in body length from 140 cm to 220 cm. Specimens were frozen immediately following euthanasia, and maintained at -20°C until immediately prior to scanning. This project was conducted with the approval of the University of Queensland Animal Ethics Committee (SAS/361/10) and Queensland Government Scientific Purposes Permit WISP09374911.

Magnetic Resonance (MR) and computer tomography (CT) Image acquisition

Magnetic resonance images of a single adult (2.7 m long) saltwater crocodile, for preliminary studies of male reproductive tract anatomy were acquired by Central Queensland Medical Imaging at the Rockhampton Mater Hospital, using a GE Excite 1.5T magnetic resonance imagining unit and an eight channel receive body coil. T2 weighted spin coronal echo images were exported from the spectrometer as DICOM format files. DICOM files were then viewed and manipulated using OSIRI X DICOM viewer software on an Apple Macintosh MacBook Pro computer.

MR images for describing the musculoskeletal system of the crocodile were also obtained from another 2.7 m long crocodile in either a 1.5 or a 3 Tesla human body scanner based in the Centre of Advanced Imaging at The University of Queensland, using flexible chest coils. A series of T1 and T2 weighted images were acquired at various body locations including, chest, abdomen and pelvis. CT images of frozen specimens were also acquired from the same animal in a 16 detector helical human body scanner (Acquilion 16). The crocodile was positioned in the isocentre of the scanner with the spinal column aligned along the ‘z’ axis of the scanner. Initial whole body scans were acquired, at 135 kV, 125 mA and a slice thickness of 1.0mm. Whole body scanning was performed using a standard helical pitch of 15 (pitch factor of 0.938) with a tube rotation time of 0.75 seconds. The resultant projection data were reconstructed to a slice thickness of 0.8 mm using a high-resolution bone weighted (FC81) and a sharp soft tissue weighted (FC19). In addition, higher resolution data were also acquired for the head, body, and tail regions, and also of disarticulated skulls and fore and hind limbs from 4 of the 6 specimens. These higher resolution scans were acquired at a helical pitch and rotation time of 11 (pitch factor = 0.688) and 1.0 seconds respectively, at a slice thickness of 5mm (reconstructed at 0.3mm slice interval). Two different kilovolt and milliamp settings were employed to
optimise between tissue type discrimination. Whilst bone weighted images were similarly acquired at 135kV, soft tissue data were acquired at 80kV, and an additional reconstruction was performed using a beam hardening corrected sharp body (FC09) kernel. CT and MR imaging data were exported in DICOM format for subsequent 3D volume rendering and surface model generation. Volume rendering of the CT data was performed using the Acquillion1 software interface, with video based anatomical segmentation achieved by direct adjustment of the displayed window level and width, 3D surface models were generated by Mimics Innovation Suit software (MIS ver 16.0)2 in accordance with the method described elsewhere (Collins et al. 2009). In brief, discretisation (segmentation) of the skeletal anatomy was based on the bone weighted reconstructed data. Bone segmentation was achieved by threshold method spanning the range of grey-scale values which matched the attenuation characteristics of cortical and trabecular bone.

**Crocodile capture and sedation**

Prior to semen collection or AI, crocodiles were caught by a snare as per the Queensland Department of Environment and Resource Management code of practice for the taking, handling and transportation of crocodiles (DERM, 2008). This initially involved enticing the crocodile to the fence of their respective enclosures with a food reward, followed by the placement of the snare over the top jaw (Figure 1.1A-D). This action would typically result in the crocodile rolling over repeatedly in a lateral aspect, thereby self-tightening the snare over both the top and bottom jaw (Figure 1.1E and F); both the top and bottom jaw were then taped closed with Duct Tape (Scotch® Tough Duct Tape, North Ryde NSW). Once the crocodile was secured, a sling was positioned around its thorax and forelimbs and it was manually loaded into a purpose built transport container (Figure 1.1G); the container was subsequently loaded by small crane onto the back of a utility vehicle for the semen collection procedure (Figure 1.1H).

Approximately 2 to 3h prior to semen collection, each crocodile was sedated using a combination of Pamlin® (Diazepam; Parnell Laboratories Pty Ltd, Alexandria, NSW, Australia) and Pavulon® (Pancuronium bromide; Teva Parenteral Medicines Inc., Irvine CA, USA), administered IM to the lateral head of the tail using a 40 mm, 16g needle; the dose rate of each compound varied depending on the body length (and approximate weight) of the crocodile (Table 1.1). The semen collection procedure commenced once the crocodile was immobilised, but still exhibited a detectable palpebral reflex (Dugdale, 2010).

Female crocodile capture procedures were similar to those described in the male but the sedation protocols were more conservative. Only in three of the 13 female crocodiles inseminated was a Pavulon® dose administered as per body length (Table 1.1), but on veterinary advice all crocodiles received an appropriate dose of Pamlin®. Following semen collection or AI, crocodiles were allowed to recover overnight within a transport container, before being released back into their respective enclosures.

**Semen collection**

In October 2011, electroejaculation was attempted on three sexually mature crocodiles, and while semen was successfully collected, the ejaculate was heavily contaminated with urates (50 – 100 mL). Serendipitously, it was noted that on insertion of the electro-ejaculation probe into the rectum without electrical stimulation, that a white viscous secretion had accumulated on the tip of the penis within the penile sulcus. Microscopic evaluation of this fluid revealed it to be a small drop of highly concentrated semen; subsequent manual digital massage of the base of the crocodile penis resulted in the recovery of a further volume of semen. In the following breeding season (2012), semen was collected by digital massage from a total of 24 animals (C1 – C24), C1 was collected 3X while C3, C8, C9, C13 and C18 were collected 2X over a 3 month period.

The semen collection protocol involved the male crocodile being place in ventral recumbency and straddled across two supporting structures with the genital opening freely accessible for manipulation
The penis was extruded from the proctodeum by placing a gloved finger into the cloaca and gently hooking out the shaft of the penis with downward pressure; this action would commonly result in the consequent release of urates and faeces that needed to be carefully wiped clean of the penis so as to not to disturb the semen that had already pooled in or flowed through the sulcus. A gloved finger(s) was then inserted past the base of the penis and into urodeum, and this region repeatedly stroked down the proximal length of penis to release a gentle flow of the white coloured viscous semen. The semen sample was allowed to run naturally along the sulcus and to drip free of the glans penis under gravity into a collection tube; any excess semen that failed to fall was recovered using a micropipette fitted with a 2 – 200µL pipette tip; to facilitate retrieval of the highly viscous seminal fluid, 3mm of the distal extremity of the tip was removed to widen the bore. The semen was collected directly into a 15 mL Falcon™ centrifuge tube (BD Australia, North Ryde, Australia) that had been purposely cut down in length (approximately 40 mm) to facilitate the “drop” of semen falling from the glans penis and reaching the bottom of the tube without sticking to the sides of the tube. Semen droplets for further processing where captured directly into a 200 µL aliquot of BEST diluent without egg yolk (Larsen et al., 1984) to prevent desiccation of the sample during the collection procedure.
Figure 1.1: Crocodile capture and restraint for semen collection. A. Crocodile is enticed to fence with food reward; B and C. Snare is initially lassoed around top jaw; D. Crocodile rolls laterally self-tightening the rope around top and bottom jaw; E and F. Second snare is secured over both jaws; G. Sling is secured around thorax and shoulders; H. Crocodile is loaded in transport box lined with rubber and the lifted by crane onto utility for transport to semen collection area.
Table 1.1: Dose rates of pancuronium bromide and diazepam used for the sedation of the saltwater crocodile for semen collection by manual massage.

<table>
<thead>
<tr>
<th>Body length (cm)</th>
<th>Approximate Body Weight (kg)</th>
<th>Pancuronium bromide (mg)</th>
<th>Diazepam (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>185 - 199</td>
<td>20 - 24</td>
<td>0</td>
<td>10</td>
</tr>
<tr>
<td>200 - 249</td>
<td>25 - 49</td>
<td>0.4</td>
<td>5</td>
</tr>
<tr>
<td>250 - 299</td>
<td>50 - 99</td>
<td>1.0 – 2.0</td>
<td>10</td>
</tr>
<tr>
<td>300 - 349</td>
<td>100 - 159</td>
<td>1.0 – 3.0</td>
<td>10</td>
</tr>
<tr>
<td>350 - 400</td>
<td>160 - 240</td>
<td>3.0 – 4.0</td>
<td>10 - 20</td>
</tr>
</tbody>
</table>

Figure 1.2: Semen collection by digital cloacal massage of the distal terminal portion of the ductus deferens. A and B Crocodile loaded on back of utility with tail supported by a brace in order gain access to the cloacal region for extrusion of the penis, digital massage and collection of semen under gravity. C. Close up of semen collection – note semen pooling in the sulcus and the droplet forming under gravity about to fall into the Falcon™ tube. Cl – cloaca; Gp – Glans penis; Ps – Penile shaft; Se – semen; Su – sulcus.
Semen evaluation

The pH of the undiluted ejaculate was determined from semen collected directly from the glans penis using narrow range pH paper strips (± 0.3; Prolabo, Paris). Following centrifugation (RCF – 1000g) of an aliquot of undiluted semen to remove spermatozoa, the osmolality of the seminal plasma was evaluated using a freezing point osmometer (Advanced Instruments Inc, Model 3320; Norwood, Massachusetts). Sperm concentration (sperm x 10⁹ ml⁻¹) was estimated using a Makler sperm counting chamber (Makler Inc. Israel) after accounting for the initial dilution of the semen sample in the collection tube with 200 µL of BEST extender. The mass activity of the neat semen sample was subjectively assessed by placing 10 µL of the neat undiluted sample on a pre-warmed (30 °C) microscope slide without a coverslip and observing under a 4X objective and scored according to the criteria used for ram spermatozoa (Evans et al. 1987). Individual sperm motility was initially assessed by dilution 1:10 in American alligator extender (Larsen et al. 1984) that was composed of 22.2 g/L BES (N,N-bis-(2-hydroxymethyl)-2-aminoetahane sulfonic acid), 6.3g/L TRIS (hydroxymethyl aminomethane), 28.6 g/L glucose and 20% (v/v) egg yolk. The percentage of motile spermatozoa was determined using a phase-contrast microscope (40X objective) equipped with a warm stage set at 30°C; rate of sperm movement was determined using criteria defined by Barth (1995). The percentage of spermatozoa with intact plasma membranes was estimated using epiflourescence microscopy (LEITZ, West Germany) and a LIVE/DEAD® sperm viability kit that utilised SYBR 14 and propidium iodide (Molecular Probes®, USA); spermatozoa with intact plasma membranes fluoresced green and were considered live, whilst dead spermatozoa with damaged sperm plasma membranes fluoresced red to orange; a total of 200 spermatozoa were evaluated. Nigrosin-eosin stained smears were prepared to describe and evaluate sperm morphology (Barth and Oko, 1986). The percentage sperm abnormalities were calculated for each ejaculate and a total of 200 spermatozoa were assessed.

Penile shaft, sulcus and seminal bacteriology

In an attempt to identify and control the microflora associated with crocodile sperm extension and preservation, the penile shaft, sulcus of the penis and undiluted neat semen of three sexually mature crocodiles were swabbed along with a swab of the shaft of the penis from a further three crocodiles. Once collected, the swabs were stored in Amies transport medium (Oxoid CM425; Oxoid Limited, Hampshire, England), maintained at 4°C and delivered overnight to the University of Queensland’s Veterinary Microbiology Laboratory at Gatton where they were processed for culture and sensitivity. Each swab was inoculated onto three plates of 5% sheep non-selective blood agar No. 2 (Oxoid CM271); one plate of MacConkey agar No.3 (Oxoid CM115) selective for Enterobacteriaceae, one plate of Columbia CNA agar (Oxoid, CM0331) plus Streptococci and Staphylococci selective supplement (Oxoid, SR0070) and one plate of Pseudomonas cetrimide agar (Oxoid CM0579) selective for Pseudomonas species. Sheep blood agar, MacConkey and Columbia agar were cultured in aerobic conditions at 37 °C overnight; Pseudomonas cetrimide plates were also incubated under aerobic conditions at 42 °C overnight. The remaining sheep blood agar plates were (1) incubated in anaerobic conditions at 37 °C overnight using Oxoid anaerobic generator in an airtight jar or in 5% carbon dioxide using an Oxoid carbon dioxide generator in an airtight jar. For growth of yeasts and fungi, the swab was plated on sabourand dextrose with chloramphenicol agar (Oxoid CM0041) and examined after 7 days incubation at 30°C. After incubation, a colony representing each type of colony was isolated, sub-cultured and identified phenotypically using the tables of Murray et al. (2007) or Winn et al. (2005). If a full identification could not be found using phenotypic characteristics, isolates were sequenced using 16s methods.

Genomic DNA was extracted from cultures grown on 5% sheep blood agar (Oxoid, CM271) at 37 ºC using PrepMan® Ultra (Applied Biosystems Inc., California, USA) according to manufacturer’s instructions. DNA concentrations of samples were determined based on absorbance at 260 nm with a Spectrophotometer ND-1000 (Nanodrop). 16S rRNA gene sequencing using the universal bacterial 16S rRNA primer 27f (5´AGAGTTTGATCMTGGCTCAG3´; Lane, 1991) and 1492r (5´TACGGYTACCTTGTTACGACTT3´; Lane, 1991) and was performed using 200 µl reaction PCR
strips containing, 2 × Amplitaq Gold® 360 Mastermix (Applied Biosystems, Inc. California, USA), 5 µM of each primer and 80 ng to 100 ng of template DNA. The PCR was performed in a Takara thermocycler TP600 (Takara Bio Inc., Kyoto, Japan) using an initial denaturing step of 94 ºC for 5 min followed by 35 cycles of 94ºC for 30 sec, annealing temperature at 48ºC for 1 minute, extension at 72ºC for 2 min and a final extension at 72ºC for 7 minutes followed by a hold at 4ºC. Injection water (Pfizer Inc., New York, USA) was used as a negative control. The amplified PCR products were purified with QIAquick PCR Purification Kit (Qiagen, Hilden, Germany). Purified 16S PCR product was extended using primer 27f and 1492r and cycle conditions as follows: an initial denaturing step of 94ºC for 5 min followed by 30 cycles of 96ºC for 10 sec, annealing temperature at 50 ºC for 5 sec, extension at 6 ºC for 4 min and on hold at 10ºC.

Sequencing product analysis was performed using the Big Dye Terminator (version 3.1) cycle sequencing kit with a capillary electrophoresis Hitachi 3730 DNA Analyzer (Applied Biosystems). Chromaspro (Chromas, Technelysium Pty Ltd, South Brisbane, Australia) sequencing software used to assemble and analyse both forward and reverse sequences. Basic Local Alignment Search Tool (BLAST) analysis was carried out through the National Centre for Biotechnology Information (NCBI) homepage. Representative organisms cultured from the samples were tested for their resistance to antibiotics using the Kirby-Bauer Disc diffusion method. Antibiotics used were TE30 (Tetracycline 30µg), SXT25 (Trimethoprim sulfamethoxazole 25µg), ENR (Enrofloxacin 5µg), CN10 (Gentamicin 10µg), AMC10 (Amoxycillin clavanic acid 10µg), KF30 (Cephatholin 30µg), P10 (Penicillin 10iu), E15 (Erythromycin 15µg), DA2 (Clindamycin 2µg), AMP10 (Amoxicillin 10µg), CIP5 (Ciprofloxacin 5µg), CAZ30 (Ceftazidime 30µg) and TIM 85 (Ticarcillin clavulanic acid 85µg).

Sperm extension and preservation studies - experimental design

After initial neat semen evaluation (pH, mass activity, % motility, rate of motility, plasma membrane integrity and preparing a nigrosin-eosin smear for morphology), the semen sample was divided into aliquots and allocated to six experiments that were carefully scheduled and conducted in parallel. Given the paucity of knowledge regarding manipulation and preservation of reptile semen, a series of experiments was conducted to investigate the survival of crocodile sperm in a range of simple diluents (including those that had previously been used for alligator spermatozoa), the effect of serial dilution, the effect of sudden temperature change, the importance and need for egg yolk in the diluent, the sperm cell’s tolerance of anisotonic media and the effect of cryoprotectant type and concentration pre and post cryopreservation.

Experiment 1 – The effect of extender type

Semen from seven crocodiles was used in this experiment. Semen was diluted 1:4 in:

1. PBS - phosphate buffered saline without Ca2+ or Mg2+ [pH - 7.2; Osmolality - 290 OsmKg-1];
2. TCG - Tris-citrate glucose [36.3 gL-1 of Tris (hydroxymethyl) aminomethane, 19.9 gL-1 of citric acid (monohydrate) and 10 gL-1 of D-glucose and adjusted to pH 7.4; Johnston et al., 2000];
3. BEST without egg yolk [22.2 gL-1 of N,N-bis-(2-hydroxymethyl)-2-aminoethane sulfonic acid, 6.3 gL-1 Tris (hydroxymethyl) aminomethane and 28.5 gL-1 D-glucose adjusted to pH 7.2; Osmolality – approximately 310 OsmKg-1; Larsen et al., 1984]; and
4. BEST with 20% (v/v) fresh chicken egg yolk.

BEST with 20% (v/v) egg yolk was used because it had been the base-extender used for initial semen evaluation (Johnston et al., 2013; this volume). Given the short period of sperm incubation and the potential confounding effect on sperm survival, no antibiotic was added to the diluents. Following extension, the diluted sample was evaluated for percentage motility, rate of motility and the percentage of sperm with intact plasma membranes after 30 mins and 120 mins of incubation, at 30ºC.
Experiment 2 – The effect of serial dilution

Semen from seven crocodiles with a mean ± SEM sperm concentration of 3.3 ± 0.2 x 10⁹mL⁻¹ (Range 2.4 – 4.2 x 10⁹mL⁻¹) was used for this experiment. Immediately after semen collection, 50 μL of a 100 μL aliquot was serially diluted (v/v) 1:1, 1:2; 1:4; 1:8; 1:16; 1:32 and 1:64 with PBS without Ca²⁺ or Mg²⁺ [pH - 7.2; Osmolality - 290 OsmKg⁻¹] and incubated at 30°C for 2 h. Each diluted sample was then evaluated for percentage motility, rate of movement and the percentage of sperm with intact plasma membranes.

Experiment 3 – The effect of a simulated rapid change of temperature

Semen from six crocodiles was used for this experiment. Immediately after semen collection, 10 μL of the neat semen sample was diluted with 30 μL of PBS without Ca²⁺ or Mg²⁺ [pH - 7.2; Osmolality - 290 OsmKg⁻¹]. A 10 μL of diluted semen sample was then placed on two pre-warmed (30oC) microscope slides on a warm plate and covered with a coverslip. One of the microscope slides was then placed directly into the freezer compartment of conventional refrigerator (-20oC) for approximately 90 seconds, while the other sample was allowed to incubate on the warm plate for the same period. The cooled sample was then allowed to re-warm to 30°C on the warm plate while the non-cooled sample was assessed for the percentage of motile sperm, rate of movement and percentage of sperm with intact plasma membranes; immediately after assessment of the non-cooled sample, the cold-shocked sample was similarly assessed for sperm survival.

Experiment 4 – The effect of egg yolk

Semen from six crocodiles was used in this experiment. Four concentrations of chicken egg yolk diluent (0, 10, 20 and 40% V/V) were initially prepared in PBS without Ca²⁺ or Mg²⁺ [pH - 7.2; Osmolality - 290 OsmKg⁻¹]. Immediately after semen collection, 50 μL of neat semen was diluted 1:3 in PBS and 50 μL of this diluted sample further diluted 1:1 with each experimental extender to give a final egg yolk concentration of 0, 5, 10 and 20% (V/V). Each extended sample was then further divided into 50 μL samples; one was incubated at 30°C for 1h and the other placed in a water jacket (initially 30 °C) and loaded into a portable refrigerator set at 4°C for a period of 24h. Following incubation and chilled storage, each diluted sample was evaluated for percentage motility, rate of movement and the percentage of sperm with intact plasma membranes.

Experiment 5 – The effect of diluent osmolality

Semen from six crocodiles was used in this experiment. Immediately following semen collection, 20μl of neat semen sample was suspended in 180μl in PBS without Ca²⁺ or Mg²⁺ [pH - 7.2; Osmolality - 290 OsmKg⁻¹] of varying osmolalities; 25, 59, 150, 225, 280, 390, 600, 880, 1237 and 1540 mOsmKg⁻¹. Hypotonic media were produced by diluting PBS with distilled water whereas hypertonic media were generated following serial dilution of a 1M sucrose PBS solution. The osmolality of each solution was determined using a freezing point osmometer (Advanced Instruments Inc, Model 3320; Norwood, Massachusetts). Spermatozoa were incubated at 30°C in each medium for a period at 15 min. This exposure represented the first or initial osmotic excursion (E); subsamples were evaluated for the percentage of motile sperm, percentage of sperm with an intact plasma membrane and the % of sperm with a coiled tail. Coiled tails were defined as the principal piece showing evidence of at least a complete 360° coiling event; flagella assessment was conducted by preparing nigrosin-eosin stain smears. The remaining semen sample was washed by centrifugation at 200g for 5 mins, the supernatant was removed and the pellet resuspended in 390 mOsmKg⁻¹ media; this media was arbitrarily chosen based on previous estimates of osmolality of the neat semen sample (335.5 ± 9.0 mOsmKg⁻¹). This exposure represented the second excursion and a return to an approximate isotonic environment (R); after 15 mins equilibration subsamples were then again evaluated for sperm survival and the percentage of spermatozoa with a coiled tail.
Experiment 6 – The effect of cryoprotectant type and concentration

Semen from six crocodiles was used in this experiment. Immediately following semen collection 300 µL of neat semen was diluted 1:10 with PBS without Ca²⁺ or Mg²⁺ or egg yolk (pH - 7.2; Osmolality - 290 OsmKg⁻¹). The semen sample was assessed for the percentage of motile sperm, the rate of sperm movement and the percentage of sperm with an intact plasma membrane. This semen sample was then placed in a water jacket set at 30°C and cooled slowly over 2 h to 4°C in a portable refrigerator. Following equilibration, the cooled semen sample was gently mixed and then divided into 10 aliquots of 300 µL. This extended sample was further diluted 1:1 with PBS containing a range of cryoprotectants so that the final extended sample contained either no cryoprotectant, dimethyl acetamide (DMA 0.68 M, 1.35 M and 2.70M), dimethyl sulfoxide (DMSO 0.68 M, 1.35 M and 2.70M) or glycerol (G 0.68 M, 1.35 M and 2.70M). After a further 5 min equilibration, 0.25 mL straws (IMV, L’Aigle, France) were prepared for cryopreservation, while a sub-sample was left in the eppendorf tube to equilibrate for a further period of 60 mins, after which time it was rewarmed to 30°C and evaluated for percentage of motility, rate of sperm movement and the percentage of sperm with an intact plasma membrane. Semen straws were sealed with PVC powder and loaded into a programmable freezer and frozen at 6°C/min (Freeze Control CL-863, Cryologics Pty Ltd., Australia) from 4 to -86oC. Straws when then removed from the freezer and plunged into liquid nitrogen (LN; -196oC) and stored for a period of 6 weeks. Straws were thawed in a 35°C water-bath for 1 min and semen samples dispensed into pre-warmed 2X 1.5 mL eppendorf tubes. Spermatozoa, still within the cryopreservation media were assessed after 10 mins incubation at 30oC for sperm survival. The remaining semen sample was immediately washed by centrifugation at 200 g for 5 mins, the supernatant removed, the pellet resuspended in PBS without Ca²⁺ or Mg²⁺ [pH - 7.2; Osmolality - 290 OsmKg⁻¹]; the sample was then incubated for 5 mins at 30°C and assessed for sperm survival.

Artificial insemination

Following dissection of cloacal anatomy of female crocodile to explore potential methods of AI, we attempted two rounds of artificial insemination procedures. The first round was conducted in late September and mid-October in 2011, and was only intended as preliminary study to determine the practicality of semen delivery. While live semen was inseminated into 10 sexually mature females that had been isolated from males prior to insemination, no attempt was made to record the seminal characteristics of the semen samples or any attention given to the reproductive status of the sexually mature females. Nevertheless, semen diluted 1:1 with BEST diluent containing 20% V/V egg yolk was inseminated into females at a time of year consistent with the peak of the natural crocodile breeding season at Koorana Crocodile Farm.

The second round of inseminations occurred in 13 sexually mature non-gravid females in late September and mid-October, and used methods of semen delivery refined in round 1. Following administration of Pamlin®, the female crocodile was placed in dorsal recumbency on a ‘tilt’ table constructed from a hospital bed. A clear plastic human vagina speculum (MedPro®, Montreal, Canada) was inserted into the cloaca to reflex the clitoris ventrally so that semen could be placed at the openings of the oviduct located with urodeum immediately cranial to the base of the clitoris. semen was deposited using either a sheep AI pipette (Figure 3; Minitube, Germany) or a 3.5 Fr gauge “tomcat” catheter (Monoject®, Becton Dickinson Labware, New Jersey, USA). Semen was collected from 12 sexually mature males and evaluated for motility (Mean ± SEM; - 54.8 ± 4.2%) and sperm concentration (Mean ± SEM; - 2.10 ± 0.29 x 10⁹ mL⁻¹) prior to AI; one male produced enough semen for two separate inseminations. A total of eight females were inseminated with 0.3 – 2.0 mL of neat semen, while 4 females were inseminated with 0.5 – 1.0 mL of semen after 1:1 dilution with BEST diluent containing 20% V/V egg yolk.

Statistical analysis

between body length and seminal characteristics from the 25 crocodiles that produced ejaculates were calculated; all seminal characters (including sperm abnormalities) were also correlated with each other to determine any intra-seminal relationships. All percentage data (motility, plasma membrane integrity and coiled tails) were arcsin transformed for the calculation of analysis variance and paired t-tests. The effect of media type, serial dilution, egg yolk, osmolality and cryoprotectant type and concentration on chilled and cryopreserved sperm on percentage motility was analysed using a single factor analysis of variance; multiple comparison tests of significant analyses were made using Fisher’s protected least significant differences (PLSD); the mean rates of sperm motility in each of these experiments were analysed individually using Wilcoxon matched pairs tests. Sperm motility and plasma membrane integrity data for the rapid temperature change experiment were compared using Two-sample T-tests assuming equal variances; the rate of sperm movement was analysed using a Wilcoxon matched pairs test. Mean motility, plasma membrane integrity and coiled tail data before and after osmotic excursion were also compared using a two-sample T-test assuming equal variances. Results are reported as mean ± standard error of the mean.

Figure 1.3: Artificial insemination of the saltwater crocodile using clear human vaginal speculum and sheep artificial insemination pipette (Minitube, Germany).
Semen collection and seminal characteristics of the Australian Salt-water Crocodile (*Crocodylus porosus*)

**Male reproductive anatomy**

The testes and reproductive tract of the male saltwater crocodile are shown in figure 2.1. Paired elongated ovoid testes were located in a retroperitoneal position and sat slightly ventral and cranial to kidneys, with the left and right testis physically separated by a prominent mesocolon. The tunica albugenia of the testis was well vascularised and the testes surrounded by a layer of adipose tissue. The ductuli efferentes and ductuli epididymides were not easily definable on gross dissection but extended from the outer lateral margin of the testis into a folded ductus epididymis that was subsequently differentiated into a ductus deferens. The ductus deferens was covered by pseudostratified epithelium and opened via a papilla at the base of a single medial groove that extended along the shaft and glans penis known as the sulcus spermaticus (Figure 2.2). The glans penis possessed a hallow cavity opening towards the tip of the penis, which was not connected directly to the medial groove (Figure 2.3). Well-developed vascular tissues, homologous to the corpus cavernosus surrounded this cavity (Figure 2.2 and 2.3).

The penis arose from the wall of the proctodeum and was stored within this chamber when not erect (Figure 2.4C); if stimulated by electroejaculation or manually palpated, it became erect and engorged with blood (Figure 2.4A and B). When the penis was erect it was possible to clearly differentiate the slightly re-curved shaft of the penis, a bulbous portion of the glans penis that resembled an inflatable cuff and a terminal extension of the glans penis (tip) (Figure 2.4A). During ejaculation, semen ran down the sulcus, which extended down the distal aspect of the penile shaft terminating at the tip of the glans. During semen collection, the semen pooled slightly retrograde under gravity creating a “drop” of semen that could be readily collected (Figure 2.4C).

One of the larger males used for semen collection showed evidence of a persistent penile prolapse in which the tissue of the glans penis was swollen, dry, grossly granulated and presumably unable to be withdrawn back into the cloaca (Figure 2.4D). The granulated tissue of the glans had resulted in multiple adhesions along the sulcus, thereby preventing sperm delivery; Figure 2.4E shows semen pooling and accumulating proximal to sulcus adhesions. Ventrolateral to the penis on the floor of the proctodeum were two glandless papillae that appeared to be patent with the abdominal cavity (Figure 2.2A).

**Success of semen collection**

Typically, semen was collected from only two to three animals per day. While this was primarily a result of the logistical operation in obtaining individual male crocodile from their respective enclosures, it was also a consequence of a variable period (120 ± 13.4 min; range 30 – 330 mins) in the time required for the crocodile to reach a sufficient depth of sedation that would allow physical manipulation of the genitalia but also account for safe human access. Once adequately sedated, semen collection was typically completed over a period of 25 minutes (range 10 – 45 mins).

Over all, semen for fertility evaluation was successfully collected by manual massage on 30/31 attempts; the only occasion on which semen was not recovered was from a juvenile male measuring 185 mm. In the majority of animals, the flow of semen down the sulcus spermaticus was greatest during the early stages of the digital massage procedure and in a number of animals, the semen had pooled at the tip of the erect penis, even before massage had commenced. semen was collected successfully from C1 three times (September 27; October 16 and December 3) and another five
animals twice over a period ranging from 18 days (C3) to 54 – 56 days (C8, C9, C13 and C16). All crocodiles fully recovered from the semen collection procedure without incident.

Figure 2.1: Gross reproductive anatomy of a sexually mature saltwater crocodile (2.7 m) dissected during July 2011. A Testis, ductus epididymis and ductus deferens suspended by peritoneal tissue in lower abdomen – note right testis located underneath the mesocolon; B. Fully dissected testis and kidney showing their respective efferent ducts and position in the lower abdomen; C. Lateral aspect of testis revealing the convoluted sperm filled region of the ductus deferens. Cl – Cloaca; Dd – Ductus deferens; De – Ductus epididymis; K – Kidney; Mc – Mesocolon; Te – Testis; Ur – Ureter. Scale bar – 2cm.
Figure 2.2: Penile anatomy of the saltwater crocodile. A. Dorsolateral aspect of penis after dissecting the dorsal wall of the cloaca – note location of the proctodeum and urodeum and two proctodeal papillae (Scale – 10 mm); B - G: Serial transversal sections of penis (Scale – 5 mm) – note transverse sections of the penis are orientated as the penile was erect and extruded from the cloaca. Ca – cavity of the gland penis; Co – collagen in penile shaft; Su – sulcus; Arrow - vascular tissues in the glans penis; Dotted line: slice line of penis; ? – proctodeal papillae.
Figure 2.3: Gross and microanatomy of the saltwater crocodile penis. A. Sagittal section of the penis (Scale bar – 5 mm); B. Macro transversal image of the base of penis after removing the right hand side (Dotted line represents the approximate position the left hand side, Scale bar – 2mm); C. Transversal section of the ductus deferens opening via a papilla into the sulcus spermaticus (Scale bar - 300µm); D. Epithelium of the ductus deferens opening into the sulcus spermaticus (Scale - 10µm. Ca - cavity of glans penis; Co - collagen in the shaft penis; Dd - ductus deferens; P – penis; VT - vascular tissues in glans penis; Arrow: papillae of the ductus deferens opening into the proximal end of the sulcus; Open arrow head - ductus deferens in the papilla located at the proximal end of the sulcus
Figure 2.4: Anatomy of the saltwater crocodile penis. A. Lateral view of erect penis; B. Head on view of the glans penis showing medial sulcus; C. MRI sagittal view of the crocodile cloaca; D. Penile prolapse; E. Close up of penile prolapse showing tissue granulation in the sulcus region of the penile shaft – note how adhesions of the sulcus are preventing the flow of semen to the glans penis. Cl – Cloaca; Gp – Glans penis; Ic – inflated cuff or bulbous region of the penis; Ps – Penile shaft; Se → - Semen; Ti – Tip of glans penis.
Seminal characteristics

The seminal characteristics of 30 ejaculates collected by digital cloacal massage from 23 saltwater crocodiles are shown in Table 2.1. Figure 2.5 depicts normal (A and B) and a variety of abnormal sperm morphologies (C) as revealed by light microscopy and the nigrosin-eosin stain. Table 2.2 reports the frequency of sperm abnormalities found in a total of 30 ejaculates from 23 crocodiles.

Table 2.1: Seminal characteristics of 30 ejaculates collected by digital cloacal massage from 23 saltwater crocodiles.

<table>
<thead>
<tr>
<th>Seminal characteristic</th>
<th>Mean ± SEM</th>
<th>Range</th>
<th>N</th>
</tr>
</thead>
<tbody>
<tr>
<td>Volume (mL)</td>
<td>0.91 ± 0.16</td>
<td>0.05 – 3.30</td>
<td>29</td>
</tr>
<tr>
<td>pH of ejaculate</td>
<td>7.3 ± 0.1</td>
<td>6.5 – 7.5</td>
<td>23</td>
</tr>
<tr>
<td>Osmolarity of seminal plasma (mOsmKg⁻¹)</td>
<td>335.5 ± 9.0</td>
<td>290 – 379</td>
<td>11</td>
</tr>
<tr>
<td>Sperm concentration (x 10⁹ mL⁻¹)</td>
<td>2.29 ± 0.26</td>
<td>0.35 – 4.0</td>
<td>21</td>
</tr>
<tr>
<td>Mass Activity (0 – 5)</td>
<td>2.2 ± 0.2</td>
<td>1.0 – 3.5</td>
<td>25</td>
</tr>
<tr>
<td>% Motility</td>
<td>50.7 ± 4.2</td>
<td>0 – 85</td>
<td>27</td>
</tr>
<tr>
<td>Rate of sperm movement (0 – 5)</td>
<td>2.6 ± 0.2</td>
<td>0 – 4</td>
<td>28</td>
</tr>
<tr>
<td>% Plasma membrane intact</td>
<td>79.9 ± 3.6</td>
<td>3 - 97</td>
<td>29</td>
</tr>
<tr>
<td>% Morphology normal spermatozoa</td>
<td>51.2 ± 5.1</td>
<td>6 – 93.5</td>
<td>28</td>
</tr>
<tr>
<td>% Cytoplasmic droplets</td>
<td>26.9 ± 4.3</td>
<td>1 - 85</td>
<td>28</td>
</tr>
</tbody>
</table>

Table 2.2: Sperm abnormalities (Mean ± SEM) found in 30 ejaculates of 23 salt-water crocodiles.

<table>
<thead>
<tr>
<th>Seminal characteristic</th>
<th>Mean ± SEM</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Macrocephalic nucleus</td>
<td>1.9 ± 0.3</td>
<td>0 - 5</td>
</tr>
<tr>
<td>Microcephalic nucleus</td>
<td>2.7 ± 0.9</td>
<td>0 - 22</td>
</tr>
<tr>
<td>Teratoid spermatozoa</td>
<td>5.2 ± 0.9</td>
<td>0 - 17</td>
</tr>
<tr>
<td>Loose head</td>
<td>7.1 ± 1.9</td>
<td>0 - 51</td>
</tr>
<tr>
<td>Damaged acrosome (missing or lost)</td>
<td>1.0 ± 0.2</td>
<td>0 – 4</td>
</tr>
<tr>
<td>Cytoplasmic droplet</td>
<td>26.8 ± 4.2</td>
<td>0 – 85</td>
</tr>
<tr>
<td>Double tail</td>
<td>0.5 ± 0.2</td>
<td>0 – 5</td>
</tr>
<tr>
<td>Bent midpiece</td>
<td>3.0 ± 1.6</td>
<td>0 - 48</td>
</tr>
<tr>
<td>Coiled principle piece</td>
<td>0.5 ± 0.1</td>
<td>0 - 2</td>
</tr>
</tbody>
</table>

Semen was collected from five crocodiles more than once, and although no statistics were conducted because of the small sample size, these ejaculates provided an opportunity to examine changes in seminal characteristics over time (early October to early December). Seminal volume increased in all 5 males in December, and 4 of the 5 males also showed an increase in sperm concentration. Interestingly, the percentage of motile sperm decreased from October to September in 4 of the 5 males, while the percentage of live and normal spermatozoa essentially remained the same. The only significant correlation between body length and a seminal characteristic was that of seminal volume (r = 0.437; p = 0.018; df = 28). Not unexpectedly, there was also a positive correlation between the percentage of live spermatozoa with motility (r = 0.63; p = 0.004; df = 26) and % normal (r = 0.41; p = 0.03; df = 28). Twenty-one of the 30 ejaculates observed for sperm morphology showed a moderate to high incidence (≥ 10 to 85%) of what appeared to be cytoplasmic droplets attached to or in close proximity to the midpiece of the spermatozoon (Figure 2.5C). In C19 and C6, cytoplasmic droplets accounted for 85 of the 94 and 60 of the 91 sperm abnormalities found in the ejaculate.
Other notable sperm abnormalities found in moderate numbers in the captive crocodile population included teratoid spermatozoa (5.2 ± 0.9%; C12 – 17%; C14 – 16%; C11 – 12%) and sperm heads without a flagellum (7.1 ± 0.9%; C11 – 51%; C12 – 29%). There were also a number of individual crocodiles that showed a moderate level of meiosis related problems resulting in macrocephalic (C1 – 5%; C8 – 7%; C13 – 5%) and microcephalic (C6 – 7.5%; C11 – 6%; C12 – 22%; C14 – 16%) sperm nuclei.

Figure 2.5: Normal and abnormal crocodile spermatozoa. A. Differential interference contrast micrograph of normal crocodile sperm; B. Nigrosin-eosin stained normal crocodile sperm. C. Panel of crocodile sperm abnormal morphology. Ac – Acrosome; Ad – Acrosomal damage (lost acrosome); Bm – Bent midpiece; Cd – Cytoplasmic droplet; Dt – Double tail; Lh – Loose head; Ma – Macrocephalic; Mi Microcephalic; Mp – Midpiece; Pp – Principal piece; Te – Teratoid. Scale bar – X µm.
Isolation of bacteria from the penile shaft, sulcus and semen

The microflora cultured from the penile shaft, sulcus of the penis and semen of three sexually mature crocodiles and swabs from the shaft of the penis from a further three crocodiles is shown in Table 2.3, along with the sensitivity and resistance of the cultured organisms to a range of antibiotics used to control growth using the disc method of Kirby-Bauer. Overall, a total of 24 species were cultured and identified from the crocodile penis and semen including one Pseudomonas species which could not be identified further. All samples submitted for culture showed evidence of bacterial growth but only a swab of the sulcus and penile shaft of one animal grew fungi. Fifteen of the 24 species were cultured from the penile shaft, 11 from the sulcus and 8 from the semen. Eight species (A. caviae, C. braakii, E. agglomerans, K. ornitholytica, K. pneumonia, P. mirabilis, P. aeruginosa and S. arizonae) were cultured only from the penile shaft and eight species (A. veronii bio sobria, B. cereus, C. meningosepticum, E. cloacae, E. avium, K. gibsonii, P. rettgeri and P. stuartii) were only found in the sulcus and semen. Results from the antibiotic sensitivities observations indicated that gentamicin was the most effective anti-microbiological for the bacteria isolated from the crocodile penis and semen, with the exception of E. faecium, which was sensitive to sulphamethoxazole/trimethoprim and amoxicillin/clavulanic.

Table 2.3: Identification and antibiotic sensitivity of microorganisms isolated from the penile shaft, sulcus and semen of the saltwater crocodile.
Extension, osmotic tolerance and cryopreservation of Saltwater Crocodile (*Crocodylus porosus*) spermatozoa

The seminal characteristics (Mean ± SEM and Range) of the ejaculates of ten saltwater crocodiles used in this study are summarised in Table 3.1.

**Table 3.1:** Seminal characteristics (mean ± SEM and range) of the ejaculates of ten saltwater crocodiles used in sperm extension and preservation experiments.

<table>
<thead>
<tr>
<th>Seminal characteristic</th>
<th>Mean ± SEM</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Volume (mL)</td>
<td>1.4 ± 0.3</td>
<td>0.3 – 3.30</td>
</tr>
<tr>
<td>pH of ejaculate</td>
<td>7.0 ± 0.1</td>
<td>6.5 – 7.5</td>
</tr>
<tr>
<td>Sperm concentration (x 10^9 mL⁻¹)</td>
<td>3.4 ± 0.2</td>
<td>2.5 – 4.2</td>
</tr>
<tr>
<td>Mass Activity</td>
<td>2.6 ± 0.2</td>
<td>2 – 3.5</td>
</tr>
<tr>
<td>% Motility*</td>
<td>63.4 ± 3.2</td>
<td>50 - 80</td>
</tr>
<tr>
<td>Rate of sperm movement (0 – 5)*</td>
<td>4.0 ± 0.2</td>
<td>2.0 – 4.0</td>
</tr>
<tr>
<td>% Plasma membrane intact</td>
<td>83.2 ± 1.3</td>
<td>75 - 86</td>
</tr>
<tr>
<td>% Morphology normal spermatozoa**</td>
<td>86.4 ± 2.2</td>
<td>83 - 95</td>
</tr>
</tbody>
</table>

*Motility assessed after dilution in PBS
**Normal sperm includes sperm with cytoplasmic droplets attached

**Experiment 1 – The effect of extender type**

The effect of diluent type on saltwater crocodile sperm % motility, rate of sperm movement and % intact plasma membranes is documented in Table 3.2. After 30 and 120 mins incubation of spermatozoa at 30°C, the motility and rate of movement of crocodile spermatozoa diluted in TRIS was significantly lower than that compared to PBS and BEST with or without 20% V/V egg yolk. The percentage of sperm with intact membranes did not change with respect to diluent type or incubation time. The presence or absence of 20% egg yolk V/V in the BEST diluent made no difference to sperm motility. PBS without egg yolk was subsequently chosen as the base diluent for experiments 2 – 6.

**Table 3.2:** The effect of extender type on saltwater crocodile sperm survival (Mean ± SEM % motility, rate of sperm motility and %plasma membrane integrity) after 30 mins (T30) and 120 mins (T120) incubation at 30°C.

<table>
<thead>
<tr>
<th></th>
<th>PBS</th>
<th>TRIS</th>
<th>BEST (0EY)</th>
<th>BEST (20EY)</th>
</tr>
</thead>
<tbody>
<tr>
<td>% Motile</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T30</td>
<td>61.8 ± 3.9^a</td>
<td>21.2 ± 5.8^b</td>
<td>42.2 ± 10.5^a</td>
<td>61.7 ± 2.9^a</td>
</tr>
<tr>
<td>T120</td>
<td>33.0 ± 9.0^a</td>
<td>5.3 ± 2.5^b</td>
<td>24.8 ± 10.4^a</td>
<td>20.4 ± 5.5^a</td>
</tr>
<tr>
<td>Rate of sperm movement</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T30</td>
<td>4.0 ± 0.2^a</td>
<td>1.6 ± 0.2^b</td>
<td>3.3 ± 0.3^a</td>
<td>4.2 ± 0.3^a</td>
</tr>
<tr>
<td>T120</td>
<td>2.6 ± 0.5^a</td>
<td>1.1 ± 0.3^a</td>
<td>2.0 ± 0.7^a</td>
<td>1.6 ± 0.6^a</td>
</tr>
<tr>
<td>% Intact plasma membranes</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T30</td>
<td>84.3 ± 1.4^a</td>
<td>84.7 ± 1.3^a</td>
<td>84.2 ± 1.1^a</td>
<td>84.5 ± 1.9^a</td>
</tr>
<tr>
<td>T120</td>
<td>79.7 ± 2.0^a</td>
<td>83.0 ± 2.6^a</td>
<td>81.5 ± 1.9^a</td>
<td>76.0 ± 4.3^a</td>
</tr>
</tbody>
</table>

Different superscripts within row indicate a significant between means at P < 0.05.
Experiment 2 – The effect of serial dilution

The effect of serial dilution in PBS on saltwater crocodile sperm % motility, rate of sperm movement and % intact plasma membranes after 120 mins incubation at 30°C is shown in Table 3.3. While there was large variation with respect to how spermatozoa of the individual crocodile responded to serial dilution, there was only significant decline in % motility at the highest dilution of 1:64. The effect of dilution on the rate of sperm movement was more pronounced, with rate of movement declining significantly after dilution at 1:8 and then further again at 1:32; there was also reduction in rate of movement in the neat semen sample compared to the dilutions from 1:1 to 1:4. The percentage of sperm with intact plasma membranes was lower in the neat semen and at a dilution of 1:64.

<table>
<thead>
<tr>
<th>Dilution</th>
<th>% Motile</th>
<th>Rate of sperm movement</th>
<th>% Intact plasma membrane</th>
</tr>
</thead>
<tbody>
<tr>
<td>1:0</td>
<td>51.7 ± 5.9&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.8 ± 0.2&lt;sup&gt;b&lt;/sup&gt;</td>
<td>65.3 ± 7.9&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>1:1</td>
<td>52.9 ± 7.5&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4.0 ± 0.4&lt;sup&gt;d&lt;/sup&gt;</td>
<td>77.9 ± 3.2&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>1:2</td>
<td>59.3 ± 6.1&lt;sup&gt;b&lt;/sup&gt;</td>
<td>4.2 ± 0.3&lt;sup&gt;a&lt;/sup&gt;</td>
<td>80.1 ± 3.1&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>1:4</td>
<td>54.9 ± 7.7&lt;sup&gt;b&lt;/sup&gt;</td>
<td>4.1 ± 0.3&lt;sup&gt;a&lt;/sup&gt;</td>
<td>81.4 ± 1.6&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>1:8</td>
<td>43.7 ± 11.5&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.9 ± 0.7&lt;sup&gt;b&lt;/sup&gt;</td>
<td>85.6 ± 2.6&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>1:16</td>
<td>37.1 ± 9.8&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.5 ± 0.4&lt;sup&gt;a&lt;/sup&gt;</td>
<td>85.4 ± 2.4&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>1:32</td>
<td>34.6 ± 9.8&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.5 ± 0.4&lt;sup&gt;c&lt;/sup&gt;</td>
<td>79.4 ± 3.7&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>1:64</td>
<td>9.1 ± 6.5&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.6 ± 0.4&lt;sup&gt;c&lt;/sup&gt;</td>
<td>75.6 ± 4.2&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Different superscripts within columns indicate a significant between means at P < 0.05.

Experiment 3 – The effect of simulated cold shock

The effect of simulated cold shock on saltwater crocodile sperm % motility, rate of sperm movement and the % of spermatozoa with intact plasma membranes are outlined in Table 3.4. Rapid change in temperature had no effect on sperm motility or rate of sperm movement but there was a significant loss in the percentage of sperm with intact plasma membranes.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>% Motile</th>
<th>Rate of sperm movement</th>
<th>% Intact plasma membrane</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>61.7 ± 2.9&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3.0 ± 0.1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>82.4 ± 2.5&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Cold shocked</td>
<td>55.6 ± 2.6&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3.0 ± 0.1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>73.0 ± 2.4&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Different superscripts within columns indicate a significant between means at P < 0.05.

Experiment 4 – The effect of egg yolk concentration

The effect of egg yolk concentration on saltwater crocodile sperm % motility, rate of sperm movement and % intact plasma membranes following either incubation at 30°C for 2h or at 4°C for 24h is documented in Table 3.5. The concentration of egg yolk (0 - 20% V/V) had no effect on the survival of crocodile sperm after 1h incubation at 30°C or after 24h at 4°C. Although the mean % motility of sperm diluted in PBS at 30°C for 2h was 10 to 18 % lower than other concentrations, there was a high degree of individual animal variability and consequently a lack of statistical significance.
Nevertheless, it was clear that egg yolk had little protective benefit for crocodile sperm survival and was, therefore, not used for subsequent cryopreservation studies (see Experiment 6).

**Table 3.5:** The effect of egg yolk concentration (V/V) on saltwater crocodile sperm survival (Mean ± SEM % motility, rate of sperm motility and % plasma membrane integrity) diluted 1:4 in PBS and incubated at either 30°C for 1h, or 4°C for 24h.

<table>
<thead>
<tr>
<th>Egg Yolk Concentration (V/V)</th>
<th>0</th>
<th>5</th>
<th>10</th>
<th>20</th>
</tr>
</thead>
<tbody>
<tr>
<td>% Motile</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>30 °C for 1h</td>
<td>59.0 ± 6.2a</td>
<td>48.2 ± 9.5a</td>
<td>42.5 ± 8.1a</td>
<td>31.2 ± 8.5a</td>
</tr>
<tr>
<td>4 °C for 24h</td>
<td>57.8 ± 0.6a</td>
<td>59.0 ± 2.1a</td>
<td>58.0 ± 1.3a</td>
<td>47.8 ± 8.3a</td>
</tr>
<tr>
<td>Rate of sperm movement</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>30 °C for 1h</td>
<td>3.2 ± 0.2a</td>
<td>3.2 ± 0.2a</td>
<td>2.8 ± 0.4a</td>
<td>2.3 ± 0.5a</td>
</tr>
<tr>
<td>4 °C for 24h</td>
<td>4.5 ± 0.3a</td>
<td>4.4 ± 0.2a</td>
<td>4.5 ± 0.7a</td>
<td>4.2 ± 0.7a</td>
</tr>
<tr>
<td>% Intact plasma membranes</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>30 °C for 1h</td>
<td>81.7 ± 2.0a</td>
<td>85.2 ± 2.2a</td>
<td>86.0 ± 2.1a</td>
<td>84.7 ± 2.2a</td>
</tr>
<tr>
<td>4 °C for 24h</td>
<td>77.3 ± 1.7a</td>
<td>83.3 ± 2.0a</td>
<td>81.8 ± 1.6a</td>
<td>84.3 ± 1.7a</td>
</tr>
</tbody>
</table>

Different superscripts within rows indicate a significant between means at P < 0.05.

**Experiment 5 – The effect of diluent osmolality**

The effect of crocodile sperm exposure to anisotonic media and their subsequent return to 390 mOsmKg⁻¹ media on sperm % motility, rate of sperm movement and the % of spermatozoa with intact plasma membranes are summarised in figure 3.1. Sperm motility varied over the range of anisotonic solutions tested both after the first (F = 42.4; P < 0.05) and second (F = 35.2; P < 0.05) excursions. While sperm motility significantly declined outside the 220 to 390 mOsmKg⁻¹ range, on return to the 390 mOsmKg⁻¹ media, there was a significant increase (P < 0.05) in motility (but not to the initial levels) of those spermatozoa originally diluted in 25, 59, 90, 150, 600 and 800 mOsmKg⁻¹.

The percentage of spermatozoa with intact membranes also varied over the range of anisotonic solutions tested both after the first (F = 2.24; P < 0.05) and second (F = 15.6; P < 0.05) excursions. The percentage of spermatozoa with intact plasma membranes diluted in the 1273 and 1540 mOsmKg⁻¹ media were significantly lower compared to those diluted in 390 mOsmKg⁻¹; however, there was no evidence of a reduction in the percentage of sperm with intact plasma membranes in hypotonic media - even for spermatozoa diluted in 25 mOsmKg⁻¹. On return to 390 mOsmKg⁻¹, there was a significant decrease (P < 0.05) in the percentage of sperm with an intact plasma membrane originally diluted in 390, 600, 880, 1273 and 1540 mOsmKg⁻¹.

The percentage of spermatozoa with coiled tails significantly increased as spermatozoa were diluted in hypotonic solutions of 150 mOsmKg⁻¹ or lower (Figure 3.2), both after the first (F = 265.7; P < 0.05) and second (F = 204.0; P < 0.05) osmotic excursions. Although the percentage of spermatozoa with coiled tails in these media significantly declined (P < 0.05 for 25, 59, 90, 150 and 220 mOsmKg⁻¹) after return to 390 mOsmKg⁻¹ media, most did not revert to their pre-treatment straight tail morphology.
Experiment 6 – The effect of cryoprotectant type and concentration on chilled storage and cryopreservation success

The effect of cryoprotectant type and concentration on crocodile sperm % motility, rate of sperm movement and the % of intact plasma membranes following chilled refrigeration for 1 h at 4°C and after cryopreservation (with and without post-thaw washing out of the cryoprotectant) are shown in Table 3.6.

Compared to spermatozoa diluted in the PBS control, there was a significant reduction in the motility of sperm diluted and chilled to 4°C in all media tested ($F = 20.3; P < 0.05$), except those extended in 0.68M DMSO. Spermatozoa diluted in the highest concentrations (2.7M) of all three cryoprotectants showed a dramatic loss of motility. In contrast, the % of sperm with intact plasma membrane was unaffected by the type and concentration of cryoprotectant ($F = 1.91; P = 0.063$).

![Figure 3.1: The effect of anisotonic PBS media on sperm survival (% Motile, % plasma membrane integrity) and % tail coiling of crocodile spermatozoa when exposed to hypertonic media for 10 mins and then returned to 390 mOsmKg⁻¹ PBS. ⊙ - indicates a significant difference ($P < 0.05$) between first anisotonic excursion and return to 390 OsmKg⁻¹; Asterisk (*) – indicates a significant difference ($P < 0.05$) between sperm survival in 390 mOsmKg⁻¹ compared to spermatozoa that have been exposed to anisotonic media and then returned to 390 mOsmKg⁻¹.](image-url)
Figure 3.2: The effect of anisotonic PBS media (25 mOsmKg\(^{-1}\) to 220 mOsmKg\(^{-1}\)) on the tail coiling morphology of crocodile spermatozoa.

Table 3.6: The effect of cryoprotectant type and concentration on crocodile sperm survival following chilling to 4°C for 2h and freeze-thaw, when the cryoprotectant was and was not washed out post-thaw.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Initial</th>
<th>Chilled (4°C)</th>
<th>Post-thaw</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Not washed</td>
</tr>
<tr>
<td>PBS (control)</td>
<td>66.7 ± 4.5</td>
<td>57.7 ± 3.6(^a)</td>
<td>0</td>
</tr>
<tr>
<td>DMA 0.68M</td>
<td>31.8 ± 4.6(^b)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>DMA 1.35M</td>
<td>6.8 ± 2.9(^c)</td>
<td>0.2 ± 0.2</td>
<td>0</td>
</tr>
<tr>
<td>DMA 2.70M</td>
<td>3.5 ± 3.5(^c)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>DMSO 0.68M</td>
<td>47.0 ± 6.6(^a)</td>
<td>0.2 ± 0.2</td>
<td>0</td>
</tr>
<tr>
<td>DMSO 1.35M</td>
<td>13.2 ± 5.4(^b)</td>
<td>0.8 ± 0.5</td>
<td>0</td>
</tr>
<tr>
<td>DMSO 2.70M</td>
<td>5.5 ± 2.5(^c)</td>
<td>2.3 ± 1.6</td>
<td>1.0 ± 0.5</td>
</tr>
<tr>
<td>Glycerol 0.68M</td>
<td>26.5 ± 8.1(^b)</td>
<td>0.3 ± 0.2</td>
<td>0.2 ± 0.2</td>
</tr>
<tr>
<td>Glycerol 1.35M</td>
<td>2.4 ± 1.2(^c)</td>
<td>0.5 ± 0.3</td>
<td>0.1 ± 0.1</td>
</tr>
<tr>
<td>Glycerol 2.70M</td>
<td>0</td>
<td>0</td>
<td>1.2 ± 0.5</td>
</tr>
</tbody>
</table>

% Intact plasma membranes
Chilled Post-thaw Treatment Initial (4 °C) Not washed Washed

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Initial</th>
<th>Chilled</th>
<th>Post-thaw</th>
<th>Washed</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBS (control)</td>
<td>84.0 ± 0.7</td>
<td>86.0 ± 0.5a</td>
<td>0.3 ± 0.2a</td>
<td>0.2 ± 0.2a</td>
</tr>
<tr>
<td>DMA 0.68M</td>
<td>85.8 ± 2.1a</td>
<td>4.0 ± 1.4a</td>
<td>5.2 ± 1.7a</td>
<td></td>
</tr>
<tr>
<td>DMA 1.35M</td>
<td>83.7 ± 2.9a</td>
<td>10.8 ± 3.2b</td>
<td>16.8 ± 3.3b</td>
<td></td>
</tr>
<tr>
<td>DMA 2.70M</td>
<td>87.5 ± 1.5a</td>
<td>17.7 ± 4.4b</td>
<td>23.8 ± 5.6c</td>
<td></td>
</tr>
<tr>
<td>DMSO 0.68M</td>
<td>80.3 ± 1.3a</td>
<td>8.5 ± 1.3a</td>
<td>13.7 ± 4.0b</td>
<td></td>
</tr>
<tr>
<td>DMSO 1.35M</td>
<td>86.3 ± 2.1a</td>
<td>16.2 ± 2.6ab</td>
<td>23.5 ± 4.8b</td>
<td></td>
</tr>
<tr>
<td>DMSO 2.70M</td>
<td>84.7 ± 1.9a</td>
<td>22.7 ± 1.4c</td>
<td>27.7 ± 6.6bc</td>
<td></td>
</tr>
<tr>
<td>Glycerol 0.68M</td>
<td>87.7 ± 0.5a</td>
<td>7.7 ± 2.3a</td>
<td>10.8 ± 2.3a</td>
<td></td>
</tr>
<tr>
<td>Glycerol 1.35M</td>
<td>81.8 ± 1.3a</td>
<td>17.8 ± 3.8b</td>
<td>26.2 ± 5.5b</td>
<td></td>
</tr>
<tr>
<td>Glycerol 2.70M</td>
<td>86.7 ± 2.5a</td>
<td>25.7 ± 6.4c</td>
<td>23.5 ± 3.5b</td>
<td></td>
</tr>
</tbody>
</table>

Different superscripts within columns indicate a significant difference between means of each dose of cryoprotectant and the PBS control and between means of each concentration within each cryoprotectant treatment at P < 0.05.

Cryopreservation of spermatozoa, irrespective of cryoprotectant type and concentration, resulted in elimination of essentially all motility, regardless of whether the cryoprotectants were retained in the media or washed out post-thaw. One crocodile had a post-thaw motility of 10% in 2.7 M DMSO. The effect of cryopreservation on the percentage of sperm with intact membranes varied depending on cryoprotectant type, concentration and whether the cryoprotectant was washed out (F = 5.63; P < 0.05) or not (F = 4.82; P < 0.05) post thaw.

Sperm membrane integrity was greatest in the highest concentrations of cryoprotectant used but there was no difference with respect to the type of cryoprotectant used. When analysed as a function of all cryoprotective treatments, spermatozoa washed immediately after thawing had a significantly higher percentage of sperm with intact plasma membranes than those not washed free of cryoprotectants (t = 1.66; P < 0.05).

There was also a degree of individual animal variation with respect to the success of the different cryoprotectants used; post thaw plasma membrane integrity ranged from 8 – 41% in 2.7M DMA, 16 – 60% in 2.7M DMSO and 13 – 37% in 2.7M glycerol. Figure 3.3 illustrates an interesting phenomenon that was commonly observed following staining of thawed spermatozoa with SYBR-14 and PI. In a high proportion of sperm, the first evidence of PI staining typically occurred at the base of the sperm head and progressively moved up towards the distal tip of the nucleus. Spermatozoa with any evidence of a damaged plasma membrane were regarded as in the process of dying, even if these spermatozoa still showed evidence of motility (Figure 3.3B).
Figure 3.3: Analysis of crocodile spermatozoa using SYBR-14 and PI stains - live (green fluorescence) and dead sperm (red-orange fluorescence). Note (white arrow) how sperm show evidence of plasma membrane initially at the base of the sperm head and then progressively along the length of the spermatozoon. D – dead spermatozoa; L – live spermatozoa; Ma – macrocephalic sperm head
Female reproductive anatomy and preliminary attempts at saltwater crocodile artificial insemination

Female cloacal anatomy

Cloacal anatomy of the female saltwater crocodile is shown in figure 4.1. A large clitoris (homologue of the penis) originates from the ventral floor of the urodeum but extends caudally into the proctodeum. Immediately cranial to the clitoris but still on the ventral floor of the urodeum are the two muscular ostia (openings) of the oviducts, the caudal extremity of which contains numerous muscular folds and is regarded by Gist et al. (2008) in the American alligator as a vagina. The uterers of the kidneys open cranial to the oviducts but are located on the dorsal wall of the urodeum.

Figure 4.1: Female cloacal anatomy of the saltwater crocodile: A. Proctodeum and urodeum opened via a cut along the dorsal wall; B. Relative locations and ostia of oviducts and ureters; C. Oviduct opened to note the presence of the muscular folds in its caudal extremity. Cl – Clitoris; Oo – Oviducal ostia; Ov – Oviduct; Va – Muscular folds of the vagina; Uo – Utereral ostia; Ur – Ureter; Ut – Uterus. Scale bar – 1cm.
Artificial insemination

A clear plastic human speculum was used to access the urodeum and reflex the clitoris to allow for deposition of semen adjacent the openings of the oviduct (Figure 4.2), but it was not possible to clearly visualise these structures. While a sheep artificial insemination pipette (Minitube, Germany) was used to conduct the initial insemination procedures, it was also possible to deliver the inseminate in a similar location by means of a 3.5 Fr gauge tomcat catheter attached to a 3 mL syringe. Females inseminated without administration of Pavulon® showed little evidence of retrograde seminal loss, as the muscular tone of the cloaca resulted in the genital slit remaining tightly closed post insemination; retrograde loss was also minimised by tilting the tail so that the semen flowed under gravity towards the openings of the oviduct (Figure 4.2).

Two female reproductive tracts from sexually mature females were dissected for anatomical description (Figure 4.1) and attempts made to pass a “tom-cat” catheter into the openings of the oviduct. Even under these conditions, when the ostia of the oviduct could be clearly identified, we were unable to physically pass the catheter through the muscular folds of the vagina, thus suggesting that manipulation of an AI catheter through in the vagina of the live animal is going to be challenging.

![Figure 4.2: Artificial insemination in the saltwater crocodile A: Use of vaginal speculum and sheep AI pipette for artificial insemination, B: Tilting the crocodile tail post-insemination to reduce retrograde semen loss.](image)

Of the 11 females inseminated in 2011, only five crocodiles laid eggs. Of these, one female produced six fertile eggs (Figure 4.3), one of which successfully hatched. In the 2012 breeding season, a total of
12 crocodiles were artificially inseminated but only four animals laid eggs. Although 150 eggs were produced, only one was fertile and the egg failed to hatch.

Figure 4.3: Fertile eggs produced by an artificially inseminated female in 2011. Note presence of opaque banding on the fertile eggs.
The musculoskeletal system of the saltwater crocodile – a preliminary study using computer tomography and magnetic resonance imaging

The resultant CT and MR data enabled visual discrimination between different tissue types and in particular differentiation between bone, muscle, fat, and skin. The bone weighted CT data enabled 3-D reconstruction of the skeletal system and differentiation of the different bone elements comprising the post-cranial skeleton. Discrimination and 3-D model generation was also possible for the cranial skeleton but it was not possible to differentiate between the different bone plates which comprised the skull.

Although muscle tissue could be readily differentiated from other tissue types, the intimate association between the respected muscle masses prevented segmentation of the different muscle groups based solely upon segmentation by grey-scale value differences. In addition, it was possible to visualise the external morphology and row structure of the individual scutes that comprised the skin of the crocodile.

Skeletal Anatomy

In general, the skeletal anatomy of *C. porosus* was typical of a tetrapod, and an archosaur in particular (Figure 5.1).

The skull displays a characteristic flattened appearance with an elongated snout (Figure 5.2). The skull is formed by the fusion of 40 individual bone plates along a series of ossified suture lines. The pitted nature of the external surface of the skull was due to the fusion with the overlying integument (skin) and partially obscured the suture lines between the respective bone plates. The premaxilla, maxilla and dentary plates bore a total of 66 to 72 peg-like conical theocodont teeth (located in sockets), with a dental formula of 5 premaxillary, 13-16 maxillary teeth, and 15 dentary teeth on either side of the midline of the skull. The upper and lower teeth alternate at occlusion, with the 4th dentary tooth fitting into a lateral notch, located near the junction between the premaxilla and maxilla bone plates. The articulation of the jaw was located caudal to the atlanto-occipital joint.

In common with all crocodilian species, *C. porosus* has 26 pre-sacral vertebrae [comprising seven cervical (Figure 5.2), 12 thoracic (Figure 5.3) and five lumbar vertebrae (Figure 5.3)], the remainder of the vertebral column consisted of two non-fused sacral vertebra (Figure 5.4), and between 30-40 caudal vertebrae (Figure 5.5). Each vertebra, with the exception of the atlas, possessed neural spines located dorsally. The atlas consists of four individual bone pieces, and the odontoid bone of the axis was not fused to the main vertebral body. Chevron bones obliquely directed in a caudal direction were attached to ventral surface of each of the caudal vertebrae.

The cervical, thoracic and sacral vertebrae bore ribs (Figures 5.2 – 5.4). The ribs articulated against a single vertebra, and whereas the ribs of the atlas and axis possessed a single articulation facet, the remaining cervical and thoracic ribs bifurcated proximally, to form separate dorsal (tubercular) and ventral (capitular) processes, which articulated on facets located on the supporting vertebrae. The ribs of the first 3 thoracic vertebrae articulated against facets located on both the transverse process and body of the vertebrae (Figure 5.3). Conversely, the articulation facets for the remaining thoracic ribs were located on the transverse process of the vertebrae. The ribs, which were robust dorsally, gradually changed shape and become blade-like ventrally. The ribs connect to the plate-like sternum via cartilaginous structures, and the ribs were typically divided into three separate osseous segments by
the presence of cartilaginous material. A fibrous membrane, containing the most superficial abdominal ribs (gastralia) connected the sternum to the pelvic girdle (Figure 5.4).

Figure 5.1: 3-D computer model of the skeletal structure of a 1.2 m male crocodile (A) Dorsal view; (B) Ventral view. The model was generated using Mimics Innovation suite software from bone-weighted reconstructed CT data acquired at 120Kv, 150mA at a rotation time of 1sec. The model shows the characteristic archosaurial-like organization of the axial (cranial and post-cranial) and appendicular skeleton of *Crocodylus porosus* and highlighting the vertebral formula interpreted in this study as: C (Cervical – yellow and fuschia) = 5, T (Thoracic – sky blue and pale green) = 12, L (Lumbar – yellow and violet) = 5, S (Sacral - purple and pink) = 2, and Ca (Caudal - peach and turquois) = 38-40. Note the presence of cervical ribs (red) on the cervical vertebrae.
Figure 5.2: 3-D computer generated skeletal model of the head and neck of *Crocodylus porosus* in dorsal (A), ventral (B) and lateral (C) view, showing the characteristic flattened appearance of the skull, the rudimentary hyoid apparatus (*star*) and the presence of cervical ribs (*magenta*) on each of the 7 cervical vertebra, and the first thoracic vertebra (*cyan*). The first cervical vertebra (atlas) is composed of 4 individual bones (*pale green, magenta, red and green*). Note also the presence of osteoderms (*yellow*) on the dorsal surface, and the conical nature of the dentary (*orange*) and maxillary and sub-maxillary (*violet*) thecodont (peg-like) teeth.
Figure 5.3: 3-D computer generated skeletal model of the thoracic and abdomen region of *Crocodylus porosus* in dorsal (A), ventral (B) and lateral (C) view, showing the pectoral girdle comprising the scapula (*orange*) coracoid (*red*) and sternum (*magenta*) which unites the forelimbs to the axial skeleton. Note the articulation of the humerus (*blue*) at the union between the scapula and coracoid. The 12 thoracic vertebrae (*green and sky blue*) support the thoracic ribs. The thoracic ribs display a complex structural organization, comprising a robust base (*cyan*) axially, and 2 flattened blade-like abaxial elements (*purple*). The base of the rib has a dual articulation to a single supporting thoracic vertebra. The first 11 ribs are fixed (united to the sternum - *fuchsia*), whilst the 12th rib is free. Note also the presence of the *gastralia* or abdominal ribs (*mustard*) located on the ventral surface of the abdomen cranial to the tips of the epipubis (*magenta*). The five lumbar vertebrae (yellow and cyan) are rib-less.
Figure 5.4: 3-D computer generated skeletal model of pelvic region of *Crocodylus porosus* in lateral (A), dorsal (B) and ventral (C) view. The pelvic girdle is comprised of 6 separate osseous elements, Os *ileum* (cyan), the Os *ischium* (yellow) and the epipubic or marsupial bones (fuchsia). The pelvic girdle is connected to the 2 non-fused sacral vertebrae (*green and cyan*), via the robust sacral ribs (partially fused to the vertebral body). The Os *ileum* and Os *ischium* form an articulation complex (the acetabulum) by which the femur (*yellow-green*) of the appendicular skeleton of the hindlimb units with the axial skeleton at the hip joint. Note also the structural organisation of the *gastralia* or abdominal ribs, located ventrally, which are comprised of an axial (cyan) and abaxial (yellow) element, the presence of osteoderms dorsally (grey).
Figure 5.5: 3-D computer generated skeletal model of cranial region of the tail of *Crocodylus porosus* in ventral (A) and lateral (B) view, showing the organisation of the caudal vertebrae (orange and cyan) and the presence of transverse processes on the first 15 caudal vertebra, which diminish in relative size caudally. Note also the presence of the chevron bones (*purple*) on the ventral aspect of each caudal vertebral body.
Figure 5.6: Figure 6 (A, B): 3-D computer generated skeletal model of pectoral (fore) limb (A) and the pelvic (hind) limb. The limbs display a pentadactyl (5-digit) structure, although digit 5 is vestigial in the hindlimb. The pectoral limb comprises the humerus (yellow), radius (cyan) and ulna (magenta) in the upper arm, the carpus contains 4 osseous elements, the fused radius-intermediate carpal bone (orange) and carpal bone 4-5 (sky blue), the ulna carpal bone (red), the accessory carpal bone (blue). The manus (hand) comprises 5 metacarpal MC bones (MC1 - pink MC2 - violet MC3 - magenta MC4 - green MC5 - fuchsia), each supporting a variable number of phalanges. The upper pectoral limb is formed by the femur proximally (magenta), and the tibia (cyan) and the fibula (magenta) distally. The tarsus comprises 4 osseous elements, the calcaneous (red), the talus (orange) talus bone 3 (blue), and tarsal bone 4 (magenta). Distal to the tarsus, the pes (foot) comprises 5 metatarsal (MT) bones (MT1 - cyan MT2 - pink MT3 – violet, MT4 – magenta, MT5 - yellow-green). MT 1-4 respectively support a variable number of phalanges.

The expansive ribs of the sacral vertebrae formed a robust structure, which connected the vertebral column to the pelvic girdle. The pelvic girdle was formed by 6 separate bone elements, the paired
ileum and ischium and pre-pubis or marsupial bone (Figure 5.4). The ilium and ischium united on either side of the body to form the articulation fossa (acetabulum) of the hip joint, through which the hindlimb (pectoral limb) was linked to axial skeleton. The pectoral girdle was formed by five bone elements, the sternum, and the paired scapular and coracoid, with the plate-like scapular and clavicle united to form the articular fossa (glenoid cavity) of the shoulder joint on either side of the body, by which the forelimb (pectoral limb) was attached to the axial skeleton (Figure 5.3).

The hindlimb was approximately twice as long as the forelimb, with the long bones of the proximal hindlimb limb (femur, tibia and fibular being larger than the corresponding bones of the proximal forelimb (humerus, radius and ulna) (Figure 5.6). The ankle or tarsus joint contained four bone elements arranged into two rows. The proximal row contained the calcaneous, and talus, and the distal row, comprised the 3rd and 4th tarsal bones. The wrist or carpal join contained four ossified bones, a proximal row comprising of the fused radial-intermediate carpal bone, the ulnar carpal bone, and the accessory carpal bone, and a distal row consisting the fused 4th and 5th carpal bone. The tarsus and carpus articulated respectively with the metatarsal and metacarpal bones and the phalanges of the digits. The hind foot (pes) had only 4 digits (digit 5 was absent – the vestigial 5th metatarsal carried no phalanges) with claws appearing on digit 2, 3, and 4. Conversely, the front foot (manus) had five digits, with digit 1, 2, and 3 each bearing claws. In addition, a series of bony osteoderms were present on the dorsal aspect of the body (Figure 5.2 – 5.4). These were arranged in discrete rows, and were intimately associated (fused) with the most prominent integumentary scutes. In general, the osteoderms decreased in size caudally. There were no osteoderms evident within the scutes that comprised the ventral integument.

Skeletal Muscles – Overview

Visualization of individual muscle groups by use of CT scanning protocols typically used for clinical veterinary examination was not achievable. However, by using ‘high resolution’ scan protocols of reduced slice thickness, lower pitch factor, and increased rotation time, it was possible to resolve skeletal muscle tissue, and obtain insight and understanding of muscle distribution within the body. Indeed, this high resolution scans, in conjunction with MR data, enabled accurate identification of several skeletal muscle groups (Figures 5.7 – 5.9). These included several key muscle groups, which constitute the principle commercial meat cuts taken from the crocodile carcass. In addition, individual muscles (sub units of the muscle groups) were recognised, and their fibre orientation, structural architecture, and tendon insertions onto the skeleton elucidated.

Muscle of the Skull

The mandibular adductor muscle group (MAM) was observed occupying the internal space between the mandible and the cranium, extending into the infra-temporal fossa, suborbital fossa, and the internal mandibular fossa, and extending caudal to the skull. It was possible to identify several of the individual muscles within the MAM, based upon differences in fibre orientation between adjacent muscles, and the presence of discernible muscle fascicles. Of particular note were M. pterygoideus ventralis (a member of the internal MAM muscles - MAMI) and the M. depressor mandibulae (a member of the posterior MAM muscles - MAMP); the two muscles that collectively form the jowl cut of meat. These muscles could be seen originating from laterosphenoid and quadrate cranial skull bones respectively, and inserting respectively onto the retroarticular and the angular and surangular mandibular bone plates. An extensive fat depot, one of few depots evident within the crocodile carcass, surrounded these muscles posteriorly. Other discernible MAM members included MAMIPtd, M. pterygoideus dorsalis running caudally from the suborbital fossa, maxilla, prefrontal and orbitonasal cranial skull bones and also individual members of the hypoglossal muscles (e.g. M. hyoglossus and M. genioglossus). It is important to note, however, that not all muscles could be adequately visualised. This was especially the case where fiber orientation of adjacent muscles was similar and where a marked dividing fascicle was absent. In addition, occasional ‘streaking’ artifacts arising from the thicker cranial bone plates masked muscle fiber orientation and prevented discrimination with certainty.
Figure 5.7: Image montage of the proximal region of the tail illustrating the inherent capabilities of the high resolution scanning protocol developed and employed in this study to visualise the musculoskeletal organisation of *Crocodylus porosus*. (A) Reference photograph showing the dorsal aspect of the proximal region of the tail spanning caudal vertebrae 1-15 inclusive. (B) Photograph of the same region with skin removed to show the epaxial musculature (M. tendinoarticularis located axially, and the larger M. longissimus located abaxially) of the proximal tail region. Note the compact nature of the muscular organisation, the characteristic pattern of the associated tendon (seen axially), which extends in a caudodorsal or craniodorsal fashion from the individual muscle elements to the dorsal spines of the caudal vertebrae and/or to the overlying osteoderms, and the oblique orientated fascia (seen abaxially) which divide the myosptal elements of the epaxial muscles. (C) 2-D transverse CT image acquired ventral to the caudal spine at the level of the ventrally located chevron bones showing the organisation and inter-relationship between the epaxial tail muscles seen abaxially, the epaxial M. caudofemoralis group located axially, the intervening fat depot (hypointense signal intensity, and the extrinsic muscles of the hindlimb in the pelvic region. (D) 2-D sagittal CT image acquired through the transverse processes of the caudal vertebrae showing the muscular organisation of the epaxial muscles of the proximal tail and the hypaxial M. caudofemoralis group, and the intervening fat depot (hypointense signal intensity). Note the progressive decrease in the size M. caudofemoralis group caudally. (E) 2-D sagittal CT image acquired immediately adjacent to the dorsal processes of the caudal vertebrae, showing characteristic dorsocranial orientation of tendons emanating from the individual elements of the epaxial M. tendinoarticularis muscle group, which insert onto the dorsal process of the caudal vertebra cranial to the muscle. (F) 2-D transverse CT image acquired at the level of the dorsal process of the caudal vertebrae showing the characteristic organisation of the tendons (thin hyperintense linear structures) located within the axially located epaxial muscles of the tail.
Figure 5.8: Image montage of the proximal region of the tail of *Crocodylus porosus* showing direct comparison between the 2-D axial slices acquired using the high resolution CT scan protocol employed in this study (right) and corresponding dissection slices (left) at 5 locations taken between caudal vertebra 1 and 15 (indicated in the reference photograph of the skinned tail A-E). There is excellent correlation between the anatomical disposition and structural organisation of the epaxial muscle groups (*M. tendinoarticularis* located axially, and the larger *M. longissimus* located abaxially), the hypaxial muscle group (*M. caudofemoralis*) and the characteristic distribution of fat depots at each location within the crocodile tail. Note the progressive reduction in cross-section size of the *M. caudofemoralis* from caudal vertebra 5 (B) to caudal vertebra 12 (D), and its absence by caudal vertebra 15 (E).
Figure 5.9: Image montage of the abdominal region of *Crocodylus porosus* to show direct comparison between high resolution CT images using the scan protocol developed in this study (right) and T2 weighted MR images (left) at 6 different locations within the abdominal region. The comparison shows good correlation both in muscular organization and anatomical disposition of the epaxial muscles of the back, the hypaxial muscles of the back, the intercostal muscles, and the abdominal muscles. Note that whilst the MR images provide good resolution of the myoseptal divisions within the axially located elements of the epaxial muscles, the corresponding CT images gives detail of the fine organisation of muscle fibres within the muscle groups.
Muscles of the Neck

Both the hypaxial and epaxial musculature of the neck were discernible, immediately surrounding the cervical vertebra; the former constitutes the neck fillet cut. The hypaxial muscles are separated from the ‘jowl’ muscles (M. pterygoideus ventralis and M. depressor mandibulae), by a marked fascicule cranially and the fat depot at the caudal limit of the jowl muscles. Caudally, at the junction between the cervical and thoracic vertebra (in the region of the pectoral girdle), the neck muscles are separated from the extrinsic muscles of the forelimbs by a second fat deposit of variable size, located axial to the coracoid and scapular. The neck muscles comprised several groups with individual muscles of differing fiber orientation. The junctions between the individual muscles, which are relatively indistinct cranially, become more pronounced caudally, where the epaxial muscle groups M. transversospinalis and M. longissimus dorsi can be readily identified. However, it was not possible to discriminate the individual subunit of these muscle groups. The hypaxial muscles, which are expansive cranially, taper progressively caudally, and appear in the region of the pectoral girdle as two cylindrically shaped muscle masses situated adjacent to the ventral spinous process of first thoracic vertebrae. There was good correlation between the musculature evident in transverse section of the high resolution CT scans and that seen in gross dissection specimens.

Muscles of the Back

The muscle groups comprising the epaxial muscles of the back were discernible along the entire length of lumber and thoracic spine. Axially the M. transversospinalis group was observed situated immediately adjacent to the dorsal spinous process, occupying the axial third (approximately) of the length of the transverse process of the thoracic vertebra. It was not possible to discriminate between the individual muscles of this group on the basis of either difference in muscle structure, fibre orientation and/or dividing fascicule. This muscle group exhibited a distinctive pattern of ligament alignment and attachment to the vertebral column and/or the overlying osteoderms. Immediately axial to the vertebral body, ligaments were orientated in a craniodorsal direction, which inserted on the spinous process of the 3rd vertebra in advance of the ligaments origin. Abaxial to this, ligaments were aligned in a caudodorsal direction inserting on the spinous process five vertebra caudal to the origin of the ligament. Abaxial to this, ligaments were aligned in a craniodorsal direction, inserting on the spinal process five vertebra cranial to the ligament origin, and also, on the subjacent osteoderm within the integumental scute.

The M. longissimus dorsi was located immediately abaxial to the M. transversospinalis group, occupying to abaxial 2 thirds of the transverse process of the transverse process of the thoracic vertebra. The M. longissimus dorsi displayed a characteristic structure of a concentric array of myosepta, which decreased in diameter caudally. Parallel to the spine, the muscle septa were of chevron appearance, orientated in a caudal direction.

A third epaxial muscle group, the M. iliocostalis is located immediately abaxial to the M. longissimus dorsi. This muscle group extended ventrally, in axial section, from the articulation between rib and transverse process of the vertebra along the length of the proximal portion of the rib. This muscle group, which is expansive at its site of origin, the crest of the Os ilium, markedly decreased in absolute size cranially.

Ventral Muscles of the Trunk

There was a discernible boundary between M. iliocostalis and the adjoining hypaxial intercostal muscle groups. The intercostal muscles formed a relatively narrow band linking and surrounding the sternal and asternal ribs, extending ventrally to the sternum and xiphoïd cartilage respectively. The intercostal musculature was located deep to a relatively well developed layer of superficial muscle, comprising the proximal part of the abdominal muscles caudally, and caudal and ventral elements of the extrinsic musculature of the forelimbs cranially. Intramuscular fat deposits marked the division between the intercostal and the overlying superficial muscles.
Muscles of the Tail

High-resolution scans were able to discriminate a number of the muscle groups, which comprise the musculature of the tail. Specifically, the epaxial *M. tendinoarticularis*, (located axially), and the larger *M. longissimus* (located abaxially) groups, and also, the hypaxial *M. caudofemoralis* group, (a major extrinsic muscle of the hindlimb). In common with the muscle architecture of the back, these muscles displayed a distinct pattern of conical myosepta, appearing as a series of concentric circles in transverse section and a chevron pattern in longitudinal section. The myosepta of both the *M. longissimus* and the *M. caudofemoralis* groups were aligned in a caudal direction, whereas those of the *M. tendinoarticularis* group were aligned cranially. The relative development (cross sectional size) of the *M. longissimus and M. caudofemoralis* was highly dependent on the actual position along the caudal vertebra. Conversely, the cross sectional size of the *M. tendinoarticularis* remained fairly constant along the length of the vertebral column. The cross sectional size of the *M. longissimus* group relative to the *M. tendinoarticularis* increased rapidly in the caudal direction, reaching its maximal relative development by caudal vertebra 5-6, and then decreased progressively in its relative size along the length of the tail. Likewise the *M. caudofemoralis* group increased rapidly in size caudally. This muscle group attained its maximal cross-sectional size by caudal vertebra two and decreased rapidly thereafter, terminating at caudal vertebra 12-13. The tail was also characterised by the presence of extensive intramuscular fat deposits most noticeable that which surrounded *M. caudofemoralis* along its entire length, and also subjacent to the dorsal process of the vertebra. The intramuscular fat, caudal to the termination of the *M. caudofemoralis* was located immediately adjacent to the dorsal process of the vertebra, and the chevron bones ventral to the body of the vertebra.

Intrinsic Muscles of the Fore and Hindlimb

The intrinsic muscle groups showed extensive development, most notable within the proximal region of the fore and hindlimb, with only a few small muscle groups present within the distal aspect of the limb (crus and manus, and tarsus and pes respectively). Differences in fibre orientation within the proximal region of the limb enabled discrimination of several individual flexor and extensor muscle groups, most notably in the larger hindlimb specimens. There was no intramuscular or subcutaneous fat deposits present within either the hindlimb or forelimb scans.

The Integument

Although this study did not specifically aim to investigate the integument of the crocodile, the resultant scans gave excellent visualisation of the skin, which allowed excellent 3-D reconstruction. The resultant 3-D models gave fine detail of the scute organization, scute size and distribution pattern for the entire body. In addition, the models permitted easy identification of those scutes associated with underlying osteoderms.
Discussion of Results

Objectives of the project:

1. 3-D MRI anatomy of sexually mature male and female crocodile
2. Reliable and safe technique for collection of crocodile semen
3. Development of methods to assess crocodile semen

Crocodilians, like chelonians, have single penis (or phallus) rather than hemipemi (Jacobson, 2007). Similar to what has been noted in other crocodilians (Moore et al., 2012; Kelly, 2013), the saltwater crocodile penis arises from the ventral wall of the proctodeum. To our knowledge, there has been no illustrative evidence of the opening point of the ductus deferens, although this information is important for the purposes of semen collection. The results we have presented in this paper clearly illustrate the exit position of the ostia of the ductus deferens as a distinctive papilla opening into the sulcus of the penis. We propose that during semen collection, we are essentially “expressing” or “milking” semen by digital palpation along the length of the distal ductus deferens and into the sulcus of the penis. We have also described an unusual pair of procotodeal papillae located either side of the base of the penis on the ventral wall of the proctodeum; these papillae were ductless but appeared to communicate with the abdominal cavity. The function of these structures remains to be determined.

We have also described a cavity formed by the glans penis that appears to be supported by a region of underlying well-developed vascular tissue. Presumably, when blood is supplied to this vascular space during erection, the cuff of the glans penis inflates (see Figure 2.4A). Kelly (2013) has suggested that erection of the alligator penis does not change its shape or bending stiffness as blood enters the vascular spaces of the penis. While such a phenomenon needs to be examined in the shaft of the penis of the saltwater crocodile, our initial observations revealed significant changes in the size and shape of the morphology of the glans penis during erection. Although the functional significance of this vascular tissue during copulation requires further investigation, we propose that the inflated cuff is associated with forming a copulatory seal within the proctodeum of the female to lock the glans penis into position, reduce retrograde loss of semen and potentially prevent the mixing of semen with water (salt or fresh), which is likely to be potentially spermicidal.

The results of this investigation have demonstrated that semen samples from the saltwater crocodile for the purposes of fertility evaluation can be readily and reliably collected (30/31). Larsen et al. (1984) have suggested that an adequate insemination dose for the American alligator is in the region of 200 – 300 million sperm cells; we therefore propose that some of the semen samples collected in this study should be of sufficient quality and quantity for the purposes of artificial insemination (AI). Previous semen collection attempts from live crocodilians have focussed on the aspiration or scraping of spermatozoa from the sulcus (Alligator mississippiensis - Cardeilhac et al., 1982; Larsen et al., 1996; Caiman latirostris - Larsen et al. 1992), but have not typically yielded adequate semen for AI, and in some cases the procedure has inadvertently resulted in penile trauma and blood cell contamination of the semen sample (Larsen et al. 1992).

In the current study, we often observed the viscous white semen already located in the sulcus of the penis prior to manual stimulation, so that it would require only gentle massage to cause the sample to “drip” under gravity from the terminal portion of the glans penis directly into the collection vessel. However, extreme care needs to be observed when initially extruding the penis from the proctodeum, in case manipulation facilitates the precipitous release of urates and faeces from the coprodeum and results in the contamination of the “clean” semen sample. This is a phenomenon which may be exacerbated when the crocodile is sedated. The ease and reliability by which semen was collected in the salt water crocodile in this study was surprising given that it has been somewhat problematic in
other crocodilians (Larsen et al., 1984); this might be accounted for by the fact that previous studies have not used physical manipulation of the ductus deferens via the urodeum to facilitate the flow of semen. While the amount and quality of semen is clearly going to be influenced by a range of factors, including breeding season, nutrition and general husbandry, it was encouraging in our study that semen could be recovered from all but the smallest male, who measured 185 cm. It should be noted that a small volume of semen was successfully obtained from an animal regarded by the crocodile farm as a juvenile male measuring only 194 cm; this suggests that male saltwater crocodiles around 200 cm could possibly be used as semen donors, at least in the peak of the breeding period. While semen collection by electroejaculation can be used to recover crocodile spermatozoa, the close proximity of the kidney and ureter to the testis and ductus deferens means that non-specific electrical stimulation could result in contamination of the semen sample with urates. Larsen et al. (1983) have also suggested that electroejaculation is not an appropriate method of semen collection for the alligator. Given that crocodiles do not possess a bladder and appear to store urine in the rectum (O’Malley, 2005), it is not surprising that insertion of the electroejaculation probe resulted in the release of waste products; perhaps if the rectum was purposely voided in advance of electroejaculation, then this technique of semen collection may be of more practical use.

If the male crocodile is able to tolerate repeated capture and sedation, it should be possible for semen to be collected regularly without any adverse effects; in our study we were able to re-collect semen from one male after only a period of 13 days. The rate-limiting step in our procedure was the initial capture of the animal (which can be unpredictable) and the depth and timing of appropriate sedation to immobilise the crocodile for safe handling and ease of manipulation. Once the crocodile was stabilised and positioned for the procedure, the majority of semen samples were obtained within 15 – 30 mins. The development of future semen collection procedures needs to be conducted in parallel with studies that allow safe crocodile handling, an appropriate depth of sedation or anaesthesia, and reduce the overall stress and impact of the capture procedure to the crocodile. Olsson and Phalen (2012) have recently reported a promising preliminary study on the immobilisation of adult saltwater crocodiles (305 cm and 460 cm) using medetomidine for at least 40 mins and reversal with atipamezole that might prove to be ideal for use with semen collection and artificial insemination.

The semen sample collected by massage from the saltwater crocodile is a white viscous fluid, which appeared to have an almost hydrophobic quality. While no physical measurements of viscosity of the semen sample were investigated, there were a number of occasions when semen pooling in the sulcus of the penis came in contact with clear, less viscous fluid released from the cloaca; it is possible this fluid is associated with exocrine glands or lymphatic aggregates located in sulcus previously noted by Moore et al. (2012) in the American alligator penis. Under these circumstances, the clear fluid appeared to be repelled by the neat semen and mixing of the fluids was prevented. The hydrophobic properties of crocodile semen need to be further investigated but may be associated with adaptation to copulation and delivery of semen into the female in the water column or for sperm storage in the female reproductive tract. The viscous nature of semen was often apparent when waiting for the semen sample to drip from the penis into the collection vessel, and may have on occasion potentially affected the quality of the semen sample by being overly exposed to air.

It is unlikely that the volume of semen collected by manual manipulation (mean – 0.9 mL) in this study was representative of the natural ejaculate, but it is difficult to envision how this parameter could be accurately determined. Similar problems occur when estimating ejaculate volume from electroejaculation procedures. John Lever (personal observation) has witnessed copulatory behaviour in crocodiles at Koorana Crocodile Farm and noted that dismount of the male is often associated with large plumes of white fluid in the water column, however this could be urates and/or faecal material voided following the mating activity. The erect engorged glans penis and tip form a hollow “cuff” which presumably fits in close apposition with the female clitoris during copulation potentially forming a tight seal during intromission preventing contamination of semen with water; it is possible that semen is deposited by the male at the base of the oviducal ostia within the proctodeum in relatively small volumes, especially given that sperm concentration is so high and sperm storage has been reported to occur at the utero-vaginal and tubal-isthmus junction of the American alligator (Gist
et al., 2008). Moore et al. (2012) has also recently described the histology of the alligator penis and noted the presence of muscularis mucosae associated with the sulcus, that maybe responsible for closing the sulcus into a tube during erection as the penis inflates, thereby facilitating movement of semen along the sulcus.

Estimates of seminal pH (mean – 7.3) and osmolality (mean - 335.5 mOsmKg⁻¹) described in this study are possibly the first for any reptile, as these values were determined directly from neat undiluted crocodile semen. While these estimates are unremarkable, they are also fundamentally similar to the physiochemical parameters of previously prepared alligator semen diluents as described by Larsen et al. (1984).

The sperm concentration of the salt water crocodile ejaculate (0.35 – 4.0 X 10⁹ mL⁻¹) was generally similar to that described for other crocodilians such as A. mississippiensis [1.8 x 10⁹ total sperm cells collected - Larsen et al., 1982; 0.3 – 0.88 X 10⁹ total sperm cells collected - Larsen et al., 1996] and C. latirostris [0.2 – 1.1 X 10⁹ total sperm cells - Larsen et al., 1992]; because of the way the sperm cells were collected by Larsen and Colleagues it was not possible to report sperm concentration as sperm per millilitre. Semen collected manually from Angolan Ptheyon (Python anchiatae) and the Timor Python (Python timoriensis; Mengden et al. 1980) and a Brazilian rattlesnake (Crotalus durissus terrificus; Zacariotti et al., 2002) all had a sperm concentration in order of 1.5 x 10⁹ mL⁻¹. In the current study, it was possible to recover semen from a large 400 cm male crocodile with a seminal volume of 3.3 mL and corresponding sperm concentration of 4.0 x 10⁹ mL⁻¹, which represented a total sperm number of 13.2 x 10⁹. This amount of spermatozoa was higher than that collected directly by dissection from the alligator ductus deferens (8.6 x 10⁹ - Larsen et al., 1982). A sperm concentration of 4.0 x 10⁹ mL⁻¹ is at the upper limit of most domestic animals (Glover, 2012), but similar to that found in poultry (4.0 – 9.5 x 10⁹ mL⁻¹; Etches, 1996).

The mass activity, gross motility or wave motion of the semen sample is a subjective estimate used in some domestic animals to provide a combined measure of sperm density and motility. Typically, it is only used in those species that produce sufficiently concentrated sperm where “eddies” of movement can be dedicated under low power magnification without use of a coverslip (Evans et al., 1987). While one crocodile ejaculate had a mass activity score of 3.5 (65% motility and 2.5 x 10⁹ mL⁻¹), the potential use of this parameter in crocodile semen evaluation needs further validation, but in theory, it could be a rapid method of semen density and motility, as it is in ungulates.

Motility of crocodile spermatozoa varied from 0 – 85% and the rate of motility from 0 – 4. The mean motility of the salt water crocodile spermatozoa observed in this study (50.7%) appeared to be substantially less than reported for semen collection in American alligators (Larsen et al., 1982; Larsen and Cardeilhac, 1996) and the Broad-nosed caiman (Larsen et al., 1994). This could simply be a reflection of the general fertility of saltwater crocodile population at Koorana Crocodile Farm but may also be related to the way the semen was collected. Previous authors have collected semen directly in extension media, where semen collection of the salt-water crocodile in this study was often associated with a semen bolus or droplet that would hang from the penis and be exposed to the air for a few minutes before falling into the collection vessel. While motility analysis was assessed after dilution of semen into BEST extender with 20% egg yolk, it is possible that the semen may have been adversely affected by air exposure before falling into collection media. In our initial observations of semen evaluation, we compared sperm motility in extenders with and without egg yolk, but quickly abandoned analysis without egg yolk, as sperm motility appeared to be suppressed without egg yolk in the diluent.

Further analysis of alternative media for sperm motility analysis is necessary, as is a better understanding of general crocodile sperm physiology, so that standard protocols for motility analysis can be developed. For example, given that crocodiles are thought to store spermatozoa in the female reproductive tract (Gist et al., 2008), an analysis of motility of recently ejaculated spermatozoa may not necessarily be representative of normal sperm function. There also needs to be parallel studies that examine the effect of temperature on sperm motility; while sperm were analysed at 30°C in this study,
sperm metabolism (and motility) is no doubt temperature dependent and could equally be affected by rapid changes in temperature.

This study appears to be the first use of SYBR-14 and PI for the assessment of plasma membrane integrity of reptilian spermatozoa using epifluorescence microscopy, and was preferred over the use of nigrosin-eosin for live-dead assessment. Although the sperm plasma membrane integrity ranged from 3 – 97%, the mean percentage of live sperm was approximately 80%, substantially higher than the mean percentage motility (50%). These observations suggest that factors other than damaged membranes are important for controlling crocodile sperm motility. The SYBR-14/PI technique will be extremely useful for future studies examining sperm osmotic tolerance, sperm storage and cryopreservation.

Although Larsen et al. (1982) noted the presence of morphologically abnormal alligator spermatozoa, there was no detailed description or quantification of these cell types. Fahrig et al. (2007) has commented on sperm abnormalities found in the semen of corn snakes (Elaphe guttata) noting the occurrence of sperm with proximal and distal droplets, detached heads, folded and coiled tails and abnormal head shapes, but unfortunately no micrographs of these sperm morphologies were provided. Essential to the establishment of a standardised spermatogram in the salt-water crocodile is the characterisation of what represents normal and abnormal sperm morphology. For the first time in a crocodile, we have noted and quantified a range of sperm morphological abnormalities, similar to what has been found in most domestic species (Barth and Oko, 1986) and humans. Given the seasonal nature of breeding activity in the saltwater crocodile, there is likely to be seasonal changes in the production and quality of spermatozoa reflecting changes in endocrine and testicular function, so these relationships will require further investigation. Equally, reproductive disease or other factors (for example, nutrition and stress) impacting on male factor fertility will be reflected in assessments of sperm quality.

Of particular interest in the ejaculate of saltwater crocodile semen was the very high incidence of spermatozoa with what appeared to be cytoplasmic droplets. While transmission electronic microscopy is required to ascertain whether these structures are in fact residual cytoplasm left over from spermogenesis, such structures have previously been reported in the turtle (Miranti et al., 1964; Kaplan et al. 1966; Gist et al., 1992) and snake (Esponda and Bedford, 1987; Fahrig et al., 2007). The significance of the presence of cytoplasmic droplets associated with crocodile sperm needs clarification as its retention in mammals has previously been linked to reduced fertility (Cooper, 2005). Gist et al. (1992) note that the spermatozoa of the painted turtle (Trachemys scripta) become detached from the sperm midpiece in a coordinated manner shortly before the commencement of autumn mating and was not observed on sperm recovered from the oviduct of females, suggesting that the loss of droplet may be associated with the final maturation of turtle spermatozoa. It will be important for assessment of crocodile sperm fertility to understand whether the high proportion of cytoplasmic droplets in the ejaculates of some individual crocodiles in this study (85%) is evidence of poor sperm maturation or simply a normal aspect of its maturation. The strategic phylogenetic position of the crocodile, make it an ideal species to investigate the evolution of sperm maturation in vertebrates.

While the therapeutic addition of antibiotics (penicillin G sodium; streptomycin sulphate and gentamicin sulphate) in alligator semen extenders has previously proven useful in preventing bacterial growth without evidence of any adverse effect on sperm survival during short-term preservation at 0°C (Larsen et al. 1984; Larsen and Cardeilhac, 1996), it was highly likely that the microflora of saltwater crocodile semen and cloaca was going to be different. The culture of microorganisms and fungi from the saltwater crocodile penis, sulcus and semen resulted in the detection of a broad range of species, many of which are likely to be faecal and/or environmental contaminants (Huchzermeyer, 2003). Apart from Enterococcus faecium, all cultured microorganisms that could potentially contaminate the semen sample during collection and extension, were sensitive to gentamicin. While the E. faecium was sensitive to sulphadimethoxazole/trimethoprim and amoxicillin/clavulanic, the cytotoxicity of these antimicrobial compounds on crocodile sperm survival still needs to be investigated.
As part of the semen collection procedures in the crocodile, this study also provided an opportunity to examine a large number of males of different ages and condition. One male presented with a prolapsed penis resulting in significant granulation of the glans penis and an inability to withdraw the penis back into the proctodeum. It was likely that this crocodile had this condition for a moderate period of time, and this pathology would have clearly interfered with its ability to achieve successful intromission. While the sulcus of this male had adhered, preventing the flow of semen to the glans penis, it was still possible to recover a sample that had pooled proximal to the adhesion; this sample could potentially be used for artificial insemination and demonstrates the importance of the procedure for genetic exchange of incapacitated males.

4. Chemical media for the preservation of crocodile semen

5. Protocols for the reliable cryopreservation of crocodile semen

The results of this study represent the first systematic investigation of the physiochemical tolerance of any reptilian spermatozoon and revealed that crocodile spermatozoa:

1. Are capable of surviving short-term manipulation and extension in a simple PBS diluent without egg yolk (ironically the BEST diluent is not the best diluent);
2. Can be diluted up to 1:16 in PBS before showing any adverse effect on sperm survival;
3. Are tolerant of rapid changes in temperature (no cold shock) without the need for any egg yolk protection of the plasma membrane;
4. Are not beneficially affected by the addition of up to 20% V/V egg yolk for both short preservation at 30°C or chilled preservation (4°C);
5. Show a remarkable ability to tolerate the impact of extreme hypotonic conditions with respect to plasma membrane integrity but not motility;
6. Show a rapid loss of motility but not plasma membrane integrity when exposed to high concentrations of cryoprotectants during the equilibration phase of cryopreservation; and
7. Show no or little post-thaw motility but moderate levels of plasma membrane integrity after cryopreservation.

Larsen et al. (1984) conducted a study to examine the suitability of 24 different extenders but the basic constituents of the buffer contained a combination of BES and TRIS; these studies determined that sperm survival following chilled storage (5°C) was highest in media which contained 20% V/V egg yolk, 20% powdered skim-milk and antibiotics. In order to examine the effect of serial dilution of crocodile seminal plasma, temperature shock, the effect of egg yolk and cryoprotectant concentration on sperm survival, we examined whether a simple medium, such as phosphate buffered saline (PBS), could be used as a standardised control extender. Sperm motility (% motility and the rate of sperm movement) and the percentage of sperm with intact plasma membranes was similar in the PBS and BEST with 20% egg yolk extenders, but sperm motility in the TRIS extender was significantly reduced; a phenomenon which surprisingly was not observed for plasma membrane integrity. Despite similar buffering capacities and osmolalities, there was a clear difference in the way the spermatozoa responded in TRIS with respect to motility and the plasma membrane. This same effect was also apparent if sperm were incubated for 2h; while there was a significant reduction in motility, particularly in the TRIS extender, the percentage of sperm with an intact plasma membrane did not decline over the same time period.

Once we had established that sperm were capable of being maintained in the PBS extender, we could then examine the effect of serial dilution. Excessive dilution of semen results in the removal of sperm surface components and changes the permeability of sperm membranes leading to sperm senescence (Watson, 1990; Johnston et al., 2000). Although there was some individual animal variability, crocodile spermatozoa incubated at 30°C for 2h retained their percentage motility up to a 32-fold dilution but the rate of movement was more sensitive, declining after an 8-fold dilution; plasma membrane integrity was reduced after 32-fold dilution. The rate and plasma membrane integrity of neat undiluted semen incubated at 30°C was also significantly lower than that of extended
spermatozoa. Larsen et al. (1984) have suggested that an adequate insemination dose for the American alligator is the region of 200 – 300 million sperm cells. Based on the mean total sperm ejaculated from saltwater crocodiles in this study (volume x sperm concentration = 4.8 x 10^9 mL^-1), and assuming that a similar total number of sperm are also required in the crocodile inseminate, a 1:16 dilution of the neat ejaculate would yield 16 X 1mL inseminates of approximately 300 x 10^6 spermatozoa. An extension of the ejaculate by 16 fold has little adverse effect on sperm survival, so that if insemination was 100% successful, then it would represent a 16 fold increase in reproductive output per male.

The results of the simulated rapid temperature change experiment in this study appear to be the first reported for any reptile and clearly illustrate the high tolerance of the crocodile spermatozoon to a rapid change in temperature both in terms of sperm motility and plasma membrane integrity. While there was a significant decline in the percentage of spermatozoa with damaged plasma membranes following exposure to cooling, this reduction was only marginal and suggests that egg yolk is unlikely to be necessary in crocodile sperm extenders used for refrigerated or cryopreservation. Our observations also suggest a high degree of fluidity of the lipid and protein domains of the plasma membrane in order to cope with temperature induced phase transitions; future studies using a controlled temperature cooling stage and fluorescent markers such as fluorescent-labeled cholera toxin B and annexin V should help reveal any evidence of respective translocations of ganglioside GM1 or phosphatidylserine across the plasma membrane (Zee et al., 2007). Most estimates of mammalian sperm motility are typically conducted on a warm-stage set at 37°C but what temperature should reptile sperm be examined? In this study we chose 30°C for our estimates of motility but Larsen et al. (1984) conducted analysis of alligator sperm at 25°C. There is a need for further investigation as to what regulates sperm metabolism and motility in the crocodile, particularly as there is evidence of sperm storage in the female American alligator (Gist et al., 2008).

Egg yolk is commonly used in sperm cryopreservation protocols of many domestic and exotic mammals, and is thought to provide protection against cold-shock (Holt, 2000). The active beneficial constituent of egg yolk is a low-density lipoprotein, but the exact mechanism of its action is controversial. Holt (2000) indicates that there is no evidence for a direct modulation of the sperm plasma membrane lipid phase transition behaviour by egg yolk and that egg yolk more likely binds to the cell surface of the sperm membrane, modifying membrane permeability and activating cellular adenylate cyclase; mechanisms that in turn might affect the sperm cell’s ability to cope with changes in osmolality and permeating cryoprotectants. Larsen et al. (1984; 1992) have suggested the use of 20% V/V egg yolk in semen extenders for the American alligator and Broad-nosed Caiman respectively but the rationale for its addition has not been justified. Using PBS as the control buffer, we systematically investigated the effect of egg yolk in a dose dependent manner when crocodile sperm were incubated at 30°C for 1h, or 4°C for 24h. The results of this experiment revealed no beneficial effect of egg yolk on the survival of crocodile sperm at either incubation temperature; consequently, given the potential complication of introducing disease from biologically derived substances (Holt 2000) and the results of experiment 2, we recommend that that egg yolk not be used for saltwater crocodile sperm preservation.

While the response of spermatozoa to the damaging effects of osmotic stress during cryopreservation can be modelled artificially by exposure of the cell to a range of osmotically defined media (e.g. Curry and Watson 1994; Meyers 2005; Willoughby et al. 1996; Johnston et al., 2006; 2012) the results reported in this study appear to be first observations for reptile spermatozoa. Crocodile spermatozoa were exposed to a broad range of osmotic media (25 to 1540 mOsmKg^-1) followed by the return of the spermatozoa to a solution similar in tonicity to crocodile seminal plasma. Sperm motility was maintained after the first osmotic excursion in the range of 220 to 390 mOsmKg^-1 but significantly and progressively declined in hypo- and hypertonic media outside this range. Interestingly, sperm exposed to the 50, 90, 150 and 880 mOsmKg^-1 media showed a significant increase in sperm motility after their return to isotonic media but did not recover fully to the level of motility observed in 280 or 390 mOsmKg^-1 media, thereby indicating that some permanent damage had been inflicted on mechanisms responsible for motility. Spermatozoa exposed to PBS measuring 220 and 390 mOsmKg^-1 showed a
decrease in sperm motility, but no loss of motility was noted for 280 mOsmKg⁻¹; these observations suggest that the optimal diluent for sperm motility is slightly hypotonic to the neat semen sample.

Probably the most remarkable finding of this study was the extreme tolerance of the sperm plasma membrane in response to exposure of hypotonic media. Even after the second osmotic excursion there was no evidence of damaged plasma membranes of sperm diluted in media from 25 to 220 mOsmKg⁻¹. While high osmolality (1523 mOsmKg⁻¹) has been reported as a possible mechanism of sperm storage in the hibernating microbat Myotis lucifugus (Crichton et al., 1993; 1994), the tolerance of the sperm plasma membrane to such extreme hypotonic media appears to be unparalleled when compared to mammals that have so far been examined. In addition, there appear to be no studies by which to adequately reference this phenomenon in the lower vertebrates. In a single study of osmotic and freezing tolerance of spermatozoa of freeze-tolerant and intolerant frogs, Costanzo et al. (1998) noted that motility and the integrity of the sperm plasma membrane of the freeze-tolerant wood frog (Rana sylvatica) decreased rapidly in media less than 110 mOsm/Kg⁻¹. However, comparisons of crocodile sperm to amphibian and fish species are not particularly instructive, as spermatozoa in these species are normally activated once released into fresh water, triggering off motility, mechanisms of capacitation and acrosome reaction in preparation for external fertilisation. Essentially, sperm in these species are released, become activated and die with minutes.

Crocodile spermatozoa also showed a propensity to respond to hypotonic media less than 220 mOsm/Kg⁻¹ by a progressive increase in coiling of the flagellum. There was an increase in the degree of intensity of this coiling with increased osmotic pressure. When the spermatozoa were returned to isotonic conditions, there was a slight decrease in the incidence of flagellum coiling in the 25, 59, 90 and 150 mOsm/Kg⁻¹ media but the majority of the spermatozoa retained their coiled morphology. These results suggest that this coiling injury was permanently inflicted. Given that the sperm plasma membrane of the majority of crocodile sperm exposed to hypotonic media retained their integrity, it is tempting to conclude that the increased water volume into the sperm cell is accommodated by an increase in flagella volume. While structural subcellular damage to the flagellum associated with water flux is likely to be the primary cause of the loss of motility in hypotonic environments, decreased motility in hypertonic environments is probably linked to osmotic injury associated with dehydration.

Crichton et al. (1994) have suggested that differences between species in their ability to respond osmotically to water volume change are likely to related to the shape of sperm and/or plasma membrane composition, noting that the sperm head of microbats are more elongated than most mammalian species. While the chemical composition of the reptile sperm membrane has not yet been described, the sperm head shape of most reptiles is filiform and so this could be contributing to their ability to withstand hypotonic excursion; it will be interesting to test whether sperm from other reptile species, including those of the American alligator have the same capacity. As sperm storage has been reported in the American alligator, the functional significance of a high tolerance of crocodile sperm membrane to anisotonic media may in fact be associated with sperm storage in this species, as it appears to be in microbats (Crichton 2000).

Studies of crocodilian sperm exposure to cryoprotectants and cryopreservation protocols are limited to observations on the American alligator (Larsen et al., 1984) and Broad-nosed Caiman (Larsen et al., 1992); both studies report the cytotoxic nature of glycerol and DMSO to sperm motility during incubation and Larsen et al. (1984) noted that either use of each cryoprotectant at 5 and 10% (V/V) resulted in poor or no post thaw motility. Unfortunately these studies did not evaluate the effect of cryoprotectant toxicity or cryopreservation on the integrity of the plasma membrane or examine whether the effects were reversible if the cryoprotectants were removed. Results of the current investigation revealed that following storage of crocodile sperm at 4°C for 1h in the various concentrations of DMA, DMSO and glycerol, there was an increasing negative effect on sperm motility with increasing concentration of cryoprotectant (except in 0.68M DMSO). Interestingly, there was no effect of cryoprotectant type or concentration on plasma membrane integrity during chilled storage. It appears therefore that the cryoprotectants used in this study were suppressing motility but not causing rupture of the plasma membrane.
Similar to what was described by Larsen et al. (1984) for American alligator spermatozoa, cryopreservation of crocodile spermatozoa resulted in a dramatic reduction in sperm motility post-thaw, irrespective of what type and concentration of cryoprotectant was used or whether the cryoprotectant was washed out or not immediately following thawing. However, the effect of cryopreservation on sperm plasma membrane integrity was more encouraging with increased protection of the plasma membrane co-incident with increasing concentration of cryoprotectant. There was no difference in the ability of DMA, DMSO or glycerol to out perform with respect to their cryoprotective ability at the highest concentration. Washing out the cryoprotectant through a centrifugation and re-suspension step resulted in an improvement of post-thaw sperm membrane integrity when data from all cryoprotectants and concentrations were analysed.

Clearly, cryopreservation has a much more adverse negative effect on the plasma membrane than that of extreme hypo or hypertonic conditions or the toxic effects of cryoprotectants, so that cryopreservation damage is most likely derived from cellular injury associated with ice crystal formation. In a high proportion of post-thaw spermatozoa, we observed that the first evidence of PI staining typically occurred at the base of the sperm head and progressively moved up towards the distal tip of the nucleus; perhaps this region of the sperm is a weak-point in the plasma membrane with respect to cryoinjury. While it is unlikely that frozen-thawed saltwater crocodile sperm recovered in this study could be used for successful fertilisation, spermatozoa do appear to require high levels of cryoprotectant for post-thaw survival, so that perhaps their general high tolerance to osmotic excursion could be an inherent advantage when designing and testing alternative methods of cryopreservation.

6. Method for crocodile artificial insemination

Given the short 12 month time frame set for this study, the major focus was on completion of objectives 1 to 5. Less attention was given to objective 6 as we decided to use observations of crocodile natural mating at the Koorana Crocodile Farm to guide us with respect to the timing of our preliminary artificial insemination (AI) attempts. The primary aims of these trials were to describe the cloacal anatomy of the female and to develop the logistics and mechanics of the insemination procedure.

The most notable feature was the difficulty in not only visualising the opening of the crocodile oviduct by means of the human vaginal speculum but also the problems we encountered in passing the AI catheter into the lumen of the vagina, given the muscular folds found in this portion of the oviduct. Even when we were able to visualise the ostia of a dissected reproductive tract from a large sexually mature female, we were unable to manipulate a “tomcat” catheter through these muscular folds.

Given the relatively simple structure of the crocodile reproductive tract, we had originally assumed that AI in this species would be reasonably straight-forward and that the rate limiting step of this procedure, based on previous research in American alligators, was going to be the collection of sufficient quantities of semen. However, in the saltwater crocodile, this assumption has proven to be quite the opposite and we now have re-think our insemination strategy if we intend to deposit semen directly inside the vagina.

Knowing now what we have learnt about the erect penis of the crocodile (see chapter 1), it is possible that the male crocodile deposits sperm under hydrostatic pressure into the female’s urodeum but not directly into the oviducal ostia. It is also possible that the viscous semen is simply ejaculated at the base of the ostia and sperm migrate into the muscular folds of the vagina where they are stored and then migrate to the site of fertilisation as is the case in the American crocodile (Gist et al., 2008). Interestingly, Limpus (1984) has described the use of laparoscopy to examine eggs within the oviduct of the fresh-water crocodile (Crocodylus johnstoni) and it is possible that this same technique could be used to inseminate semen directly into the lumen of the oviduct.

No doubt the greatest limitation to the successful production of offspring by AI in this study was that all inseminations were conducted without reference to timing of ovarian activity of the individual crocodiles. While a total of 23 crocodiles were inseminated, only nine animals laid eggs and two
produced fertile eggs. Clearly the factors that control the timing of ovarian follicular activity and ovulation in the salt water crocodile will need to be resolved before AI is going to be routinely successful. In this study we judged the timing of insemination naively based on observations of natural mating of other breeding pairs in Koorana Crocodile Park but future studies will need to map the profiles of reproductive hormones or follow ovarian activity by means of ultrasound or laparoscopy. Larsen et al. (1982) has also used combinations of GnRH and PMSG to stimulate follicular activity and ovulation and perhaps this approach can be used for improving the timing of insemination. However, the precise timing of AI may still not be a major issue in the crocodile, if it is determined that sperm can be stored in the female reproductive tract for weeks at time.

Another factor that may have contributed to the failure of ovulation and fertilisation in our study is that capture and artificial insemination is likely to be a stressful event. Franklin et al. (2003) have previously shown that manual restraint of the saltwater crocodile (noosing with ropes) causes a significant increase in haematocrit, haemoglobin, glucose, lactate and corticosterone concentrations in comparison to immobilisation by electro-stunning. It is likely that the manual restraint used in our study had a significant negative effect on the hypothalamic-pituitary-gonadal axis, which may have interfered with reproductive function of the females and lead to the low incidence of ovulation.

7. Musculoskeletal system

This is the first paper to report on the CT and MR musculoskeletal anatomy of saltwater crocodile C. porosus. This study has used advanced imaging and computer modelling techniques to give new understanding and insight of the musculoskeletal system aimed specifically for those involved in the commercial production of this species. Indeed this study provides a standard reference text as advocated by Huchzermeyer (2003), which future work can build on. In general, the findings of this study are consistent with that reported in recent times for the crocodilian skeleton by Brinkman (1981), Muller and Alberch (1990), Grigg and Gans (1993), Meers (2003) and Bona and Desojo (2011). Likewise, anatomical information for muscles disposition and organization of the head, back and tail region is in broad accordance that given in more focused crocodilian anatomical studies by Busbey (1989), Frey and Riess (1989), Endo et al. (2002), Organ (2006) and Tsai and Holliday (2011). The agreement between this study and former research lends further support to the assertion that advanced imaging modalities (CT and MR) are valuable tools for the acquisition of anatomical knowledge.

This study has employed advanced imaging techniques to provide a basic anatomical description of the axial and appendicular skeleton, and generated 3-D computer models to aid better understanding of the skeletal system. These models are readily converted into interactive 3-D PDF file (see submitted accessory files) format enabling the widespread dissemination of information to commercial producers, and those involved in the husbandry and management of this species. In addition, focus has been given to provide detailed information pertaining to the muscle groups associated with the commercial meat cuts typically taken from the crocodile carcass. Information has also been provided regarding their anatomical disposition relative to the skeleton, and their structural organisation, appearance, and/or association with intramuscular fat.

Specifically, this study has focused upon: the cranial muscles used for the preparation of “Jowl” cuts (M. pterygoideus ventralis and M. depressor mandibulae), the neck muscles used for “Neck Fillets” (hypaxial and epaxial muscle groups), the back and trunk muscles included in the “Backstrap” (M. transversospinalis and M. longissimus dorsi), “Rack of Rib” (M. transversospinalis and M. longissimus dorsi M. iliocostalis and intercostal muscles) and Body Meat cuts (M. iliocostalis, intercostal muscles abdominal muscles) the tail muscles used for preparation of the “Tail Eye” (M. caudofemoralis), “Tail Fillet” (M. longissimus and M. tendinoarticularis), “Tail” and “Tail Steak” cuts M. longissimus, M. tendinoarticularis and M. caudofemoralis), and finally, the limb muscles used in “Leg Meat” cuts (intrinsic muscles of the limb).

An intimate knowledge of the relationship between the skeleton and skeletal muscle groups is essential to ensure efficient and optimised methods for meat recovery. For example, an awareness of the dual faceted articulation between rib and supporting vertebral body is needed for efficient preparation of the
“Rack of Ribs” cut. To disarticulate the rib, two incisions have to be made, the first, an abaxial incision, can be identified by visual inspection of the inside of the eviscerated carcass, the second, an axial incision, needs to be made in a blind manner at the site of the second articulation point.

The inability of CT scanning protocols typically used for clinical veterinary examination to differentiate the musculature of the crocodile, despite using soft tissue weighted reconstruction algorithms, is of interest. This probably results from factors relating both to the anatomy of the crocodile, and factors relating to the parameters used for scanning. Tissue discrimination and elucidation of structural organisation of the tissue is reliant upon differences in the signal intensity between tissues and/or structures. It is this difference in signal intensity that, in part, accounts for contrast evident within the resultant images. The crocodiles examined in this study had little intramuscular fat. In fact, crocodile meat is renowned for its relatively low fat content (Hoffman et al. 2000). In addition, individual muscle groups were tightly apposed with thin fascicles and small amounts of connected tissue. Hence, the inherent body composition of the crocodile itself presents an imaging challenge.

Contrast is also dependent on the signal to noise ratio of the scans, and clinical scanning protocols are designed to give an acceptable signal to noise ratio. Signal to noise ratio is influenced by the parameters selected for scanning (Ryberg et al. 2000, Primak et al. 2006). In respect of CT scanning this is determined by tube current (mA), slice thickness, rotation time, and helical pitch. Clinical scans typically employ relatively moderate current (120mA), large slice thickness (>1mm), fast rotation times (<1.0 sec) and a high pitch factor. This results in signal being generated from a relative large tissue slice. This leads to a trade-off which impacts on the ability to discriminate between tissue boundaries and fine detail of structural organisation. As the signal is generated from a relatively large tissue slice, it represents the average signal intensity for the entire tissue slice. Thus boundary detail and structural detail can be obscured when these features are not aligned orthogonal to the direction of slice acquisition. This is of particular relevance given the characteristic of the skeletal musculature evident in C. porosus, including closely opposed muscle groups, thin fascicles and conical shaped myosepta. The ‘high resolution’ scans employed in this study, which reduced the slice thickness (0.5) were able to reduce the volume averaging effects and enabled muscle tissue discrimination where convention protocols failed. The inevitable reduction in signal and signal to noise ratio resulting from a 50% reduction in slice thickness was compensated for, by increasing tube current (135mA), rotation time (1.0 sec) and decreasing the helical pitch factor. Collectively, these measures resulted in marked improvements in skeletal muscle discrimination, and elucidation of fine structure.

It is anticipated that with further optimisation of scanning parameters employed in the high resolution, combined with contrast agents, further gains in muscle discrimination will be achieved. These gains would offer a number of exciting possibilities into the future that would benefit all involved in the commercial production of this species. Firstly from an education perspective, it should be possible to generate comprehensive 3-D models for the entire musculoskeletal system, similar to those produced in this study for the skeleton of C. porosus. Secondly, in a similar manner to that currently been employed in commercial sheep and pig production (Haynes et al. 2010, Bunger et al. 2011), CT scanning could prove to be a most valuable tool for the evaluation of body composition and meat quality. Furthermore, given the ability of CT scanning also to visualise and accurately document scute organisation and row characteristics of the skin, CT scanning could be a valuable tool for the crocodile skin industry. Indeed, by inclusion of this advanced imaging technique as part of a holistic scientific approach involving molecular genetics and reproductive biology, it may be possible to develop a highly effective selective breeding program for the industry. This would have a focus on using relevant gene marker data, reproductive characteristics and skin characteristics, body composition and meat quality to prospectively identify individuals most likely to deliver improvement in ‘quality’ of commercially produced crocodiles. Quality is defined here as increased growth potential, reduced generation time, improved reproductive fecundity, and superior skin grade, body composition and meat quality. Achieving this objective would offer the industry clear financial benefits, in an increasingly competitive market place.
Finally, increased knowledge and understanding of the CT anatomy of *C. porosus* offers the potential to bring about improvements in the veterinary care and health management of this species and in delivering further advances in husbandry. More detailed understanding of the normal anatomy allows advances to be made in the ability to detect and diagnose disease, disorders and pathology. An incidental finding of this current study was the presence of foreign bodies within the stomach of five out of six crocodiles. In two cases, enteoliths (formed around the foreign bodies) were seen to occupy the entire stomach. It is not known whether this is consequence of commercial production; nevertheless, this finding has implications both in respect of husbandry and veterinary care, and could represent an important limitation on growth rate and well being that warrants further investigation. This study provides valuable baseline anatomical information for *C. porosus*. This information is of benefit to those specifically involved in commercial production of this species, and provides insight and understanding of the muscular skeletal system relevant for commercial meat production. The imaging protocols developed in this study can serve as a valuable adjunct to bring about improvements in commercial production into the future.
Implications

1. The successful development of a reliable, repeatable and safe semen collection procedure in the saltwater crocodile will now allow:
   - breeding soundness evaluation in male crocodiles that can be used to diagnosis infertile and sub-fertile males and thereby improve reproductive management of breeder males on farm
   - removal of unsatisfactory male breeders so as to eliminate maintenance of unproductive animals and free up space for new fertility tested or proven breeding stock
   - better characterisation of male reproductive seasonality and puberty and exploration of external factors such as nutrition, climate and husbandry that impact reproductive potential
   - further characterisation and optimisation of methods for short-term and cryopreservation of crocodile spermatozoa
   - the development of artificial insemination procedures and the associated genetic management benefits that flow from this technology, including use of wild genetics and the dissemination of highly desirable genotypes
   - application of fertility assessment and breeding technology to endangered crocodilian species in the wild and in captivity.

2. A database for the establishment of spermatogram will now allow:
   - development of standards and norms for the assessment of crocodile sperm quality
   - selection of males with high reproductive potential.

3. The documentation of cloacal and seminal microflora will now allow:
   - control of bacterial contamination for crocodile sperm extension and preservation
   - antibiotic therapy to prevent transmission of venereal disease or other pathogens via artificial insemination.

4. Characterisation of the physiochemical environment of the crocodile sperm will now allow:
   - for the production of customised semen extenders for short-term chilled and cryopreservation
   - a better understanding of factors that control sperm motility and sperm storage.

5. Baseline studies of short-term and cryopreservation will now allow:
   - refinement of chilled semen extension for semen transport and artificial insemination
   - development of new approaches to cryopreservation for long-term storage and genome banking.

6. An improved understanding of female cloacal anatomy will now facilitate:
   - the development of more informed methods for artificial insemination.

7. 3D documentation of the whole body and musculoskeletal system of the saltwater crocodile by CAT and MRI scan and 3D modelling will now allow:
   - an improved understanding of the muscle used in commercial crocodile meat production, and how to butcher those muscles for maximal yield
   - identification of muscle masses that could lead to co-selection of body morphs that increase total surface area and higher skin yields or increase growth rates
   - calculation of total surface area of skin and the most optimal cut patterns to maximise skin yield and reduce offcuts
   - better veterinary practice through an improved understanding of anatomical structures for surgery and treatment.
Recommendations

Further research is necessary on the following:

Semen collection and evaluation

- Determine better methods of capture and restraint that allow for animals to be processed for semen more efficiently
- Use semen collection techniques along with endocrine and ultrasound assessment of the testis to characterise seasonal and pubertal changes in male reproductive function—especially the possibility of asynchrony between testicular development and sperm storage in the vas deferens
- Conduct broad scale breeding soundness evaluations in all commercial crocodile farms to remove unsatisfactory breeders, using the new skill of semen collection
- Explore the use of this technique in endangered crocodilians species to identify male infertility in order to make more informed breeding recommendations, using the new skill of semen collection
- Determine the possibility of collecting semen from juvenile males slaughtered for their skin; it may be possible to recover sperm from these animals and thereby shorten generation intervals to hasten genetic gain.

Semen preservation

- Test that the addition of gentamicin and sulphamethoxazole/trimethoprim and amoxicillin/clavulanic to semen extenders is not spermicidal to crocodile spermatozoa
- Design experiments that explore those factors which control sperm motility—for example, studies that explore mitochondrial membrane potential and the reactivation of motility following exposure to cryoprotectants or cryopreservation
- More fully explore the effect of environmental temperature on crocodile sperm metabolism
- Determine the optimal concentrations of DMA, DMSO and glycerol prior to cytotoxicity of the sperm plasma membrane, and whether the adverse effect of cryoprotectants on sperm motility prior to cryopreservation is reversible
- Explore a greater range of cryoprotectant types including propylene glycol, ethylene glycol, methanol and DMF.

Artificial insemination

- Develop ultrasound procedures for assessing ovarian follicular activity, detecting eggs within the oviduct
- Determine the most appropriate timing of insemination by designing experiments to examine seasonal changes in female reproductive function (n = 10 sexually mature females) such as steroid reproductive hormones, ovarian follicular dynamics and reproductive behaviour
- Determine the most appropriate timing of semen collection by designing experiments to examine seasonal changes in male reproductive function (n = 10 sexually mature male) such as steroid hormones, testicular volume, seminal characteristics and reproductive behaviour.
- Explore the use of GnRH and eCG to induce oestrus and ovulation
- Explore further the phenomenon of sperm storage in the female reproductive tract
- Follow the passage of sperm up the female reproductive tract following artificial insemination by means of histological evaluation of the oviduct
- Explore the use of laparoscopic insemination directly into the oviduct
- Determine the efficacy of inseminating neat or diluted crocodile semen.
Accessory files

The following files are available on the RIRDC website, along with this publication:

1. **Accessory file #1** – PowerPoint Presentation - Advanced Imagining and Computer Modelling of the Skeletal and Musculoskeletal Anatomy of *Crocodylus porosus*.
2. **Accessory file #2** – 3D PDF of crocodile limb as an exemplar of educational package
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Development of breeding techniques in the crocodile industry

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This report describes successful preliminary investigations into the development of assisted breeding technology in the Australian saltwater crocodile industry, with a particular focus on semen collection and preservation. Semen collection and preservation is the first step towards the establishment of artificial insemination, the successful implementation of which has the potential to revolutionise crocodile farming with respect to reproductive and genetic management.

This report is targeted at crocodile farmers, veterinarians and zookeepers, to illustrate the application of semen collection and preservation to their industries.

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