15 February 2008

Australian Centre for Applied Marine Mammal Science
2007 Funded project update

PROJECT TITLE: Determining critical reproductive parameters for a subtropical dugong population.

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PROJECT INTRODUCTION:

This PhD project is developing methodologies to assess the reproductive state of wild dugongs via non-invasive hormonal profiles in exhaled air and faeces, and plans to calibrate these profiles against blood samples. These data will be used along with gender, body size and social association data gathered during mark-recapture studies, to determine the reproductive status of individual dugongs. Life history parameters determined during this study will be incorporated into a population study of dugongs in Moreton Bay, southern Queensland. An understanding of reproductive behaviour and biology is crucial if we are to develop useful models for management strategies.

PROJECT OBJECTIVES:

The overall aim of this project is to obtain essential conservation data on the structure and reproductive patterns of a dugong population adjacent to areas of rapid urban development in southern Queensland: Moreton Bay. We will develop and use alternative sampling methods to collect exhaled blow, faecal and blood samples from dugongs to determine reproductive status. We are already studying the Moreton Bay dugong population through a hands-on mark-recapture program (Lanyon *et al*. 2002), and the comprehensive data set (including identity, size, gender) on this population provides the basis for this research into life history.

The objectives of this project are to:

1. *Sample blow, faecal and saliva samples from wild dugongs in Moreton Bay*. This project will obtain essential data on reproductive status and rates of wild dugongs through collecting samples of exhaled blow, faeces, saliva and blood to measure reproductive hormones.

2. *Sample blow, faecal, saliva and blood samples from captive dugongs.* This project will sample blow, faecal, saliva and blood from captive dugongs to (i) quantify reproductive hormone concentrations, (ii) compare concentrations from the four sources, (iii) biologically validate levels.

3. *Analyse reproductive hormones in exhaled blow, faecal, saliva and blood samples of captive and/or wild dugongs* with a view to obtaining seasonal and size-related life history data for individual dugongs.

4. *Correlate reproductive status of individual dugongs (see above) against body size, gender and condition*

Objective 1. Sample blow, faecal and saliva samples from wild dugongs in Moreton Bay.

(a) Blow samples

Methodology for collection of blow samples

Exhaled air samples have previously been collected from captive and wild bottlenose dolphins (Hogg *et al*., 2004) and more recently from humpback whales (Hogg *pers. comm*.). The collection method is species-specific: gauze collecting pads have been trialled for wild cetaceans and 50ml centrifuge tubes for captive and immobilised dolphins. We trialled a variety of devices and materials for collecting blow in dugongs, which had previously been used by Hogg *et al.* (2004).

Since dugongs exhale a large number of times during the restraint period of up to 5 mins, a centrifuge tube proved to be the most effective and easiest device. Exhaled air from the blow of the dugongs was collected by holding a sterile 50 ml polypropylene jar above a single nare of the dugong. The open jar was held inverted over the dugong's nare at a distance of 2-3 cm during one or more exhalations. The exhaled air condensed on the inside of the tube.

Another sample collection material was trialled which used inert nylon stockings. For the nylon stocking trial, Koltex nylon knee high stockings (Koltex, Australia) were cleaned by sonication for 15 minutes with 0.5 % methanol (MeOH) and then sonicated for a further 15 minutes with Milli-Q water, changing the water every 5 minutes. This stocking was then stretched over a plastic container with a diameter of 10 cm. This device was then suspended over the nares of the dugong during exhalation. This method was not as convenient as using the sterile jars because of the cleaning process involved, and therefore was abandoned.

To date, we currently have a collection of 15* exhaled air samples from individual wild dugongs. The gender and size class of these samples are summarised in Table 1. Collection of blows in wild dugongs will be ongoing throughout the duration of this project.

(* We have fewer samples than anticipated because the very wet and windy weather in south-east Qld over the past 6 months has made field sampling very difficult. Coastal waters have been so turbid that dugong sampling has been severely impacted)

Table 1: Summary of blow samples collected from wild dugongs in different gender and size classes.

	Adult	Sub-adult	Juvenile	TOTAL
Female	2	1	3	6
Male	5	2	2	9
TOTAL	7	3	5	15

Laboratory analysis of blow using LCMS

The samples of exhaled air will be analysed in collaboration with Alun Jones at the Institute for Molecular Bioscience, University of Queensland. A pilot study analysing ten faecal samples has been run using the liquid-chromatography mass-spectrometry technique. These samples are still awaiting final analysis and will help us determine which hormone metabolites are present and their molecular weights.

Unfortunately, this aspect of the project using LCMS laboratory techniques has proven more time consuming than originally anticipated due to problems with calibration of steroids. However, this work is progressing and we anticipate meeting this objective by July 2008.

(b) Faecal samples

Methodology for collection of faecal samples

It is not uncommon for faecal material to be produced by restrained animals including dugongs, and this provides the opportunity to collect a faecal sample. Should defecation not occur during restraint, then a soft latex tube (veterinary yearling stomach tube) was introduced into the distal part of the rectum to collect a faecal core. We have had a lot of success with faecal sample collection using this methodology. To date, we currently have a collection of over 140 faecal samples from individual wild dugongs in Moreton Bay (Table 2).

Table 2: Summary of faecal samples collected from wild dugongs in different gender and size classes.

	Adult	Sub-adult	Juvenile	TOTAL
Female	36	22	12	70
Male	33	24	13	70
TOTAL	69	46	25	140

Laboratory analysis of faecal samples using EIA

We have trialled various hormonal assay kits which are available commercially to measure steroid hormones in faecal samples. These trials not only included kits from various companies but also different kits using different immunoassay techniques.

Initially, we planned to analyse our faecal and blood samples using radioimmunoassay (RIA). The technique of radioimmunoassay (RIA) had previously been successful with dugong faecal samples (Lanyon *et al.* 2005). For this project, we trialled oestrogen and oestrogen-sulfate RIA kits purchased from *Diagnostic Systems Laboratory, Inc*. However, the results of these two kits did not give reliable results, with significant variation (>10%) shown between duplicate samples. Whilst results weren't clear, it was suspected that equipment error was possibly the cause and this problem has since been corrected.

The essence of any immunoassay is the competition between added labelled antigen ('tracer') and unlabelled antigen (i.e., hormone in the sample) binding to an antibody. In the RIA procedure the tracer is radioactively labelled. Another immunoassay technique is enzyme-linked immunoassay (EIA). An RIA laboratory needs to be licensed for the use of radioisotopic tracers, and gamma and beta scintillation detection equipment are comparatively expensive. By contrast, EIAs do not require special precautions for using radioactivity, equipment is less expensive, no sophisticated apparatus is required and reagents are easy to prepare, are highly stable and have a long shelf-life. Many EIAs are now as sensitive as RIAs and so are gaining in popularity.

Since the EIA technique has significant advantages over the previously used RIA, we also trialled the use of enzyme-linked immunoassays (EIA) to detect hormone levels in dugongs.

Analyses were preformed using commercial EIA kits produced by *Diagnostic Systems Laboratory, Inc.* and *Diagnostic Biochem Laboratories.* Whilst these kits proved successful in detecting hormone levels in dugongs (Objective 3), these kits were logistically challenging because of import Quarantine permits and shipping costs.

As an alternative to purchasing and importing EIA kits, we also trialled the methodology and endocrinology setup used at the Animal Reproductive Centre, Western Plains Zoo. The Animal Reproductive Centre is an existing hormone laboratory which specialises in enzyme immunoassays on faecal samples from wild and captive species. This centre is an established laboratory which has all the equipment and prepares its own reagents necessary to run EIA assays (it' an alternative to purchasing commercial kits which come complete with equipment and reagents). Using their laboratory set up and protocol, we are now able to analyse our entire faecal sample collection of captive dugong faecal samples and wild dugong faecal samples against four hormone profiles (oestrogen, progesterone, testosterone and cortisol). Rather than importing individual kits from commercial companies, collaborating with the Animal Reproductive Centre allows us access to all the equipment and reagents needed to conduct assays. This has proved to be a more cost-effective and time-efficient method. This has resulted in a significant increase in sample size from the 75 dugongs originally proposed to being able to analyse the entire sample collection of 140 dugongs.

The methodology and anti-body reagents used at the Animal Reproductive Centre has been shown to reliably detect hormone levels in dugong faeces. During this project, the EIA techniques used in the Animal Reproductive Centre laboratory have been trialled by Burgess and have proven to be effective for determining hormone concentrations in dugong faecal extracts. For example, Figure 1 and 2 depict a parallelism between the standard reference serum and the serially diluted serum sample curve produced from dugong faecal extracts (Brown *et al.* 2005). These curves must be parallel to support the assumption that the antibody-binding characteristics are similar enough to allow the determination of antibody levels in the diluted serum sample. Across all assays these curves are parallel and therefore, we are confident that this methodology is valid for samples of dugong faecal extracts.

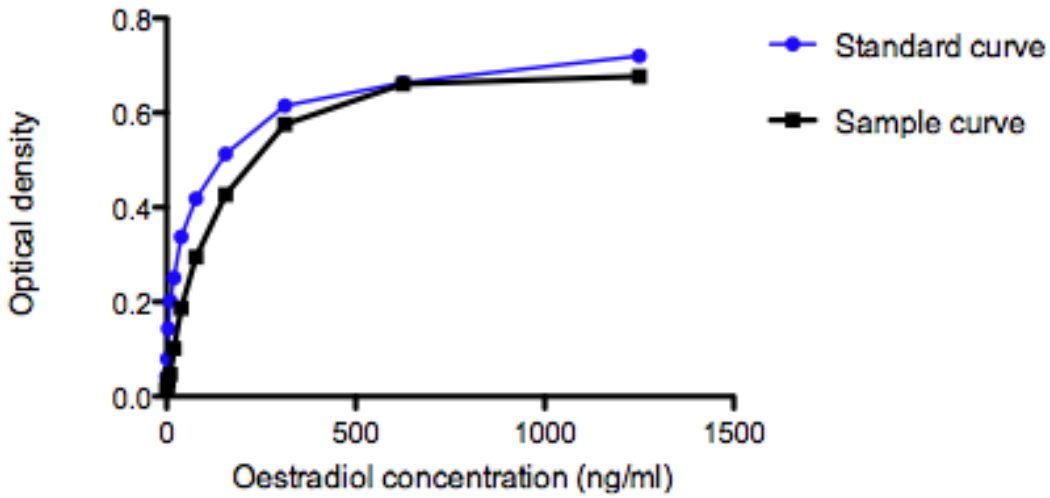

Figure 1: Comparison of Oestradiol standard reference curve and a curve produced from a serial dilution of dugong faecal extracts, showing parallelism between the curves.

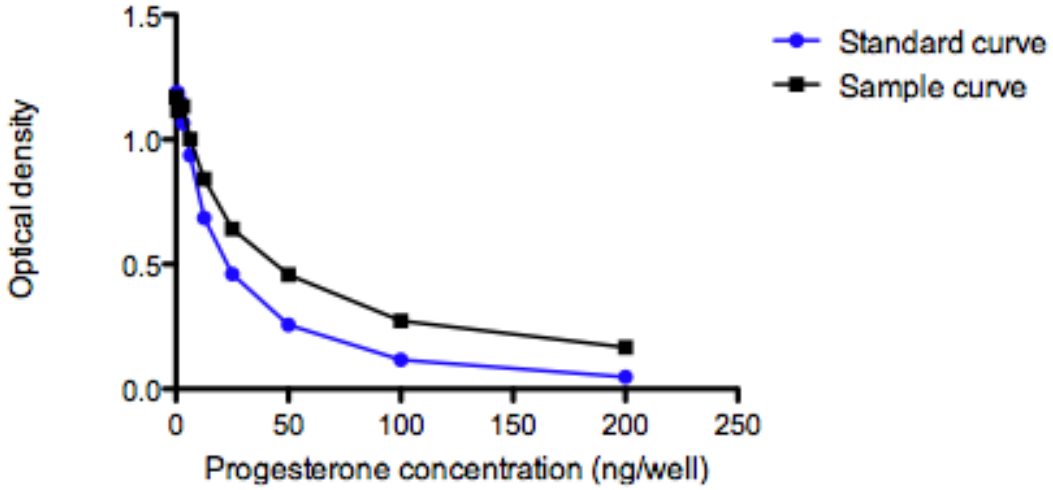

Figure 2: Comparison of Progesterone standard reference curve and a curve produced from a serial dilution of dugong faecal extracts, showing parallelism between the curves.

(c) Saliva samples

Methodology for collection of saliva samples

Previous to this project, the collection of saliva samples had not previously been attempted in live wild dugongs. We trialled collection of saliva using a large cotton gauze swab (7.5 cm x 7.5 cm x 4 ply) on three dugongs. However, we were unsuccessful in reaching the salivary glands located at the rear of the mouth. The unique morphology of the downturned rostrum, small gape and massive jaw-closing muscles in the dugong made it difficult to access the salivary glands. Furthermore, working with the dugong restrained in water, also made saliva collection difficult.

In conjunction with another research aim, we plan to restrain a number of dugongs (up to 30) onboard the research vessel, *Sea World I.* This use of this vessel fitted with marine mammal hoist and holding facilities, will allow for an opportunity to better trial the collection of saliva in a restrained animal out of the water.

To collect the saliva we have developed a suitable collection device, which consists of an extended flexible rod with cotton gauze end. The gauze material will be securely to the end of the strip and will absorb the saliva from the back of the palate with gentle insertion from the side of the mouth.

Laboratory analysis of saliva using LCMS

When saliva is successfully collected, it will be analysed in collaboration with Alun Jones at the Institute for Molecular Bioscience, University of Queensland. Saliva will be extracted from the gauze medium by spinning the sample in a centrifuge. The extracted saliva will then be analysed using the chemistry technique of liquid-chromatography mass-spectrometry.

(d) Extra media samples

While we have not been successful in obtaining samples of saliva from wild dugongs, we have successfully obtained vaginal swabs from female dugongs in the wild. To trial this methodology, we went through the required Animal Ethics and Scientific Permits process and approval.

This is an innovative methodology to be used on a wild, marine mammal species, though it is routinely used by zoos for assessing their captive, terrestrial mammals. The vaginal epithelium of many mammalian species is responsive to circulating oestrogen concentrations. The value of vaginal cytology in monitoring the oestrous cycle of rodents and domestic animals is widely recognised. Using this technique involves the vaginal insertion of a sterile cotton swab to collect a small amount of cells and mucus from the vaginal wall. The epithelial cells are then examined as a slide smear under a microscope. We also plan to attempt to extract mucus from these swabs, and analyse the mucus for reproductive hormone levels using the enzyme immunoassay or liquid-chromatography mass-spectrometry technique. We will trial both analytical techniques to determine which is the most efficient.

We currently have vaginal swabs from six female dugongs and this is now a routine sampling procedure for female dugongs. Swab sample collection will be ongoing throughout the duration of this PhD project.

Objective 2. Sample blow, faecal, saliva and blood samples from captive dugongs.

Methodology for collection of samples from captive dugongs at Sea World, Gold Coast
The two dugongs held at Sea World, Gold Coast are Pig (immature, male) and Wuru (immature, female). Over a seven month period, Pig and Wuru were sampled once a week for exhaled blow and faeces, and once every three months for blood and body measures (to coincide with routine health checks of the dugongs performed by Sea World).

(a) Blow samples

Without restraining the dugong, exhaled air from the blow of the captive dugongs was collected by holding a 50 ml centrifuge (polypropylene) tube above a single nare of the dugong as the animal swam passed the edge of the tank. The tube was held inverted over the dugong's nare at a distance of 2-3 cm during an exhalation.

(b) Faecal samples

Captive dugongs were observed throughout the day for defecation. A handheld pool net on a 6m pole was used to retrieve samples either directly from the animals as it was voided or from the water within minutes of excretion.

(c) Saliva samples

We hoped to obtain some saliva samples from the Sea World dugongs during their routine health checks out of the water. Unfortunately, no saliva has been collected due to logistic constraints which did not permit this sampling to be attempted. But we continue to work with Sea World and hope to be able to get some saliva samples from their two captive dugongs.

(d) Blood samples

During routine health checks of the dugongs, Sea World restrained the dugongs outside the water. This allowed for blood samples to be taken by veterinarian, Wendy Blanshard. Blood

samples were collected from the anterior palmar surface of the pectoral flipper between the distal parts of the ulna and radius using a 21 gauge 1.5 in. needle and syringe.

Summary of samples collected from captive dugongs

Between 24 July 2007 and 8 February 2008, weekly samples of faeces and exhaled air have been collected from a male dugong, Pig, and a female dugong, Wuru. A total of 222 faecal and 151 exhaled air samples have been collected in the 29 weeks (Table 3 and 4). Over this period, 4 weigh days provided an opportunity to collect blood samples and tear samples from Pig only. Unfortunately, Sea World has not been forthcoming with being able to take blood samples from Wuru. We continue to work in collaboration with Sea World and anticipate that we will be able to get the full collection of samples from Wuru at the next routine health check in April 2008 and then at following 3 monthly periods.

Table 3: Summary of sample collection from captive dugong, Pig (male).

		SAMPLES COLLECTED				
Week	Date	Faeces	Blow	Blood	Tears	Body measures
1	24-Jul-07	3	5	1	3	yes
2	31-Jul-07	4	3			
3	7-Aug-07	5	3			
4	14-Aug-07	5	3			
5	21-Aug-07	7	3			
	25-Aug-07	4	2			
6	28-Aug-07	5	3			
7	4-Sep-07	3	3		nasal mucus	
8	12-Sep-07	4	2			
9	19-Sep-07	3	2			
10	25-Sep-07	4	2			
11	1-Oct-07	2	3	1	2	yes
12	9-Oct-07	3	2			
13	17-Oct-07	6	2			
14	24-Oct-07	3	2			
15	30-Oct-07	1	3			
16	6-Nov-07	6	2			
17	13-Nov-07	3	3			
18	20-Nov-07	5	2			
19	28-Nov-07	2	3	1	1	yes
20	4-Dec-07	5	2			
21	13-Dec-07	3	2			
22	17-Dec-07	2	3			
23	27-Dec-07	3	2			
24	2-Jan-08	3	2			
25	8-Jan-08	3	2			
26	15-Jan-08	3	3			
27	24-Jan-08	3	3	1	1	yes
28	30-Jan-08	6	2			
29	3-Feb-08	8	3			
	TOTAL	117	77	4	7	

Table 4: Summary of sample collection from captive dugong, Wuru (female).

		SAMPLES COLLECTED				
Week	Date	Faeces	Blow	Blood	Tears	Body measures
1	24-Jul-07	2	5			yes
2	31-Jul-07	4	3			
3	7-Aug-07	5	3			
4	14-Aug-07	8	2			
5	21-Aug-07	6	3			
	25-Aug-07	3	2			
6	28-Aug-07	5	3			
7	4-Sep-07	3	3			
8	12-Sep-07	3	2			
9	19-Sep-07	3	2			
10	25-Sep-07	3	2			
11	1-Oct-07	2	3			yes
12	9-Oct-07	3	2			
13	17-Oct-07	6	2			
14	24-Oct-07	2	2			
15	30-Oct-07	1	2			
16	6-Nov-07	5	2			
17	13-Nov-07	1	3			
18	20-Nov-07	5	2			
19	28-Nov-07	2	2			yes
20	4-Dec-07	4	2			
21	13-Dec-07	2	2			
22	17-Dec-07	1	2			
23	27-Dec-07	2	2			
24	2-Jan-08	3	2			
25	8-Jan-08	2	3			
26	15-Jan-08	3	3			
27	24-Jan-08	2	2			yes
28	30-Jan-08	6	3			
29	3-Feb-08	8	3			
	TOTAL	105	74	0	0	

Objective 3. Analyse reproductive hormones in exhaled blow, faecal, saliva and blood samples of captive and/or wild dugongs.

Analysis of samples from captive dugongs

Captive animals at Sea World, Gold Coast have provided long-term repeated samples for monitoring fluctuations in hormone concentrations over time. To date, preliminary hormone profiles have been run to assess progesterone and oestradiol levels in Wuru's faeces over 18 weeks. Figure 3 and 4 show minor fluctuations in hormone levels, which is to be expected, and provides an idea of hormonal range within an individual. These results are preliminary and show that the EIA technique was able to detect fluctuating hormone levels in dugongs. Further, these levels represent baseline hormonal levels in this immature dugong.

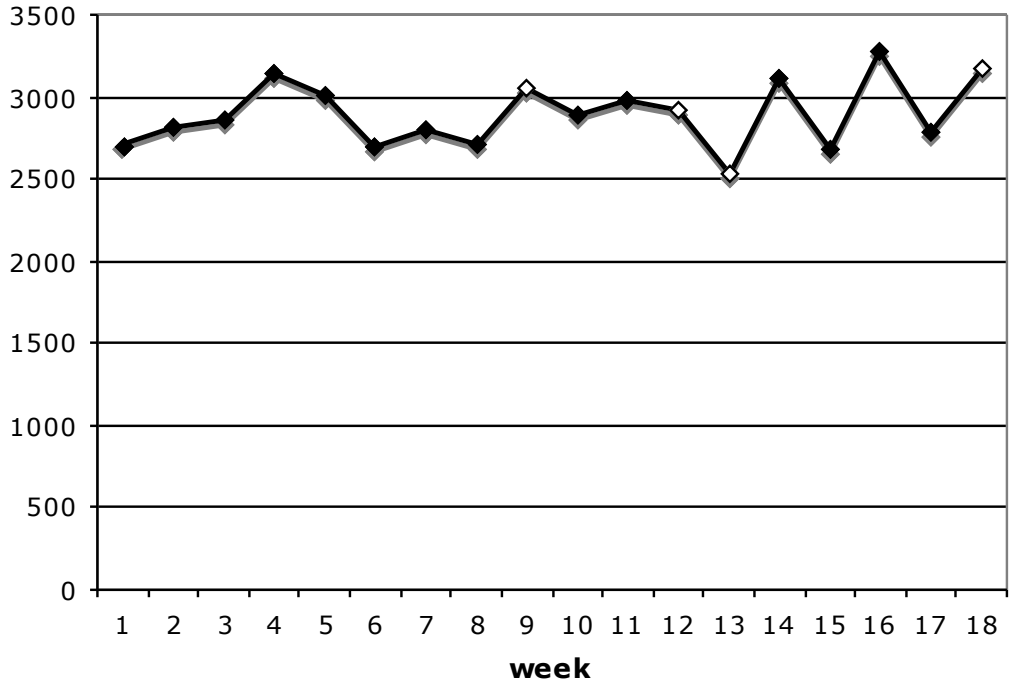

Figure 3: Oestradiol hormone concentrations for Wuru, using faecal samples collected weekly, between 24 July - 20 November 2007, at SeaWorld. White dots indicate that sample was not confirmed to be from Wuru.

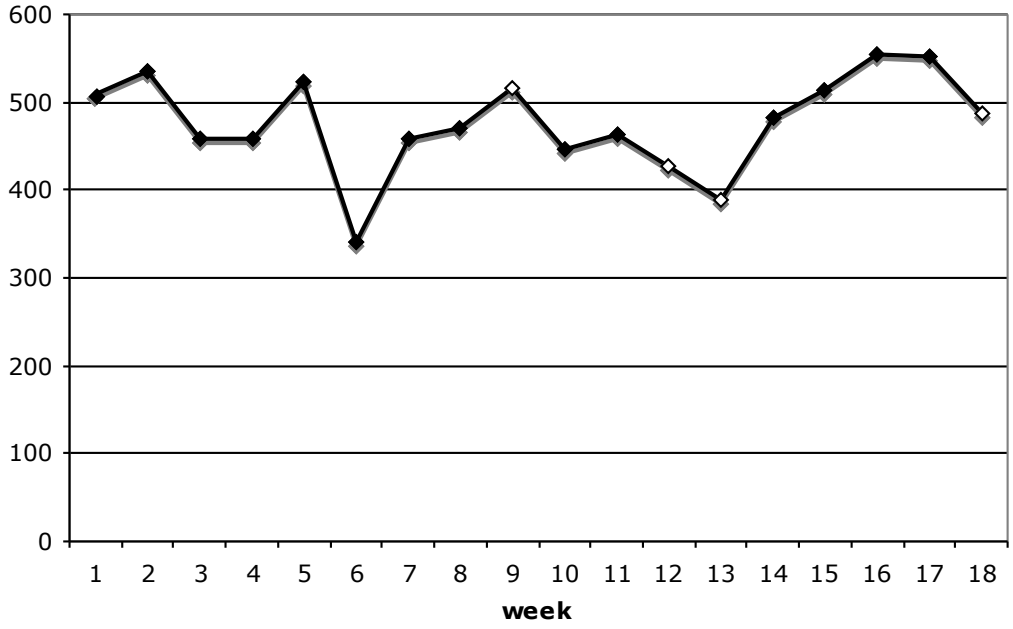

Figure 4: Progesterone hormone concentrations for Wuru (female), using faecal samples collected weekly, between 24 July - 20 November 2007, at SeaWorld. White dots indicate that sample was not confirmed to be from Wuru.

Analysis of samples from wild dugongs

Immature males, immature females and adult males have lower levels of progesterone in their faeces than do adult females. Adult females on average have higher progesterone levels than other age and gender classes, but also show a higher variation between samples. This may be due to females being sampled at different periods during their cycle (progesterone increases after ovulation) or may be because of pregnancy (maintains a high level during gestation).

We are continuing our laboratory analysis of faecal samples using the enzyme immunoassay technique. Figure 5 shows the results analysing progesterone using a commercially available kit produced by *Diagnostic Systems Laboratory, Inc*.

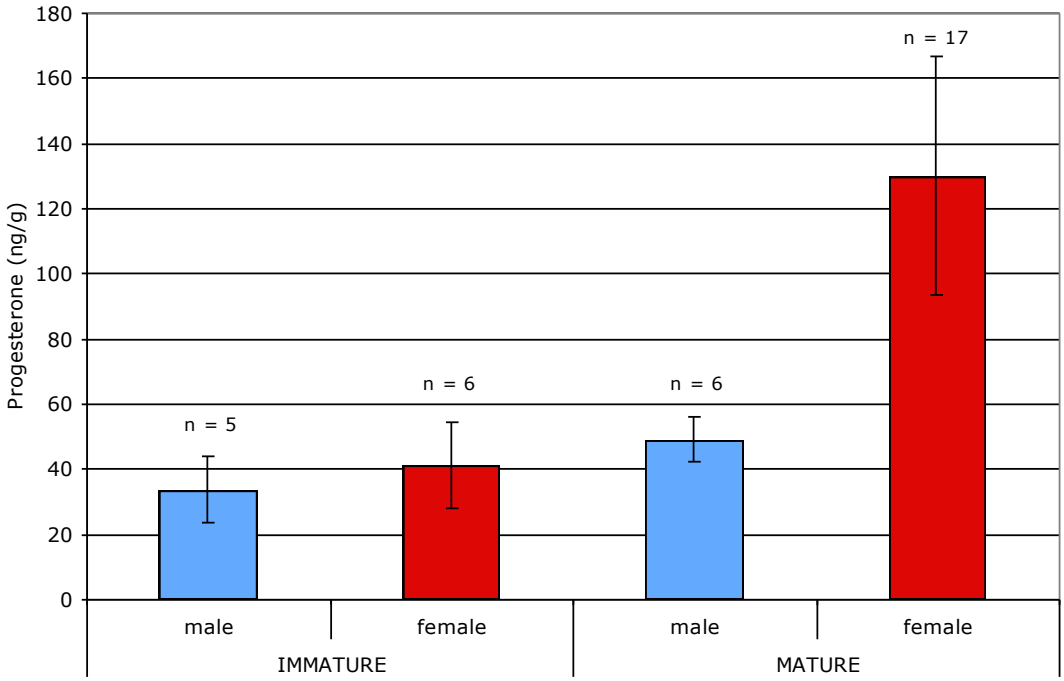

Figure 5: Progesterone hormone concentrations for wild-caught dugongs in various age class and gender categories (n = 34). Bars indicate standard errors.

In this analysis, one of the samples was from a confirmed pregnant female (#06513). Five months after a faecal sample was collected from dugong #06513, she was sighted with a first-year calf. Dugong #06513 had progesterone levels significantly elevated, at approximately three times higher than the mean progesterone concentration across all dugongs sampled. Figure 6 plots the range of adult female dugong progesterone levels. It could be possible that levels higher than 200 ng/g indicate pregnancy, as the progesterone hormone is elevated during pregnancy to support gestation. When females are in the follicular phase of cycling, progesterone levels are similar to adult males. The 11 females with progesterone levels below 200 ng/g have a mean of 28.7 ± 3.6 ng/g. Our mark-recapture program provides the on-going opportunity to sample females in different reproductive phases. Sampling of mothers with calves allows us to retrospectively determine which of past samples were from pregnant females.

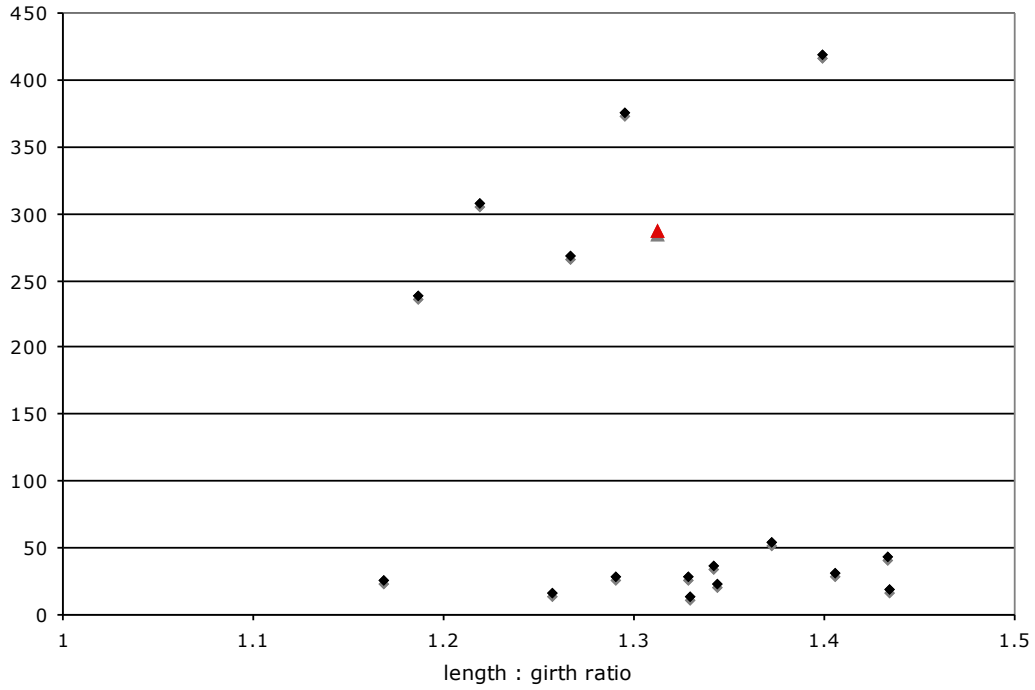

Figure 6: Progesterone hormone concentrations for adult female wild-caught dugongs (n = 17) compared to their length to girth ratio. Red triangle is a confirmed pregnant female (Dugong #06513). It is possible that the females with higher progesterone levels here may be confirmed as pregnant at the time of sampling, if they are accompanied by a calf at later recapture.

Calibration of hormone levels in various sample media

To date, no comparison has been possible between the hormone levels in blood samples versus other media. Taking blood samples from the captive dugongs held at Sea World has been restricted. We have collected four blood samples from the male, Pig over a period of seven months. Unfortunately, no blood samples have been taken from Wuru, because of restrictions imposed by Sea World. We plan to continue collaborating with Sea World over the next two years of this project, and hope that with a longer time projection we will be able to collect more blood samples from both Pig and Wuru.

Collecting blood from wild dugongs has also proved challenging when using the capture protocol of holding the dugongs in the water whilst sampling (Lanyon *et al*. 2006). To alleviate this problem, we will collaborate with Sea World to sample dugongs in the wild using their research vessel *Sea World I*. Dugongs will be brought up on to the vessel *Sea World I* and hoisted on-board. The dugong will be held temporarily on deck to allow for a blood sample to be taken. By making use of this vessel, we plan to collect blood from a sample of 30 adult and sub-adult animals from both sexes. This sample size will enable us to compare the hormone levels between blood and faeces. We will also obtain samples of urine, exhaled blow, tears and vaginal mucus for steroid hormone analysis at this time.

Objective 4. Correlate reproductive status of individual dugongs against body size, gender and condition

Investigating correlations between reproductive status of dugongs and life history parameters (such as body size, gender and body condition) has not yet been attempted. We will wait for further results from Objective 3 to achieve this aim.

REVIEW OF PROJECT OUTPUTS:

Expected outputs	**Date of completion (mm/yy)**
1. Evaluation of methods to assess hormonal profiles in dugongs	12 / 07
2. Scientific publication of methodology and results	12 / 09
3. Completion of PhD thesis	12 / 09

Output 1 which involves the evaluation of methods to assess hormonal profiles in dugongs is partially completed. We are confident that the enzyme-linked immunosorbent assay (EIA) will be reliable in assessing progesterone, oestradiol, testosterone and cortisol in dugongs. We have also confirmed that the EIA technique used by the Animal Reproductive Centre, Western Plains Zoo will be used for all our dugong faecal and blood assays to allow for comparison between samples.

Evaluating the methods for Liquid-chromatography mass spectrometry (LCMS) to detect trace hormones in exhaled air needs more laboratory work, and this is ongoing. We anticipate that results will be produced by a completion date of 06 / 08.

Output 2 and 3 are on target, with the completion of a scientific publication of methodology and results by 12 / 09 and completion of a PhD thesis by 12 / 09.

SUMMARY OF PROJECT UPDATE:

- **Sampling:** In summary, we have achieved a large collection of samples (especially faecal samples) from individual wild dugongs within Moreton Bay and from captive dugongs over time (including recaptured animals) which will enable us to assess reproductive parameters in subtropical dugongs.

- **Analysis:** We have trialled several different radio-immuno and enzyme-linked immunosorbent assay techniques and kits to measure steroid hormones in faecal samples, and have trialled several methods of hot and cold extraction of hormones. We have concluded that the most efficient method is to use the EIA techniques already established at the Animal Reproductive Centre, Western Plains Zoo.

- **Results:** We have results on the fluctuations of female hormone levels in an individual female dugong over 7 months and other samples from a male captive dugong await laboratory analysis. We have analysed the progesterone levels of wild dugongs across immature and mature age classes and across both genders. We also have preliminary results on progesterone levels that may indicate pregnancy in wild dugongs. Our laboratory analyses are still ongoing, but we still anticipate an output of scientific publications on this research by December 2009.