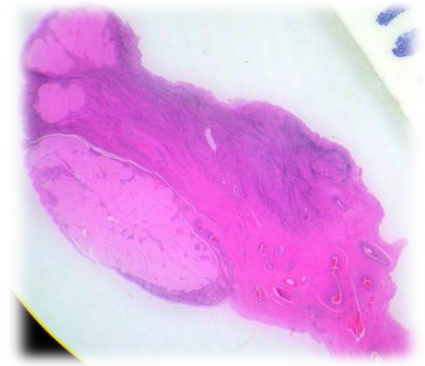
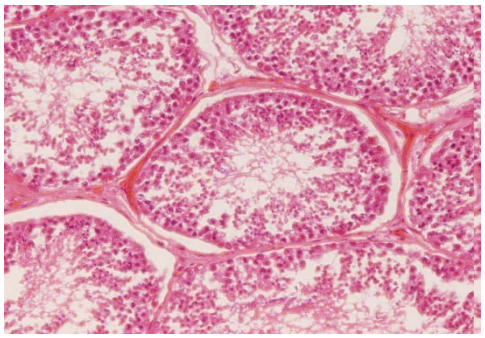
Report of 2009 Bottlenose Dolphin (*Tursiops truncatus*) Life History Workshop: Expanding Network Capabilities

July 6-9, 2009

Compiled by Margaret Lynott
Contributors: Megan Stolen, Wayne McFee, Wendy Noke Durden, James Powell, Teresa Mazza, Susan Barco





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ACKNOWLEDGEMENTS

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Images used in this document were provided by the contributors (Stolen, Durden, and Powell).

EXECUTIVE SUMMARY

The Virginia Aquarium & Marine Science Center Foundation's Stranding Response Program (VAQS) was awarded a grant in 2008 to conduct life history analysis on over 10 years of *Tursiops truncatus* teeth and gonad samples from stranded animals in Virginia. A major part of this collaborative grant included a workshop involving life historians from Hubbs-Sea World Research Institute (HSWRI), NOS, Texas A & M University (TAMU), and University of North Carolina Wilmington (UNCW). The workshop was held at the NOAA Center for Coastal Environmental Health & Biomolecular Research in Charleston, SC on 7-9 July 2009. The workshop convened to 1) address current practices among the groups conducting life history analysis, 2) decide on protocols to follow for the collaborative Prescott grant between VAQS and HSWRI, 3) demonstrate tissue preparation techniques and discuss shortcuts and pitfalls, 4) demonstrate data collection from prepared testes, ovaries, and teeth, and 5) discuss data analysis and prepare an outline and timeline for a future manuscript. The workshop concluded with discussions concerning the current collaborative *Tursiops* Life History Prescott grant award and the beginnings of a collaborative Prescott proposal with members of the Alliance of Marine Mammal Parks and Aquariums to further clarify reproductive analyses. This technical memorandum serves as a record of this workshop.

INTRODUCTION

Life history sampling of cetaceans is a fundamental component of population assessments but is complicated due to sampling limitations of live free-ranging animals, and the fact that these animals spend the majority of their lives underwater. While not an exact replication of the live population, consistent data and sample collection from stranded cetaceans can provide an efficient and cost-effective method of obtaining life history samples for analysis and management purposes.

The most important data involved in life history analyses include age, morphometrics and reproductive status and history. In odontocete cetaceans, teeth provide information on age, while gonads provide information on reproductive status. Population parameters based on life history analyses include: longevity, reproductive history, and social structure of specific groups (*e.g.* mass strandings, distribution, animals with human interaction). Finally, changes in life history data over time, coupled with information on population stressors such as disease, fishery by-catch and other human interaction, can be detected by monitoring parameters such as age at sexual maturity and calving intervals (Reynolds *et al.,* 2000).

The definitive life history study on western north Atlantic bottlenose dolphins (*Tursiops truncatus*) was conducted by Mead and Potter (1990) and included dolphins from what are now considered several stocks/management units along the entire Atlantic coast of the United States. Growth curves and ages at sexual maturity published by Mead and Potter differ from more recently published growth curves for resident *Tursiops* populations in the Sarasota Bay and Indian River Lagoon regions of Florida (McFee *et al.,* 2009; Stolen *et al.,* 2002; Read *et al.,* 1993).

The most recent international cetacean life history workshop was conducted by the International Whaling Commission (IWC) in 1980. Scientists convened at the Scripps Institution of Oceanography in La Jolla, California in September of 1978 to discuss findings and techniques on both aging and reproductive analysis of marine mammals. This workshop was summarized in two publications, both by the IWC (Perrin and Myrick, 1980; Perrin *et al.,* 1984). Since that time, cetacean life history analysis has been conducted by a relatively limited number of scientists. This workshop was planned in part to increase the number of investigators in the eastern United States who are capable of preparing and analyzing life history data, and to develop a consortium of researchers who will collaborate on future marine mammal life history projects.

Wayne McFee of the Charleston NOS lab in South Carolina offered to host this *Tursiops* Life History Workshop. At this workshop, the participants discussed life history analysis techniques and received training in gonad analysis and tooth preparation. Wayne McFee, Megan Stolen of HSWRI, and their staffs provided the technical expertise for the five day training event. The goals of the workshop included: a comparison of current practices (among HSWRI and NOS), demonstrations and discussions of tissue preparation techniques, demonstration of data collection from prepared testes, ovaries and teeth and a discussion of data analyses for future manuscript(s).

Techniques covered at the workshop included: mounting and sectioning teeth on an Isomet© saw; decalcifying teeth; sectioning teeth on a sledge microtome; staining teeth; mounting tooth sections on a slide; gross sectioning of formalin-preserved ovaries; gross preparation of testes of different sizes; and histology of reproductive samples.

WORKSHOP PRESENTATIONS

Dolphin Tooth Anatomy and Sectioning Methods, Wayne McFee

Wayne McFee provided a background in dolphin tooth anatomy and sectioning methods. Methods covered staining thin sections, formic acid etching and xylene-based etching techniques. McFee described standard procedures for sectioning and staining according to Myrick *et al*. (1983) and Hohn *et al*. (1989). He covered common concerns with tooth selection such as mandibular location and shape. Traditionally teeth #13-16 of the lower left jaw are selected (Perrin and Myrick, 1980). Straight teeth cut along the bucco-lingual axis best capture the post-natal dentine necessary for accurate age estimation. Mounting, cutting, and decalcification techniques were all discussed.

McFee also briefly discussed formic acid and xylene-based etching procedures and the potential, novel use of laser ablation-inductively coupled plasma mass spectrometry (LA-ICPMS for age estimation. LA-ICPMS provides lateral and depth resolved trace element analyses in solid samples and could be used to validate classical aging techniques.

Reading Teeth for Age Estimation, Megan Stolen

Megan Stolen presented standard procedures, common pitfalls and recommendations for most efficiently estimating age once samples are prepared.

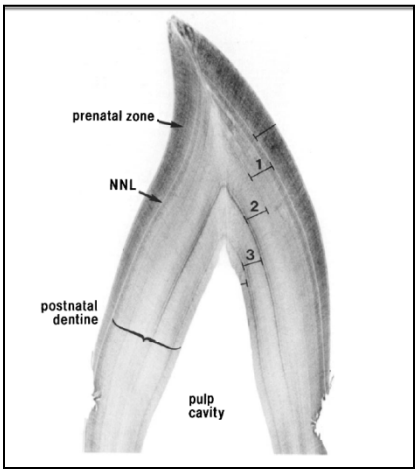

Figure 1: Growth layer groups and dental anatomy. Hohn *et al.* (1989)

Stolen cited the procedures employed by Myrick *et al*. (1983) for *Stenella* spp. and Hohn *et al*. (1989) for *Tursiops* sp. (Figure 1). In addition, she described her process for verifying and resolving

any discrepancies in readings which includes multiple blind readings spaced several days to weeks apart. If two of the three readings agree, provided the third reading is close, this duplicative reading would be considered the estimate. If the three readings are close (within two growth layer groups (GLG)), the median is used as the estimated. If larger discrepancies occur (three or more GLGs apart), a fourth reading is used to resolve the issue. Any tooth that cannot be resolved is recut and the process starts over. Several teeth were presented in slides for demonstration of this process.

Problems described for readings included intensity of stain, off-center sectioning, misinterpretation of accessory layers as GLG's, restructuring, and damage sustained by the tooth either from extraction or wear and attrition. Again, Stolen used slides of stained teeth to demonstrate these common problems.

Evaluating Female Reproductive Maturity in Stranded Cetaceans, Wendy Durden

Although the determination of female reproductive maturity is straightforward, the interpretation of ovarian scars is convoluted and not definitively understood. Reproductive biology and age at maturation are critical to managing cetacean stocks. Wendy Durden discussed this and began her presentation with a general description of the female mammalian reproductive cycle, citing Akin *et al*. (1993). She stressed the complications of cetacean biology and the lack of knowledge on the persistence of ovarian scars throughout life. One question that arises with cetaceans is whether, unlike other mammals, the *corpus albicantia* increase in length and persist with age (Mackintosh and Wheeler, 1929; Dempsey and Wislocki, 1941; Miyazaki, 1977; Marsh and Kasuya, 1984; Akin *et al*., 1993, Boyd *et al*., 1999; Halldorsson and Vikingsson,

2001). Durden suggested that the presence of scars can determine the onset of reproductive maturity but research has found that some cetaceans absorb the scar tissue with time (Harrison, 1949; Sergeant, 1962; Harrison *et al*., 1972; Harrison *et al*., 1981; Brook, 2002; Dabin *et al.*, 2008) and that they are likely not representative of ovulation rate (Benirschke *et al*., 1980; Kirby and Ridgway, 1984; Schroeder, 1990; Brook, 2000; Robeck *et al*., 2005; Sawyer-Steffan and Kirby, 1980; Yoshioka *et al*., 1986). One problem that photo-identification (i.e – sighting) data represents for studies on these animals, even for well documented groups such as the Indian River Lagoon population, is that calves that die in dystocia may not have been documented and thus cannot be correlated to a scar observed during post-mortem analyses once the mother dies. A suggestion for testing the hypothesis that scars persist post-parturition in *Tursiops* would be to study animals born in captivity whose reproductive behavior is well documented. Upon death, the scars from these animals could be compared with the number of known births.

Durden stressed that the entire female reproductive tract should be collected upon necropsy and stored in formalin with the left and right ovaries marked for later examination. This protocol would provide the opportunity to examine ovaries as well as vascular changes within the uterine horns and body indicative of previous pregnancy (Meisner and McFee, 2004). A datasheet (Appendix 3) and method for processing ovaries by 2 mm sections was presented to the group. *Corpora lutea* were classified based on the typical presence of a globular bulge and "yellow-colored" granulosa cells, while *corpora albicantia* were typed (Perrin *et al.*, 1976) 1-6 based on internal and external appearance and diameter (internal) (Akin *et al.*, 1993).

Gross and histological descriptions for *corpora lutea* and *albicantia* were covered during the presentation. The two descriptions were then compared for accuracy. The benefits of histological preparation seemed to outweigh the cost since 9% of *corpora* are missed during gross examination alone.

Male Reproductive Development and Testis Histology, James Powell

James Powell presented on research findings for cetacean testes, basic anatomy and spermatogenesis, brief background on male reproductive studies, current South Carolina research, sampling techniques, histology techniques, and examples of histology findings.

Much life history information can be obtained by studying the testes, including: the age at maturity, the best location for testis sampling, and whether bottlenose dolphins exhibit reproductive senescence. Due to the abundance of *Tursiops* samples from Virginia and other regions, it would be possible to compare the age at maturity between various populations, geographical regions, and areas with differing anthropogenic environmental effects. Testis sampling would provide descriptions of the differences between testes of different species, between individuals or even between testes within the same animal.

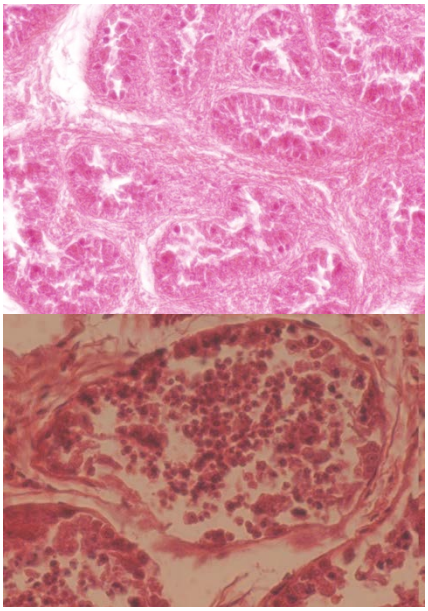

Figure 2: Histological preparation of an immature (top) and mature (bottom) *Tursiops* testis. H&E stain

Historically, age at attainment of sexual maturity in cetaceans has relied on data from captive animals or on the presence or absence of sperm with limited histology collected.

In this workshop, past research on male reproductive development in *Tursiops* was discussed, including life history studies by Harrison and Ridgway (1971) and Sergeant *et al.* (1973). Harrison and Ridgway found that male *Tursiops* reach sexual maturity at a mean length of 260 cm and 10 years of age. Sergeant *et al.* found that male *Tursiops* reach sexual maturity at a mean length of 245 cm and at 13 years of age. Both studies had a relatively small *n* (11, 31, respectively) which alone would not be representative of the much larger populations. Other studies discussed lag time between spermatogenesis and the appearance of sperm in the epididymis demonstrated with short-finned pilot whales (*Globicephala macrorhynchus*) and zonality and asymmetry of testicular development and maturity which has been demonstrated in sperm whales (*Physeter macrocephalus*), sei whales (*Balaenoptera borealis*) and long-finned pilot whales (*Globicephala melas)* (Kasuya and Marsh, 1984; Best, 1969; Masaki, 1976).

The NOS laboratory in Charleston has initiated research which includes age estimates on all archived male *Tursiops* teeth and two separate phases of gonad analysis. Phase I will consist of an age-based analysis of development on all archived testes and Phase II will be a longitudinal gradient study on whole testes from future strandings.

Powell discussed problems concerning storage based on the size of testis. He also presented sampling protocols which would maximize data that can be obtained from the testis while minimizing their storage size (Appendix 4). A brief overview of the histology technique for sampled sections was detailed during the talk and a datasheet with sampling protocols was developed (Appendix 4). Powell concluded the presentation with histology slides detailing anatomical features indicative of sexual maturity (presence of

spermatogenesis, decreased abundance of interstitial tissue) and discussing signs of decomposition which could compromise analysis (Figure 2).

DEMONSTRATIONS

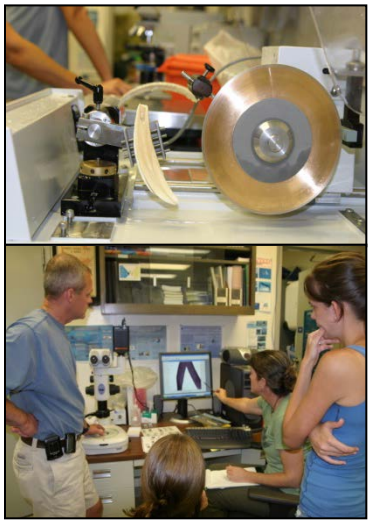

Figure 3: A sectioned sperm whale tooth (top). Stolen demonstrating tooth reading techniques and common complications (bottom).

Following the presentations, the workshop continued in the life history laboratory, which is led by Wayne McFee. VAQS had purchased an Isomet© 4000 precision saw which was brought to the workshop for training purposes. While both NOS and HSWRI utilize the slow cut precision saw manufactured by Buehler, teeth sectioning was demonstrated with both saws. McFee, Stolen and Lynott cut *Tursiops* teeth and a sperm whale tooth using the larger Isomet 4000 (Figure 3). Powell and McFee compared and discussed techniques with HSWRI using the slower precision saw, such as plating of samples and angles of blade contact with the samples.

Megan Stolen discussed proper tooth selection. Examples of angled teeth, tooth damage and problems associated with the age of the tooth were presented to the group (Figure 5). Stolen provided suggestions on how to overcome and avoid these problems when selecting and cutting such teeth. McFee demonstrated the process of formic acid etching with teeth from a young *Tursiops* that stranded in Virginia.

James Powell provided the group with a demonstration of the testis sampling protocol employed in the male reproductive research being conducted by NOS. Testes from a male *Tursiops* that stranded in Virginia were used for the demonstration. A tour of the lab and a demonstration of the histology process at the South Carolina Department of Natural

Resources Marine Resources Laboratory were presented that afternoon. The group was shown how to plate and stain reproductive samples for histological review. That afternoon, Powell presented several slides from male *Tursiops* of different stages of sexual maturity and discussed identifying factors such as the presence of sperm, the size and appearance of seminiferous tubules, and Leydig cells. He also discussed and presented examples associated with advanced decomposition and poor preservation techniques.

Wendy Durden and Teresa Mazza demonstrated their protocol of analyzing and documenting scars on cetacean ovaries on the last day of the workshop. The ovaries from a mature female *Tursiops* that stranded in Virginia were used as an example (Figure 4). The ovaries were photographed, measured, and each external scar was measured and documented. One ovary was cut into 2mm sections and scars within each section were measured and documented. Each section was placed in an individually labeled cassette and stored in formalin. The animal (VAQS20091041) was found to have nine total scars on this particular ovary.

Figure 4: Ovaries from a mature *Tursiops.*

DISCUSSION AND CHALLENGES

Blind Age Estimation

McFee, Stolen, Chris Marshall and Rachel Nueunhoff participated in the blind readings of 32 sets of teeth from *Tursiops* that either stranded (*n*= 30) or were caught during a capture-release study (*n* = 2) along the southeastern United States. Slides were provided by McFee, Stolen and Nueunhoff. Each participant separately read each tooth to the nearest growth layer group (GLG) using a Nikon 1500 stereomicroscope. Ages were estimated using the model for *Tursiops* by Hohn *et al.* (1989). Once ages had been estimated for all teeth by one reader, the next reader completed readings for all teeth. Following the completion of the blind readings, discrepancies were discussed among readers. A Correlation Report (Table 1) was compiled from the results of all readers to demonstrate similar technique and reliability of the methodology. The analysis showed strong concordance among all readers (between 0.93 - 0.98 correlation coefficient). The two most experienced readers showed the strongest concordance between them (0.98). Differences in readings among readers generally occurred for very young animals (<3 y) and older animals (>25 y). This pattern is not an uncommon occurrence among readers of varying experience, especially

Table 1: Correlation Report for Blind Age Estimation Exercise

Correlation Coefficients Matrix:

	Stolen	McFee	Nueunhoff
McFee	0.9832		
Nueunhoff	0.9762	0.9794	
Marshall	0.9482	0.9336	0.9369

Probability Matrix:

	Stolen	McFee	Nueunhoff
McFee	0.0000		
Nueunhoff	0.0000	0.0000	
Marshall	0.0000	0.0000	0.0000

z Value Matrix:

	Stolen	McFee	Nueunhoff
McFee	2.3861		
Nueunhoff	2.2098	2.2824	
Marshall	1.8136	1.6854	1.7118

Degree of Freedom Matrix:

	Stolen	McFee	Nueunhoff
McFee	27		
Nueunhoff	27	27	
Marshall	27	27	27

for young animals as the first GLG is sometimes difficult to identify. These problems are further discussed in Hohn and Fernandez (1999).

Inter-laboratory comparisons are important to determine what biases or techniques may produce disagreement between readers. The results from this comparative study show a strong agreement between readings by Stolen and McFee and a reasonable correlation with Nuenhoff followed by Marshall. This may be due to the greater expertise by McFee and Stolen or due to tooth preparation technique differences between the HSWRI and NOS labs and the lab at TAMU.

Common Challenges and Road Blocks in Age Estimation

Stolen presented possible age estimation pitfalls in her presentation which included: intensity of stain, off-center sectioning, misinterpretation of accessory layers as GLG's, restructuring, and damage sustained by the tooth either from extraction or wear and attrition (Figure 5). Stolen demonstrated the problems that these issues present to the reader with slides and a stereomicroscope in the lab.

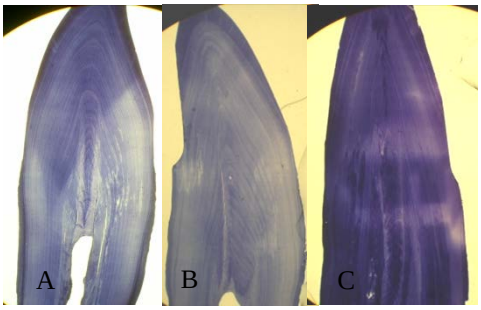

Figure 5: (right to left). *Tursiops* teeth showing (A) accessory layers, (B) wear and (C) dark staining.

Problems with Female Reproductive Analysis

The driving force for research on female cetacean reproductive analysis is to address the lack of concrete evidence on the persistence of ovarian corpora. It is unclear how long

they persist and if these scars are true representations of the animals' complete reproductive (ovulation) history.

Durden also discussed the inconsistency in corpora counts between gross and histological assessments. Histological preparation techniques can make corpora appear more visible (Figure 6). Durden found that 9% of corpora were missed prior to the histological examination, suggesting that gross examination may underestimate corpora counts. While we can assess ovaries to determine age at reproductive maturity, further research could better describe the production and persistence of corpora.

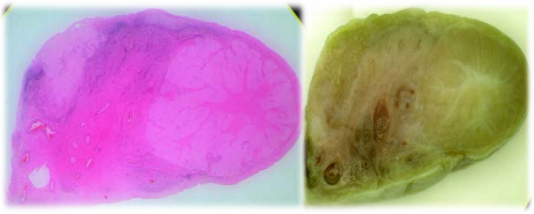

Figure 6: Comparison of histological preparation (left) and the gross view (right) of the same *Tursiops* ovary.

Problems with Male Reproductive Analysis

James Powell described complications that accompany the analysis of male reproductive organs in cetaceans including size, preservation, and decomposition. Mature testes can be large and present logistical problems for long-term storage and preservation. Powell described a sampling protocol of the anatomical areas in the testes that may provide the most information about the cetaceans' reproductive status and functionality yet overcome the challenges in storage and preservation. The large amount of formalin that is needed to preserve testes can be logistically problematic and, if insufficient, would result in sample decomposition. Decomposition renders the samples useless for histological examination and prohibits the assessment of seminiferous tubules and detection of sperm presence within the cells.

FUTURE RESEARCH

The meeting concluded by discussing possible future research projects to better understand *Tursiops* life history. It was suggested that we continue to investigate ovarian scars and male senescence and begin to investigate neonatal characteristics using captive animals as controls. The reproductive history of captive and previously studied free-ranging animals is well documented (Read *et al.,* 1993, Urian *et al.,* 1996). Therefore, it was suggested that samples from these animals be used to test hypotheses on the retention of corpora, senescence, and zonality and asymmetry in testes. Arrangements were made to contact members of the Alliance of Marine Mammal Parks and Aquariums (AMMPA) for the availability of such samples from captive and free-ranging animals.

In 2009, VAQS submitted a collaborative Prescott with HSWRI, NOS, Chicago Zoological Society, and the Mote Marine Laboratory titled *The evaluation of corpora persistence, senescence, and variation in sperm production and their influence on the assessment of reproductive maturity in bottlenose dolphins stranded along the Southeastern United States.* This grant was not funded but received excellent comments and scores. Mote Marine Laboratory submitted a similar proposal to the John H. Prescott Grant Program in 2010 for further collaborative life history work. That project was funded and the work is currently being conducted.

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APPENDIX 1

Life History Workshop Proceedings

July 7- 9, 2009
Charleston, SC

Day 1 (July 7th)

- Presentations
  - Dolphin Tooth Anatomy (McFee)
  - Sectioning and Methods (McFee)
  - Reading Teeth for Age Estimation (Stolen)
  - Evaluating Female Reproductive Maturity in Stranded Cetaceans (Durden)
  - Male Reproductive Development and Testis Histology (Powell)
- Lunch
- Lab
  - Equipment Setup
  - Selecting the best tooth for aging purposes
  - Preparing teeth for sectioning
  - Demonstrations on reading teeth and common roadblocks in the process
  - Tooth Sectioning practiced (bottlenose dolphin teeth)

Day 2 (July 8th)

- AM
  - Continue Tooth Sectioning (sperm whale tooth)
  - Formic Acid Etching practiced
  - Blind Readings conducted
    - McFee, Stolen, Marshall, Neuenhoff
- PM
  - Testis sampling protocol (Powell)
  - Testis histology preparation tour (Powell)
  - Identifying signs of sexual maturity in Testis (Powell)
  - Discussion of Blind Readings, common mistakes

Day 3 (July 9th) Pabst in attendance

- AM
  - Overview of female reproductive sampling protocol
    - Uterine samples (McFee)
    - Ovaries (Mazza/Durden)
  - Sectioning and describing ovaries and scars
  - Reading age of a tooth prepared with formic acid
- PM
  - Discussion of future publication (NOAA Tech Memo)
  - Discussion of collaboration with AMMPA members for “ground truth”-ing reproductive analysis and neonatal characteristics

APPENDIX 2

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APPENDIX 3

Female Reproductive Sampling Datasheet

Field Number: _____
Photos: _____
Date of Stranding: _____
Code: _____
Total Length: _____

Follices Present in Sections: _____

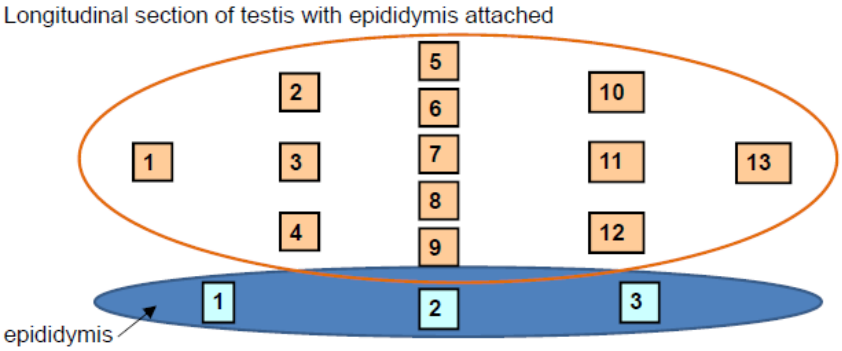
Left ovary sketch:

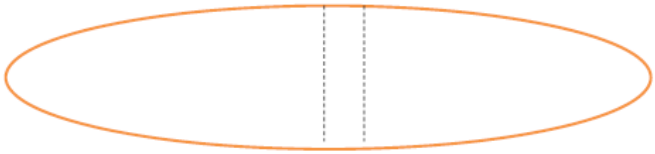
Ovaries: **Left :**
Length (cm) _____
Width (cm) _____
Height (cm) _____
Weight (g) _____
Uterine Body Sampled Y/N
Left horn diameter _____

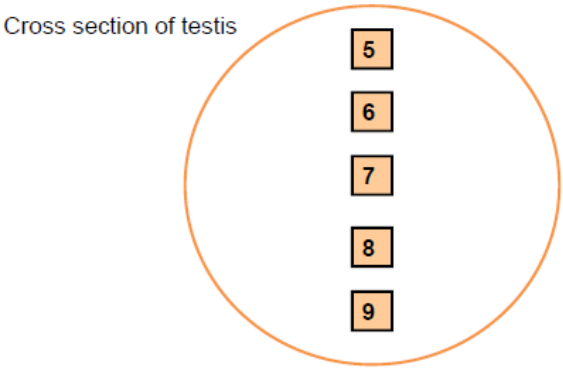
Ca/Cl #1 Present in: Cassette Letters: _____ Diameter (external-mm) _____ Internal: _____ Measured in section: _____ Type: _____ Description:	**Ca/Cl #2** Present in: Cassette Letters: _____ Diameter (external-mm) _____ Internal: _____ Measured in section: _____ Type: _____ Description:
Ca/Cl #3 Present in: Cassette Letters: _____ Diameter (external-mm) _____ Internal: _____ Measured in section: _____ Type: _____ Description:	**Ca/Cl #4** Present in: Cassette Letters: _____ Diameter (external-mm) _____ Internal: _____ Measured in section: _____ Type: _____ Description:
Ca/Cl #5 Present in: Cassette Letters: _____ Diameter (external-mm) _____ Internal: _____ Measured in section: _____ Type: _____ Description:	**Ca/Cl #6** Present in: Cassette Letters: _____ Diameter (external-mm) _____ Internal: _____ Measured in section: _____ Type: _____ Description:

APPENDIX 4

Male Reproductive Sampling Protocol







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