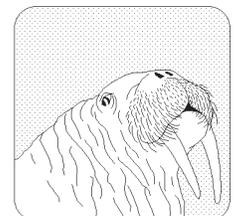
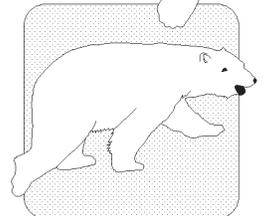
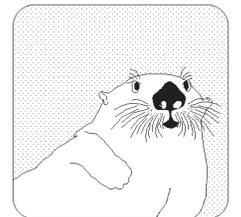
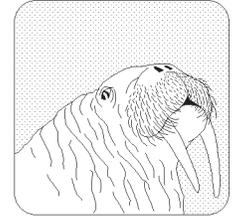
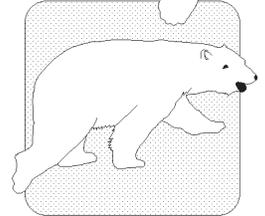
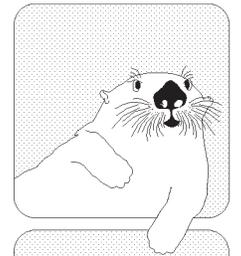


Analysis of the Sea Otter (*Enhydra lutris*) Reproductive Tract: A Methods Manual

Vanessa von Biela and Verena A. Gill

September 2007

MARINE MAMMALS MANAGEMENT
Fish and Wildlife Service
Region 7, Alaska
U.S. Department of the Interior



**Technical
Report
MMM 2007-02**



DEPARTMENT OF THE INTERIOR
U.S. FISH AND WILDLIFE SERVICE
REGION 7
Technical Report

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A Methods Manual**

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INTRODUCTION

Reproduction in the female sea otter, *Enhydra lutris*, was relatively unstudied until Sinha et al. (1966) examined 140 reproductive tracts collected 1955-62 and used their findings to describe sea otter reproductive anatomy and biology. Two years later Sinha and Conaway (1968) published a more detailed paper on the ovary of the sea otter. These descriptive papers have been used as the basis for all subsequent studies of sea otter reproductive tracts.

During biological collections of sea otters in the 1960s and 70s a large number of female carcasses became available to wildlife biologists. Using Sinha's research, Schneider (1973) analyzed 1,482 female reproductive tracts to determine the timing of reproduction, gestation period, age of sexual maturity, fetal sex ratio and growth rate of otters in the Aleutian Islands. A similar study was conducted by Bodkin et al. (1993) on a sample of 177 females collected after the 1989 *Exxon Valdez* oil spill. Recently (von Biela 2007) examined 134 reproductive tracts obtained from beachcast and harvested otters across the three Alaskan population stocks as part of a Master's thesis.

As with most life history data, comparisons among and within populations that differ in status relative to equilibrium densities provide useful data with which to test hypotheses about the cause and effects of changes in demographic rates such as reproductive rate. However, in order to make such comparisons, methods used in different periods must be comparable. The purpose of this manual is to explicitly describe how to collect and analyze sea otter reproductive tracts for the determination of reproductive rate, pregnancy rate, percentage of mature females, and timing of reproduction so that the data will be directly comparable to that collected in the past. The techniques presented in this manual have been used to study sea otter populations over the last 50 years, and maintaining such consistency is essential to comparisons in the future.

This manual is based on the methods of previous researchers and draws heavily on the published and unpublished works of James Bodkin, Karl Kenyon, Calvin Lensink, Daniel Mulcahy, Karl Schneider, and Akhouri Sinha. Most invaluable to the production of this manual were the direct communications with Karl Schneider and Dan Mulcahy. In each instance, researchers have communicated with each other to attain comparable methods. Recognizing that researchers in the future may not have this luxury, this guide has been produced to preserve the technique. In addition to using this manual, researchers should consult with colleagues experienced in the analysis of mammalian reproductive tracts, preferably specific to sea otters. Individuals are encouraged to contact V. von Biela with any questions.

Sea otter reproductive tracts have most commonly come from either intentional sampling through harvests (Sinha et al. 1966, Schneider 1975) or unintentional large scale mortalities (e.g. the 1989 Exxon Valdez oil spill) (Bodkin et al. 1993). Carcasses and reproductive tracts can also be obtained through the collection of fresh beach cast

carcasses. Analysis of reproductive tracts should consider the source of carcasses as samples representing either the “living” or “dead” sea otter population, as they may differ in reproductive parameters. In most cases the reproductive tracts are fixed in formalin or frozen (minimum of -20°C) immediately after collection; both methods are acceptable for later analysis of the tissue. Immediate fixation is preferred as it is a necessary step in analysis. Uteri and ovaries are then examined to determine the current and past reproductive history of each individual. This manual also includes an example datasheet (Appendix A) and glossary (Appendix B).

TISSUE COLLECTION

1. The reproductive tracts should be collected from any beachcast or hunted female carcass with proper authorization from the U.S. Fish and Wildlife Service.
2. To access the tract, place the carcass on its back and cut through the abdominal wall. The reproductive tract is located deep in the abdominal cavity (Figure 1), behind the intestines (Figure 2) and is distinguishable by its “Y” shape (Figures 2 and 3). Be certain that the tract is removed intact (vagina, 2 uterine horns and 2 ovaries).

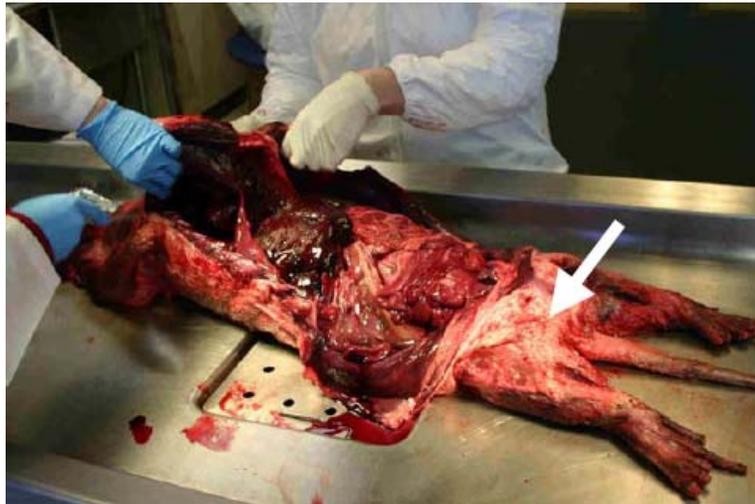


Figure 1: The reproductive tract is located ventrally in the abdominal cavity (arrow indicates the general location of the reproductive tract).



Figure 2: Ventral view of a fresh reproductive tract in the abdominal cavity and after intestinal removal (sample ID: FW05062).

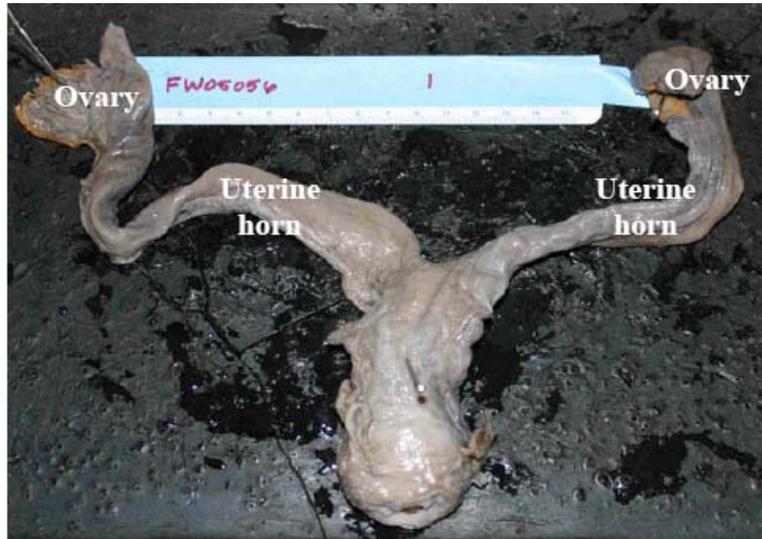


Figure 3: Removed and fixed reproductive tract of a mature female sea otter. Gray coloration is an artifact of the preservation method, when fresh tissue is pink (FW05056).

SAMPLE STORAGE

1. Once the entire reproductive tract (uterus and ovaries) is removed from the sea otter, the tract should be cleaned of excess connective tissue and rinsed in water to clear any fluid.
2. Store and fix the reproductive tract by placing it in a plastic container with 10 parts 10% neutral buffered formalin to 1 part tissue immediately after harvesting. Formalin is a hazardous chemical that should only be used in a fume hood and while wearing protective clothing (nitrile gloves, lab coat, and goggles). Remember to follow the formalin protocols of the lab where the work is being conducted and review the Material Safety Data Sheet for formalin.
3. Label the storage container with the following information:
 - Sample number
 - Reproductive tract in 10% formalin
 - Enhydra lutris*
 - Fixation date
 Keep in mind that if other information is added to the label such as collection location or age, the person analyzing the reproductive tract is no longer blind to those details. In some instances it is important for the research to remain blind to certain details if they will be used for comparisons, such as comparing reproductive parameters by location.
4. Formalin fixed samples should be stored in plastic bottles within a flammable safe cabinet.

5. Tracts that have been stored in formalin for at least 2 weeks can be moved to nalgene plastic containers with 70% EtOH (10 EtOH:1 tissue). This procedure allows tissue examination to take place without formalin exposure, but does not change the presentation of the tissue.
6. If the reproductive tract cannot be immediately placed in formalin, it should be frozen at -20°C. Prior to analysis, the reproductive tract must be fixed in formalin. To properly fix a previously frozen sample, the sample must be completely thawed before being placed in 10% neutral buffered formalin.

GENERAL ASSESSMENT OF TISSUES

The general assessment is an important first step in analysis. The researcher should check that the tissue is a complete reproductive tract and determine if it is immature or mature. Incomplete reproductive tracts are not useful to this analysis. For example, if only one ovary has been collected, it is impossible to determine the past reproductive history of the otter, but it may be suitable for determining maturity.

1. Remove tissue from fixative in a fume hood, and rinse the tissue of excess formalin or EtOH with tap water. Do not dispose of the jar or fixative, as the tissue will be stored in the same container after analysis.
2. Determine if the reproductive tract is intact. At the bottom of the “Y” shape is the muscular vagina, from this point there is a bifurcated uterus with an ovary at the top of each horn (Figure 4). Since ovaries are enclosed in epithelial tissue and often fat, they are most easily found by touch. Ovaries have a glandular feel that will distinguish them from the surrounding tissue.



Figure 4: The same reproductive tract from the previous page with the ovary on the left exposed (FW05056).

3. Use the general appearance of the tissue to determine if the reproductive tract is immature or mature. Record information on the datasheet (see Appendix A).

- Color: immature tracts are usually white or light pink while mature tracts are gray. Color is also dependent on carcass decomposition state, so this trait should only be used in combination with other information from the tract to determine maturity.
- Horn thickness: when laying flat, immature reproductive tracts have thin uterine horns with a depth of <2cm, while mature reproductive tracts are usually at least twice as thick.
- Size: determine if the entire reproductive tract is large or small. Immature tracts are small and will fit in a closed hand.
- Enlargements: note any enlarged areas on the datasheet.

Tracts of nulliparous animals are usually light in color, thin walled, small and with out enlarged areas (Schneider 1973) (Compare figures 4 and 5 in this manual). Further investigation will help you to determine whether enlarged areas of the uterine horns are pregnancies (Figure 14 and 15) or abnormalities (Figures 20, 21, and 22).



Figure 5: Example of a nulliparous tract. Note the thin uteri, light color and overall smaller size compared to the tract in Figure 1 (500014).

UTERINE HORNS

Placental scars are located within the lumen or interior wall of the uterine horns. They are markers of recent (within ~1 year) implantation sites. While they are evidence of past pregnancy they do not necessarily represent births. To locate and identify placental scars:

1. Make an incision along the uterine horn.
2. Open the uterine horn by slowly running the thumb up the lumen.
3. Placental scars may be detected by texture or appearance.
 - Touch: the tissue of placental scars will feel harder to the touch (D. Mulcahy pers. comm.).

- Thickness: the wall of the uterine horn is often thickened at the attachment point of a placental scar (D. Mulcahy, personal communication, Figure 6).
- Color: the lumen wall may also be discolored and/or irregular (Bodkin et al. 1993, Figures 6 and 7).

Use all three methods for the most accurate results.

4. Typically no more than one scar is found in a tract and not all mature tracts will have scars.

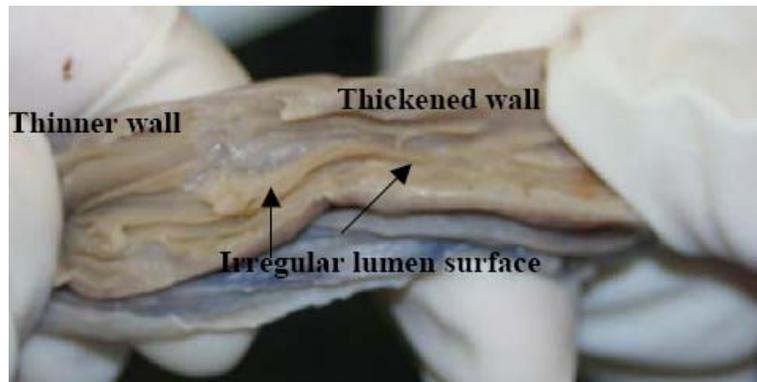


Figure 6: Uterine horn with placental scar detected due to changes in the lumen surface (PW96212).

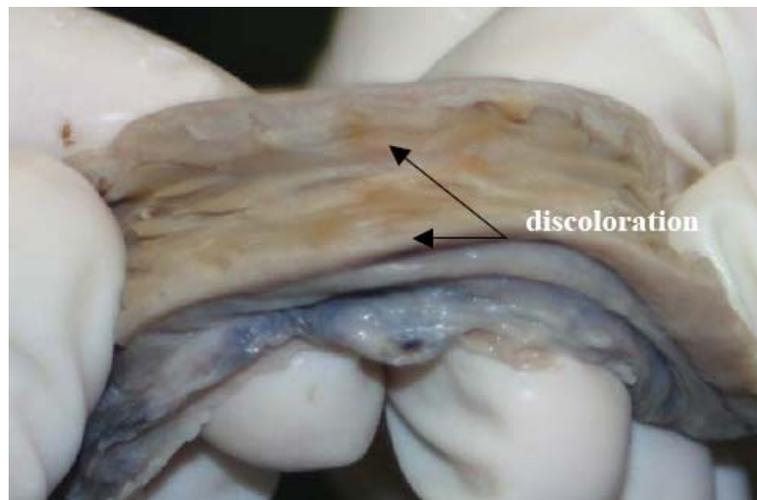


Figure 7: Discoloration in the lumen of the uterine horn indicates a placental scar (PW96212).

OVARIES

Examination of both ovaries can provide data on current and past pregnancies. As with placental scars, each ovarian structure represents a pregnancy event that may or may not have resulted in a birth.

1. Excise the ovaries from the top of the uterine horns. Grasp the top of the horn between the thumb and index finger until the position of the ovary is evident. Cut into the epithelial tissue surrounding the ovary to excise.
2. Record the general appearance and measurements of each ovary
 - Texture: immature ovaries are smooth while mature ovaries are wrinkled. Circle smooth or wrinkled on the datasheet.
 - Size: mature ovaries are much larger (11-23 mm, Sinha and Conway 1968) than those of immature ovaries (7-11mm, Sinha and Conway 1968). Measure the length, width, and depth in centimeters with calipers and record.
 - Mass: mature ovaries generally have a mass >0.50 g (von Biela et al. unpublished). Record a mass for each ovary in grams on the datasheet.
 - Enlarged areas: record the presence of enlarged or white areas on the surface of the ovary (Figure 9).



Figure 8: Notice the difference in color and texture between the ovary of an immature one-year-old animal on the left (500014) and a mature fourteen-year-old female on the right (BS00024).



Figure 9: Mature ovary with enlarged area that was a corpus luteum (BS98003).

2. Make ~1 mm thick “breadloaf” slices (Figure 10). To breadloaf make thin slices, but do not cut through the entire tissue so that each “slice” remains in the correct order.



Figure 10: Breadloaf slices in an ovary (480001)

3. Look in each slice for structures.
 - Larger (5-17mm), round, discolored, filled areas often with a cortex and medulla are corpora lutea (CLs, Sinha et al. 1966, Figure 11).
 - Small, round or oval, white filled areas are corpora albicantia (CAs, Sinha et al. 1966, Figures 12 and 13).
 - Open circles are recently released follicles (Sinha et al. 1966, Figure 13).
4. Record the number of CAs and CLs in each ovary.
5. Measure the diameter of the largest ovarian structure (CL, CA or follicles) and record on the datasheet. Measurements can be used for fine scale classification of reproductive status.

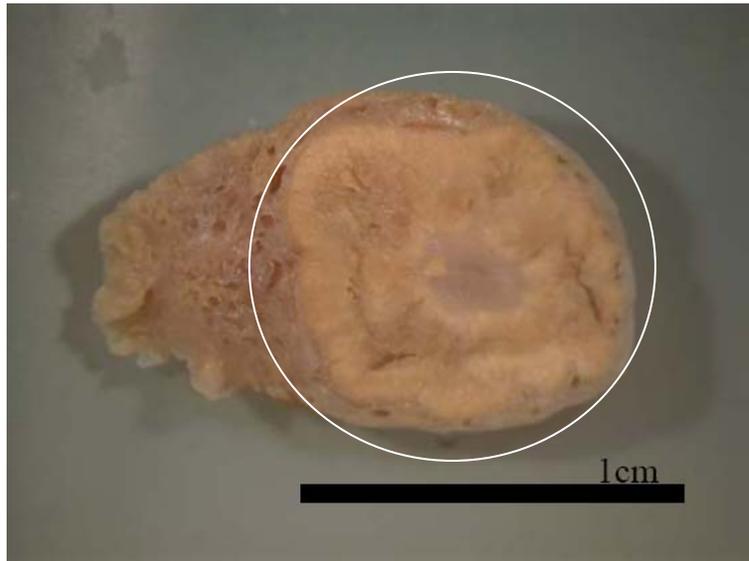


Figure 11: Cross section of a mature ovary with large corpus luteum (see white circle, BS04029).

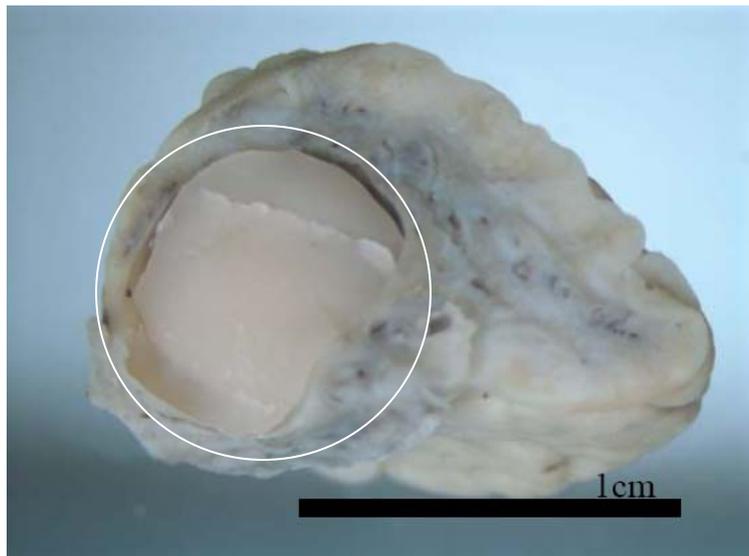


Figure 12: Cross section mature ovary with new corpus albicans (see white circle, structure is regressing from CL, BS04061).

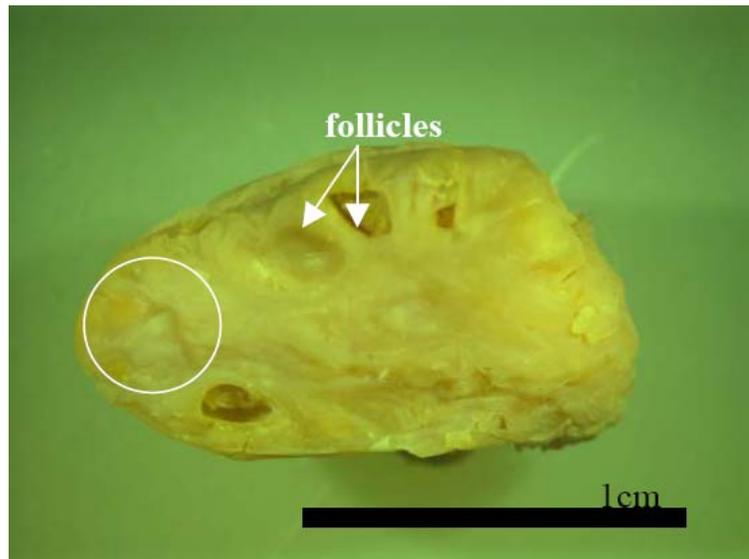


Figure 13: Cross section of a mature ovary with follicles (arrows) and a small corpus albicans identifiable by scar-like appearance (inside circle, BS97036).

PREGNANCIES

The stage of pregnancy can be determined by the corpus luteum size and the state of the fetus.

1. If a CL is present without an enlargement of the uterine horn or a fetus, the female is considered unimplanted pregnant.
2. If a CL is present, and there is an enlargement of the uterine horn or a fetus is present, then the fetus has implanted and the female is classified as implanted pregnant (Figure 14).



Figure 14: An intact reproductive tract from an implanted pregnant sea otter (480001).



Figure 15: Fetus in a bisected uterine horn (480001).

3. If a fetus is visible (Figure 15) it should be removed, separated from the placenta, and weighed without placenta.
4. The straight line distance from the tip of the nose to the end of the tail should be measured while the fetus is on its back. This is called the standard length and is the preferred measurement (Figure 16). Record this measurement in centimeters on the datasheet. It may also be necessary to measure and record the curvilinear length (Figure 17) if this data is going to be compared to a previous study that did so, such as Schneider's study (1973).



Figure 16: Measuring the standard length (tip of nose to tip of tail) of a fetus (480008).



Figure 17: Measuring the curvilinear length (crown of head to crown of rump) of a fixed fetus (480008).

5. If the fetus is at least 5 cm long sex can usually be determined by the presence or absence of a baculum. The baculum, or penile bone, is an elongated raised area located on the midline of the abdominal cavity. (Figure 18).

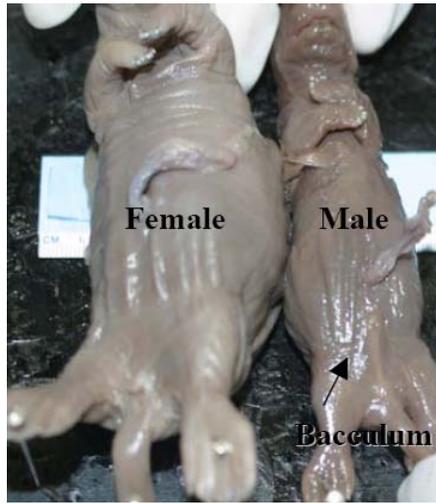


Figure 18: A fixed female (480004) and male (480008) fetus.

ASSESSING REPRODUCTIVE STATUS

Depending on the objective of the study, reproductive tracts can be classified at broad or fine scales. A broad scale is appropriate for determining the reproductive rate or age at first reproduction in a population. However, if the aim of the study is to define the timing of reproduction or compare birth pulses between regions a fine scale of classification is most suitable. Fine scale studies will require a large sample size and samples from all seasons.

Broad Scale

1. Use the data collected from the general assessment, uterine horns and ovaries to classify the current reproductive status as one of the following (from Bodkin et al. 1993):
 - Non-reproductive: no corpus luteum present
 - Unimplanted pregnant: corpus luteum is present, but fetus or swelling of the uterine horn is not.
 - Implanted pregnant: corpus luteum and fetus or swelling of the uterine horn are present.
 - Post partum: female is lactating, a corpus albicans that still contains luteal tissue (Figure 12), or a distained uterine horn is present.
2. Classify the lifetime reproductive status (from Schneider 1973):
 - Nulliparous: no ovarian structures are present.
 - Primiparous: only one ovarian structure (a corpus luteum or a corpus albicans) is present. uterine horns and ovaries are mature.

- Multiparous: multiple ovarian structures present with mature uterine horns and ovaries.

Fine Scale

1. Use the data collected from the general assessment, uterine horns and ovaries to classify the current reproductive status (Sinha and Conway 1968) as one of the following:
 - Anestrus: no corpus luteum or follicles >3.5 mm.
 - Proestrus: no corpus luteum, but follicles >3.5 mm are present.
 - Estrus: no corpus luteum, but one large follicle is present (~10mm).
 - Unimplanted pregnant: corpus luteum is present, but fetus or swelling of the uterine horn is not.
 - Implanted pregnant: corpus luteum and fetus or swelling of the uterine horn are present.
 - Post partum: female is lactating, a corpus albicans that still contains luteal tissue, or a distained uterine horn is present.
2. Classify the individual by lifetime reproductive status in the same manner as if conducting a broad scale study.

LIMITATIONS OF THIS METHOD

Researchers should be aware of the following limitations.

1. Each pregnancy will not result in a birth and the percentage of failed pregnancies cannot be determined. Thus pregnancy rates cannot be equated with parturition rates.
2. Animals that have recently given birth may be accidentally classified as unimplanted pregnant if the animal was collected before the CL could regress to a CA.
3. Many of these mistakes can be avoided by noting a hollow enlargement of the uterine horn that lacks a fetus, or from field notes that indicate the female was lactating or accompanied by a pup.

The technique is dependent on the researcher's consistency in identifying all relevant structures. To ensure data quality all personnel should re-analyze a blind, random, subsample of reproductive tracts and statistically compare the results to estimate consistency. This can be used to assess differences within and among observers.

Because of potential biases identified above, analysis of reproductive tracts may not provide data that are directly comparable to other methods used to obtain reproductive vital rates, such as the longitudinal study of living individuals over time.

ABNORMALITIES

Schneider's 1973 study documented three major types of reproductive abnormalities in sea otters: ovarian cysts, tumors, and resorption events. Overall, the frequency of abnormalities was low (<5% of tracts examined), although rates did increase with age (>10 years). In general, the cause and side effects of the abnormalities are unknown. In a few instances large tumors are associated with reproductive failure (Schneider 1973). Pictures and descriptions of known abnormalities are presented below. If an abnormality is found, a veterinary pathologist should be contacted for specific sampling protocol. If one is not available, find an area where normal and abnormal tissue are juxtaposed and cut out a small area of the tissue containing the normal and abnormal sections. Make sure that the smallest dimension is less than 1 cm. Store this subsample in a plastic container with 10 parts 10% formalin to each part tissue for later analysis by a pathologist. If a camera is available pictures should be taken of all abnormalities.

Ovarian cysts

1. Para-ovarian cysts are fluid filled sacs located near the oviduct (Figure 19, M. Miller pers. comm.).
2. The specimen pictured below was implanted pregnant at the time of analysis. Ovarian cysts may not cause reproductive failure, but the effects of para-ovarian cysts on reproductive rate are currently unknown.
3. If ovarian cysts are present, count the number on each ovary and record the number of cysts in the 'abnormalities' section of the datasheet.



Figure 19: Location of two para-ovarian cysts in a mature female sea otter (480008).

Tumors

1. A tumor is an abnormal mass of tissue that results from excess cell division. Like tumors in other species, it is assumed that tumors in sea otter reproductive tracts can be benign or malignant.
2. Tumors have been located throughout the reproductive tract (Figures 20, 21, and 22).
3. If tumors are present, measure the length, width and height of each tumor and collect a subsample as described in the first paragraph of this section.



Figure 20: A small tumor located in the left uterine horn of a female sea otter (BS97049).



Figure 21: A large tumor in the uterine horn of a female reproductive tract (500027).



Figure 22: Bisected tumor from mature female. Note the lack of definition that distinguished this mass from a developing fetus (500027).

Fetal Resorption

Resorption is the process by which a developing fetus is aborted and the tissue is reabsorbed by the mother. Schneider (1973) found a few tracts that showed evidence of fetal resorption based on the orientation or condition of the fetus.

1. Resorption can be identified by the presences of some developed feature (such as bone) in otherwise undifferentiated tissue (Schneider 1973).
2. Possible reason for resorption were outlined by Schneider (1973):
 - Strangulation with the umbilicus.
 - Too many fetuses in a single uterine horn: Two fetuses in a horn were found late in development, but any numbers of fetuses greater than two were being resorped. This suggests that in sea otters a single uterine horn cannot support more than two offspring.
 - Tumor in the uterus.
 - Fetal resorption is most commonly found in regions where otters are in poor body condition.

ACKNOWLEDGEMENTS

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Appendix A. Datasheet

SEA OTTER REPRODUCTIVE TRACT DATASHEET

ANIMAL NUMBER: _____

LOCATION: _____ **DATE:** _____

RESEARCHER(S): _____

GENERAL ASSESSMENT:

Color (circle one): **WHITE** **LIGHT PINK** **GRAY**

Uterine horn thickness (circle one): **THIN** **THICK**

Size (circle one): **SMALL** **LARGE**

Enlargements (circle one): **YES** **NO**

 IF YES, describe: _____

UTERINE HORN 1 (circle one): **RIGHT** **LEFT** **UNKNOWN**

Number of Scars: _____ Comments: _____

UTERINE HORN 2 (circle one): **RIGHT** **LEFT** **UNKNOWN**

Number of Scars: _____ Comments: _____

.....
OVARY 1 (circle one): **RIGHT** **LEFT** **UNKNOWN**

Texture (circle one): **SMOOTH** **WRINKLED**

Number of CLs: _____ Length: _____ Width: _____ Depth: _____ cm Mass _____ g

Number of CAs: _____ Largest Structure: _____ Diameter: _____ cm

Comments: _____

OVARY 2 (circle one): **RIGHT** **LEFT** **UNKNOWN**

Texture (circle one): **SMOOTH** **WRINKLED**

Number of CLs: _____ Length: _____ Width: _____ Depth: _____ cm Mass _____ g

Number of CAs: _____ Largest Structure: _____ Diameter: _____ cm

Comments: _____

.....

Presence of a fetus: **YES** **NO**

 If YES then: **MALE** **FEMALE** **UNKNOWN**

 Standard Length (snout to tail): _____ cm

Lactating (from field notes): **YES** **NO**

Current Reproductive Status (circle one):

Non-reproductive **unimplanted pregnant** **implanted pregnant** **post partum**

Lifetime Reproductive Status (circle one):

Nulliparous **Primiparous** **Multiparous**

.....

ABNORMALITIES:

Ovarian cysts (circle one): **YES** **NO**
 IF YES, how many on left: _____ right: _____

Tumors (circle one): **YES** **NO**
 IF YES, describe: _____

 Length: _____ cm Width: _____ cm Depth: _____ cm

Resorption (circle one): **YES** **NO**
 IF YES, describe: _____

Appendix B. Glossary

Anestrus- a phase of the reproductive cycle distinguished by the absence of a corpus luteum and follicles over 3.5 mm.

Breadloafing- to make incomplete slices through an organ. Most commonly used when searching for tissue structures or abnormalities.

Corpus albicans (singular), corpora albicantia (plural)- a regressed corpora luteum. Identified by light color and scar like texture.

Corpus luteum (singular), corpora lutea (plural)- an ovarian structure distinguished by it's large size, darker color, and soft texture that is necessary to sustain a pregnancy.

Estrus-a phase of the female reproductive cycle when the corpus luteum is absent and a large follicle (~10mm) is present.

Implanted pregnancy- the period when a fetus has implanted in the uterine wall of a female. Distinguished by the visible swelling of the uterine horn, presence of a corpus luteum. Fetus is not always visible.

Multiparous- an animal that has had multiple pregnancies. Multiparous animals are distinguished by the presence or multiple ovarian structures, multiple placental scars, or a fetus and one or more corpora albicantia.

Nonpregnant- an animal that is not pregnant, determined by the lack of a corpus luteum.

Nulliparous- an animal that has not reproduced, determined by the absence of any ovarian structures or placental scars.

Ovarian structures- any corpora lutea or corpora albicantia.

Post partum- an animal that has recently given birth, determined by lactation, presence of a corpus albicans that still contains some luteal tissue, a rough placental scar, or distained uterine horn.

Primiparous- an animal that has only had one pregnancy, determined by a single corpus luteum or a single corpus albicans.

Proestrus- a phase of the reproductive cycle when the corpus luteum is absent and follicle(s) are over 3.5mm but not as large as estrus follicles.

Unimplanted pregnancy- a term used during the first ~4 months of pregnancy before the fetus has attached to the uterine wall. This stages is determined by an active corpus luteum and absent swelling of the uterine horn.