Research and Management Techniques for the Conservation of Sea Turtles
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IUCN/SSC Marine Turtle Specialist Group Publication No. 4, 1999

Reproductive Cycles and Endocrinology

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Introduction: Why and When to Study Reproductive Systems?

The study of sea turtle reproductive cycles and their endocrine control falls primarily into areas often thought of as basic research. Should this type of research be done at all on threatened or endangered species? This is an important concern that must be considered very carefully before starting any such project. The answer must be "sometimes yes and sometimes no." Four questions should be answered in the affirmative before initiating any study of reproductive physiology of marine turtles:

(1) Has the investigator received scientific peer approval to do the research through proper institutional, state, national, and international permitting agencies? In other words, scientifically speaking, is the project a high priority?

(2) Does the investigator have the technical skills to safely undertake the project from the standpoint of both the animal's and the human investigator's welfare?

(3) Is the proper equipment on hand or available to safely handle the turtles and complete the protocols and analyses?

(4) Since it is expensive, are the financial resources available to do this type of research?

If the proposed project can meet these standards, then the research should go forward. Are these standards too high? They do not appear to be, since at least five separate labs in Australia and the USA have clearly met these standards and made significant contributions to sea turtle endocrine/reproductive science without harm to study animals.

Identifying projects which might be appropriate for sea turtles is critical since one does not want to attempt a protocol which could further damage an already depleted population. For this reason it is useful for the physiologist to collaborate with a conservation biologist (they could be the same person) whenever possible. A set of guidelines were suggested several years ago which still have some application in terms of justifying new research projects in this area (Owens, 1995). They are: (1) identifying critical and possibly unique reproductive processes of major concern to species survival; (2) developing improved techniques for accomplishing high priority applied and basic research; and (3) moving vigorously ahead in basic reproductive physiology research, especially where critical areas have been identified.

Research Potential: What Uses Are There for this Type of Study?

Sea turtles have been surprisingly useful models for reptilian reproductive biology because, despite their awkwardly large size, blood is easily obtained for hormone studies and sea turtles are good surgical patients. The first relatively complete reptilian hormone cycles were documented in green sea turtles from the Grand Cayman Turtle Farm (reviewed in Owens, 1997). Where solid ecological data are also gathered on free-living populations, important life history questions can be addressed, such as, sex ratio of immature populations, chronology of ovulation for the female, fecundity within a season, percent of a population that is reproductively active at one time, and questions of time and age at reproductive maturity. Because of several unusual life cycle traits seen in sea turtles *(e.g.*, late maturity, long life, variable breeding cycles, temperature dependent sex determi-

nation), population modelers need the kind of precise reproductive information that can result from combining detailed ecological (field) studies with carefully designed physiological investigations. The important studies at Heron Island, Australia, show the full potential of such hybrid studies *(e.g.,* Limpus, 1985; Wibbels *et al.,* 1990).

With increased capabilities for capturing and tracking individuals (see S. Eckert, this volume), it should be possible in the near future to vastly improve our understanding of migration controllers and mating systems, as well as nutritional dynamics and stress during reproduction. While an improved understanding of captive breeding potential was an initial motivation as well as a clear product of this research field, the successes of these programs (Wood and Wood, 1980) have actually reduced the need for intensive research in captivity except as it can relate to gaining a better understanding of basic physiology.

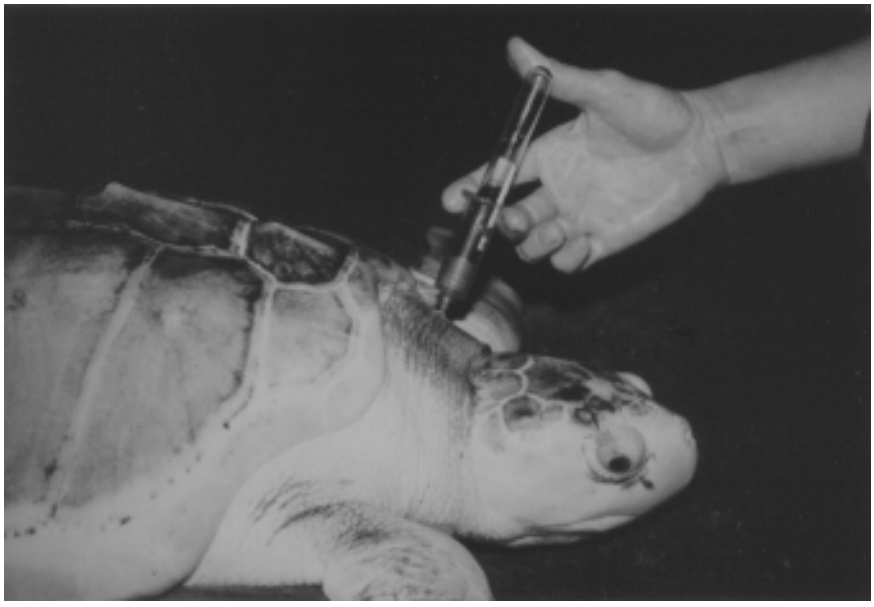

Figure 1. Blood sampling from the dorsal cervical sinus in a Kemp's ridley using a vacutainer system vacuum collection tube. Careful cleaning of the neck region is required prior to sampling.

Specific Techniques

There are four techniques that are often used in endocrine studies: blood sampling, hormone radioimmunoassay, laparoscopic surgery, and ultrasonography. The applications, strengths, risks, and analysis of each will be discussed.

Blood Sampling

Blood is considered a body tissue, as are muscle and bone. The advantage of blood is that it is easy to sample and can provide, through its subcomponents, excellent indicators of many aspects of an individual's health and reproductive status. Taking a blood sample from the sinuses in the dorsal side of the neck is now routine (Owens and Ruiz, 1980). After a modest amount of practice it is possible to obtain a blood sample at least 95% of the time (Figure 1).

Either a syringe and needle or a vacuum tube, needle, and holder system work well for drawing blood. With practice the sample can be taken within 30 seconds. For turtles less than 0.5 kg, a 23 gauge 1/2 inch needle works best. For turtles from 0.5-5 kg, a 1 inch 21 gauge needle is satisfactory, while a 1.5 inch 21 gauge needle works well in most larger animals except leatherbacks (*Dermochelys coriacea*; see below). Lithium or sodium heparin is best for an anticoagulant. EDTA (also an anticoagulant) should be avoided since it causes hemolysis in sea turtle blood. It is important to position the turtle so that the sinus fills with blood. For this reason, consistent results have been obtained when the turtle's head is lower than the body. An angled restraining rack, a slanted table or bench, or an inclined nesting beach (with assistants doing the restraining) all work well. Always carefully clean the neck with alcohol (containing at least 70% concentration of ethanol), or other antiseptic prior to sampling.

The sinus is on either side of the midline of the neck about 1/3 to 1/2 way toward the back of the head from the anterior edge of the carapace. Depending on the size of the turtle, the sinus is from 0.5-3 cm lateral to the midline. There is some variation in individuals, thus is it not unusual to have to insert the needle three to five times in order to locate the sinus. If one side of the neck does not produce blood, try the opposite side. Always insert the needle vertically (90 degrees to the plane of the neck) into the neck and *do not* move the needle laterally to locate the sinus. This will cause unnecessary tissue damage. Once the needle is inserted, apply suction and move the needle slowly up and back down until the sinus is located. *Do not* remove the needle from the neck while still applying

suction as this can damage your sample.

Centrifuge the blood immediately or keep it on ice until centrifuging. Separate the plasma from the red cells and save both fractions, preferably at ultracold temperatures, for research and archiving. Several brands of small, portable electric centrifuges are available from biomedical and scientific supply companies. Ultracold temperatures (< -50°C) are preferred for storing blood products because these cold temperatures reduce protein changes. For the short term, ultracold temperatures may be achieved by dry ice or liquid nitrogen. Long term storage in an electric ultra cold freezer or liquid nitrogen freezer are recommended.

Because their neck sinuses are larger, both species of ridley (*Lepidochelys* spp.) and loggerhead (*Caretta caretta*) turtles (particularly immature individuals) are more easily sampled than are green (*Chelonia mydas)* and hawksbill (*Eretmochelys imbricata*) turtles. Adult nesting females of all species can be problematic to sample after they have been crawling on a beach. The sinus appears to be more constricted than in an animal taken freshly from the water. Sampling leatherbacks is more difficult because their necks are very large and require a long (3 – 3.5 in) spinal tap needle. The leathery skin is exceptionally difficult to penetrate and there are several procedural challenges, including clotting in the long needle (the inside of the needle should be coated with anticoagulant before sampling). A promising alternative technique for sampling leatherbacks from their rear flippers has been described (Dutton, 1996). As a general rule, extracting blood from a female in the process of covering her eggs can be difficult because the sinus is reduced in size and hard to locate. Sampling is easier if one can anticipate the end of oviposition and obtain the sample before covering begins; however, a drawback of this technique is that the turtle may abort the end of her clutch.

Hormone Radioimmunoassay

Just as blood chemistry work for ions or sugars can give an indication of an animal's health status, the specific levels of certain hormones in the circulation can also provide clues as to the precise reproductive or behavioral status of the individual. Many hormone (endocrine) assays are now available in kits from several companies such as ICN Pharmaceuticals (Costa Mesa, California USA) or Diagnostic Products Corporation (DPC) (Tarzana, California USA). Many steroids can be analyzed at veterinary teaching hospitals or specialized labs. Each assay should be validated (proven to work) for the species and hormone being studied. Several U.S. labs currently have good experience with sea turtle assays (see Guillette *et al.,* 1991; Owens, 1997; Wibbels *et al,* 1990). The testosterone-based sex determination technique (Owens *et al.,* 1978) requires a special assay for testosterone which is set sensitive enough to detect hormone at the very low levels seen in young animals (see Wibbels *et al.,* 1993 and Wibbels, this volume). This sensitive assay is not a routine procedure.

Laparoscopy

This form of surgery uses a miniature telescope to directly view inside the peritoneal cavity. It is a potentially dangerous procedure and should not be attempted until proper veterinary training has been obtained (Wood *et al.,* 1983). Laparoscopy can be used to determine the sex of immature turtles or the reproductive status of adults (see also Wibbels, this volume). It can also have value in diagnosing liver, lung, bladder, and intestinal tract problems; however, these types of evaluations require considerable veterinary experience and should not be undertaken by the novice. The minimum equipment necessary is a laparoscope, trocar, sleeve, fiber optics projector, and standard surgical instruments (Figure 2). The estimated minimal cost for this equipment is now about US$ 4000, depending on the size and options of the equipment purchased.

Complete familiarization with sea turtle anatomy is essential prior to doing surgery. In addition, the surgery should be performed in collaboration with a veterinarian until proficiency is developed. It is important to use aseptic techniques at all times to prevent infections. Following a surgical scrub (three alternating applications of 70% ethanol and a surgical iodine soap), the animal is restrained in an inverted position and a local anaesthetic injected into the muscle and dermis of the peritoneal wall of the inguinal area. A 1-2 cm incision is then made just through the skin and the trocar and sleeve used to push through the muscles and peritoneal wall into the body cavity. Particular caution is necessary to avoid an entry that is too far posterior (where the trocar might strike the kidney) or an entry that goes too deep (where the trocar might strike the lung or gut). After entry into the peritoneal cavity is achieved, it should be verified with the laparoscope prior to inflating the body cavity with filtered air. Inflation (known as insufflation) is necessary to visualize the internal organs. When the exami-

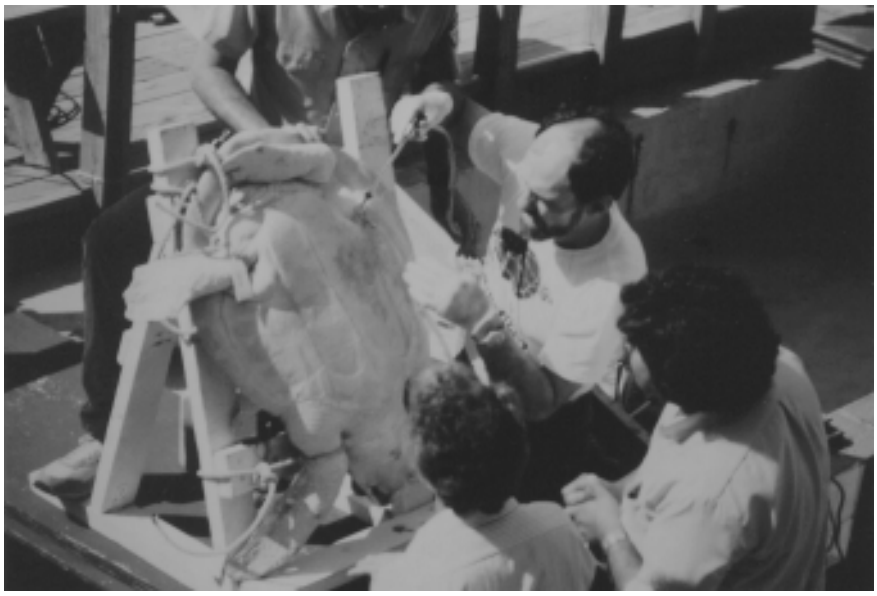

Figure 2. After local anaesthesia the turtle is restrained for laparoscopy in the inverted position. Sterile surgical techniques are required in this procedure to prevent infection. The laparoscope is being inserted into the sleeve which has been introduced into the peritoneum.

nation is complete, all air must be removed prior to suturing the wound. A single deep suture and two superficial sutures are usually adequate to seal the wound.

It is currently common practice to avoid the use of general anesthetics (with veterinary approval) for this particular surgery since a local anaesthetic incurs less risk of mortality, is adequate for reducing apparent pain, and allows a much shorter post-operative observation period (Wood *et al.,* 1982; Wibbels *et al.,* 1990).

Striking vital organs during trocar entry has the potential of inducing severe bleeding and mortality. In sea turtles, even experienced laparoscopists can expect a mortality rate in the order of 1-2%, under good conditions. The two most common causes of mortality include excessive bleeding due to poor trocar placement and death due to non-specific symptoms in a turtle that has already been compromised due to other conditions. For example, an overheated turtle may have a gas-distended lung or gut which can easily be perforated even with the best of technique. In addition, sea turtles with a heavy parasite load, a severe bacterial infection or acute obesity may succumb very easily during surgery. Captive animals are particularly susceptible to infection in the area of the sutured wound. Animals with any of the above-mentioned compromising symptoms must not be subjected to this type of surgery. If a turtle does die during the operation, it is essential to have an independent veterinarian conduct a necropsy to determine the cause of death.

Ultrasonography

The use of ultrasound imaging (Rostal *et al.,* 1990) has proven ideal in the rapid evaluation of an adult female's ovarian condition (Plotkin *et al.,* 1995). While additional research is needed to realize the full potential of this technique, it has some clear advantages over laparoscopic surgery in some situations. Most importantly, it does not require the aseptic technique, incisions, and sutures of surgery. Thus ultrasonography is generally fast, very safe, and non-invasive. An additional advantage is that the reflected images (sonograms) can be stored as video or still frames and exact measurements of structures such as follicles or eggs can be made from the real-time image or from the saved video. The disadvantages compared to laparoscopy are that one does not see the real tissue color or the smaller figures where the structures naturally lack heterogenous densities. For example, it has been difficult to distinguish immature ovaries from immature testes, a task that is easily done using the surgical approach. Other disadvantages are that the instrument, which is essentially a microcomputer with sensing probe, costs several thousand US$ when purchased new and requires a safe and dependable power supply. The ideal field system links a generator to an Uninterrupted Power Supply (UPS) and then to the instrument.

In ultrasounding the ovary, the turtle is placed on its carapace in a comfortable position for restraint (Figure 3). An assistant can easily restrain one of the smaller species (such as a ridley) in an automobile tire while the ultrasound is done. Larger turtles require more assistants to ensure the safety of the turtle, researchers, and machine. Ultrasound has not been very useful in males, but one unusual circumstance should be noted in case it might be of use in sea turtle anatomy and physiology studies. Adult males show a softening of the medial plastron (Wibbels *et al.,* 1991). With ultrasound, one can directly visualize the heart beating as well as blood flow in the major vessels. This is not possible in immatures or females due to their dense plastron shell.

Figure 3. An ultrasound evaluation of the ovary is possible using the ultrasonic probe placed on the inguinal area just posterior to the plastron. No anaesthetic is used as three assistants restrain the turtle on an automobile tire.

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