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To: Sheryan Epperly
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Southeast Fisheries Science Center

From: Jeanette Wyneken
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RE: Summary report on sex identification in *D. coriacea, C. mydas*, and *C. Caretta*

Attached you will find a series of products from our studies of sex identification in neonate leatherback and green turtles and comparisons of the criteria that identify males vs. females. We examined live turtles using laparoscopy as well as dead-in-nest turtles that were preserved in formalin and unpreserved dead-in-nest hatchlings to identify criteria that might also serve in laparoscopic sex identification. Because of their endangered (rather than threatened) status, our permit restricts us from biopsying gonads of leatherbacks and green turtles for sex verification during laparoscopy, as we did in our studies identifying sexes in young loggerheads (Wyneken et al. 2007). Instead, we explored indirect verification using criteria established from the study of dead-in-nest turtles, adding data from a few turtles that were examined laparoscopically shortly after natural mortality unrelated to our studies, and from a few the veterinary biopsies of several lymphatic "cysts" that included a small section of normal gonad.

The examinations of dead-in-nest hatchlings were partitioned into two manuscripts because preservation of the dead turtles resulted in slightly different criteria than we found useful in unpreserved turtles. The results of the preserved turtles studied are published: Ceriani, S.A. and Wyneken, J. 2008. *Comparative morphology and sex identification of the reproductive system in formalin preserved sea turtle specimens*. Zoology 111(3):179-87. We presented the results of that study combined with the unpreserved turtle study at the 28th Symposium on Sea Turtle Biology and Conservation. The presentation is available online at: http://www.seaturtle.org/ists/PDF/final/2468.pdf. The extended abstract for the Proceedings (currently available at http://iconferences.seaturtle.org/preview.shtml?event_id=14amp;abstract_id=2780) entitled *Sex-Specific Morphology Of Neonate Sea Turtles: Methods For Identifying Sex In Formalin-Preserved and Fresh Dead Hatchlings and Posthatchlings* by Ceriani, Wyneken, and Wibbels is included. (I noted that the online abstract comes up several ways: one lists it as a 2005 abstract, another lists it as 2007 presentation, and a third lists it as 2009 presentation.)

We also wrote and submitted a manuscript describing this study for Endangered Species Research. Following peer-review, we were given a choice of publishing the work after revising the title to include the phrase "Preliminary methods…" and having the paper accepted as such or after bolstering our sample sizes of males. We chose to increase the sample sizes. You have a draft of that revised manuscript (*Sex-*

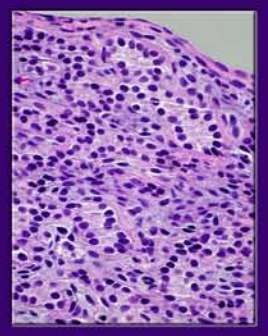
Specific Morphology of Neonate Sea Turtles: A Field Method to Identify Sex In Fresh Dead Hatchlings of Three Species by Ceriani, Wyneken and Wibbels) which we expect to complete when my co-authors return to their respective campuses in a few weeks.

Interestingly, some characteristics that we find in dead turtles do not appear in live turtles (for example scalloped edges of the gonads in dead green turtle hatchlings are not found in most laparoscopies). Similarly, some important characters that we observe in live turtles, particularly in leatherback, such as the completeness of the paramesonephric duct, or the duct's mobility, may be obscured by decomposition in the dead-in-nest turtles. While gonad color and "surface pattern" such as granularity look like promising characters in some sexes of some species, they is not a characters that we can fully assess with current sample sizes.

We are slowly building a videotaped library of laparoscopic exams of leatherback, green turtle, and loggerhead gonads so that the experience one develops in one year, and needs to train one's eye, is not lost between years. Based on the leatherback and green turtle laparoscopies, we drafted an initial incomplete manuscript on laparoscopic sex identification in neonates (currently labeled *Draft Cm & Dc Lap ms Wyneken & Ceriani, others?*). We hope to bolster the sample sizes, particularly for leatherbacks, before submitting this ms, as our statistical power is currently weak.

I provided an Excel spreadsheet with the criteria we used to identify laparoscopically the sexes of leatherbacks and green turtles and the data collected to date. The samples sizes for green turtles remain larger than those for leatherbacks because green are hardier in the laboratory, require considerably less room to grow to a size suitable for laparoscopy (120 g), and because the leatherbacks we bring into the lab are not all good candidates for captive rearing or are good surgical candidates (laparoscopy is a minor surgical procedure).

Overall, we have found the cheloniids show greater gonadal and accessory duct dimorphism than *D. coriacea*. Leatherbacks are far more difficult to sex because some features such as gonad shape and attachment are not reliably dimorphic in this species. Leatherbacks gonads remain at a very early stage of differentiation, even in turtles of 6-8 weeks of age. Several of the dead turtles we examined had both very early testicular and ovarian tissue present in the same gonad.



Comparative Morphology of the Reproductive System in Neonate Sea Turtles

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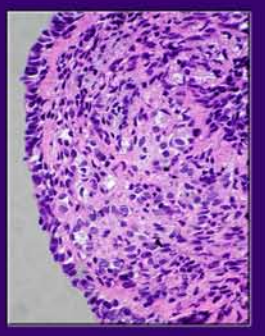

ABSTRACT

We compared the morphology of the gonads, kidneys and reproductive ducts of hatchling and posthatchling loggerheads *(Caretta caretta)*, green turtles *(Chelonia mydas)*, and leatherbacks *(Dermochelys coriacea)* to identify differences in these structures that may be useful in determining sex. *Caretta caretta* served as the baseline against which the other species were compared. Previous studies identified the value of using relative gonad size, shape, gonad attachment and several paramesonephric duct characters to discriminate males from females. These same characters may be used in *C. mydas* with some modifications. The paramesonephric ducts of *C. mydas* are less mobile than those in *C. caretta*. In *D. coriacea* gonads and reproductive ducts are less developed than the same structures in the cheloniid species we examined.

INTRODUCTION

The sex of young sea turtles is difficult to determine because, as in many other reptiles with environmental sex determination, they lack heteromorphic sex chromosomes. Additionally, they are not externally dimorphic until near puberty.

Attempts have been made to visually distinguish between ovaries and testes by using gross gonad morphology[1,2] in untreated gonads or fixed gonads with glycerin clearing[3,4]. However, the two techniques failed to provide consistent results. Some authors consider gonadal histology to be the only reliable method to determine sex in neonate sea turtles[4,5]. Recently, a laparoscopic study[6] using live *C. caretta* posthatchlings (~ 120 g) demonstrated that gross gonad and accessory duct characteristics assessed together are reliable for sex identification.

Here we studied the same gross characteristics used in the laparoscopic study and assessed their value in dead hatchlings and posthatchlings using both unpreserved and preserved specimens. We verified the reliability of the characters with histology, and compared their similarities and differences in loggerheads *(C. caretta)*, green turtles *(C. mydas)*, and leatherbacks *(D. coriacea)*.

METHODS

A. Samples

Our samples consisted of hatchling and posthatchling turtles from Florida, U.S.A. (*C. mydas* [46.5-88.6 mm SCL]; *D. coriacea* [58.4-90.9 mm SCL]), and from Florida and North Carolina (*C. caretta* [39.2-48.7 mm SCL]). Samples sizes were: *C. mydas*, n = 24; *D. coriacea*, n = 15; *C. caretta*, n = 14.

When possible, individuals were observed shortly after death. All the samples were preserved in 10% buffered formalin and examined using a dissecting microscope (magnification 8-26X).

B. Sex-specific Characteristics

The gonads and accessory ducts (**Fig. 1**) were compared among the species. Eleven characteristics were considered (**Table 1**). To compare sizes, a scale was positioned inside the animal at the gonad level and digital photographs were taken. The gonad size was measured using Image J software (Image J v.1 33u, NIH, USA).

C. Histology

To verify sex, gonad samples were prepared as paraffin sections (10 µm) and stained with Gill's hematoxylin and eosin for light microscopy. Sex was identified using established criteria[7, 8, 9].

D. Analysis

Data were compared graphically and statistically. The relationships between gonad size and body size were assessed by Spearman Rank Correlation tests. The sex-specific characters were classified by presence or absence.

RESULTS

We found that gonad size tended to increase with body size in cheloniid hatchlings; however, the correlation was statistically significant only for *C. mydas* (Spearman Rank Correlation test: *C. mydas*: r_s = 0.832, $p<0.05$; *C. caretta*: r_s = 0.529, $p<0.10$). Gonad size and body size are not related in *D. coriacea* (r_s =0.35, n.s.), (**Figure 2**).

Most Florida sea turtle nests are strongly female biased. As a result, our samples are composed of more females than males (*C. mydas*, 23F:1M; *D. coriacea*, 13F:2M; *C. caretta*, 9F:5M).

We determined that the morphology of the gonads and ducts differs between males and females. Four of the 11 gonad and accessory duct characteristics (paramesonephric duct size, mobility, complete lumen, and gonad mobility – if it was tightly adhering to the body wall along its length or not) reliably predicted sex in both Cheloniidae and Dermochelyidae. Two additional characteristics (gonad shape and edge form) were reliable for determining sex only in *C. mydas* and *C. caretta* (**Table 1**).

The morphological characteristics that were sex-specific are detailed in **Table 2** and **Figure 3** for each species. Cheloniids tended to be more similar to each other than to *Dermochelys*. Character presence or absence is summarized in **Table 3**. Gonad size, color, attachment to the mesentery, and surface texture (smooth or granular) were unreliable in these size ranges of turtles.

FIGURES AND TABLES

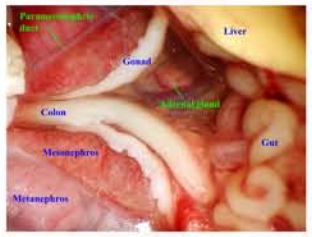

Figure 1. Overview of a sea turtle hatchling urogenital system: gonad and accessory duct structures are found caudal to the lung and attached to the body wall overlying the kidney (mesonephros and metanephros). The paramesonephric duct is lateral to the kidney and is attached to the body wall by a mesentery that is either wide enough to allow movement or short so the duct remains immobile. The paramesonephric duct becomes the Müllerian duct in females and regresses in males.

STRUCTURE	CHARACTER	CRITERIA
Paramesonephric Duct	Size	Small vs. large*
	Duct Presence	Complete vs. incomplete
	Lumen	Complete vs. incomplete*
	Mobility	Mobile vs. low mobility*
Gonad	Shape	Fusiform vs. irregular*
	Edge	Smooth vs. scalloped*
	Mobility	Mobile vs. low mobility*
	Attachment	Asymmetric vs. symmetric
	Size	Small, medium, large[1]
	Surface	Granular vs. smooth
	Color	White, cream or pink

Table 1. *Structures, characters, and criteria developed to distinguish sex in hatchling and posthatchling turtles.*

[1]Measurement or proportional length or area. *Reliable characteristics in both Cheloniidae and Dermocheliidae; *Reliable only in Cheloniidae.

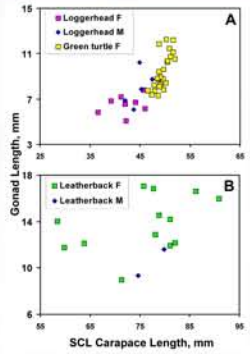

Figure 2. A: Gonad length as function of body size (SCL= straight carapace length) in *C. caretta* and *C. mydas* hatchlings. F, female; M, male. In the *C. mydas*, the relationship is significant (Spearman-Rank Correlation r_s = 0.743, $p < 0.05$). For *C. caretta*, r_s = 0.529, $p < 0.10$. B: Gonad length/body size relationships in *D. coriacea* varying in age between 1 day and 7 weeks. Analysis reveals no significant correlation between body size and gonad length (Spearman-Rank Correlation r_s =035, n.s.).

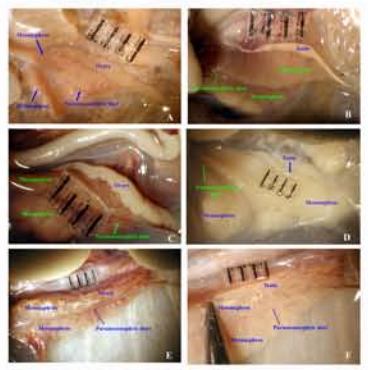

Figure 3. Gonad and accessory duct gross morphology by sex among the 3 species. *Caretta caretta*: female (A), male (B); *Chelonia mydas*: female (C), male (D); *Dermochelys coriacea*: female (E), male (F). **Gonad characteristics**: Cheloniid females (A, C) have an irregular gonad shape with a scalloped edge. Dermochelyid females (E) have long elliptically shaped gonads with smooth edge. Males of the three species have a regular and fusiform gonad shape with smooth edge (B, D & F). **Paramesonephric duct (PD) characteristics**: Females of the three species have a large and complete PD with complete lumen (A, C & E). Males of the 3 species have a small PD with incomplete lumen (B, D & F). Cranial is to the right in each picture. The kidney (mesonephros and metanephros) is used as landmark to locate the gonad. (**Scale** = 3 mm).

STRUCTURE	SEX	*Caretta*	*Chelonia*	*Dermochelys*
Paramesonephric Duct	Female	· Large · Complete · Mobile · Complete lumen	Same as *Caretta*	Same as *Caretta*
	Male	· Small · Complete (n = 4) or incomplete (n = 1) · Absent or low mobility · Incomplete lumen	Same as *Caretta* but only incomplete PD (n = 1)	Same as *Caretta*
Gonad	Female	· Irregular shape · Scalloped edge · Mobile	Same as *Caretta*	· Regular & fusiform · Smooth edge · Mobile
	Male	· Regular and fusiform shape · Scalloped or smooth edge · Low mobility	Same as *Caretta* but only smooth edge found	Same as *Caretta* but only smooth edge found

Table 2. *Morphological characteristics of the gonads and accessory ducts by sex and species.* (PD = paramesonephric duct).

Species	Sex	Paramesonephric Duct Characteristics				Gonad Characteristics		
		Large size	Complete duct	Complete lumen	Mobile	Fusiform shape	Smooth edge	Very Mobile
Caretta	F	+	+	+	+	-	-	+
	M	-	+/-	-	-	+	+/-	-
Chelonia	F	+	+	+	+	-	-	+
	M	-	-	-	-	+	+	-
Dermochelys	F	+	+	+	+	+	+	+
	M	-	+/-	-	-	+	+	-

Table 3. Comparisons among species of occurrence of sex-specific characteristics.

DISCUSSION AND CONCLUSIONS

(1) Cheloniid hatchlings and posthatchlings (represented by *C. mydas* and *C. caretta*) show greater differentiation of the gonads and accessory ducts than do *D. coriacea* young. Despite these differences, several morphological traits are reliably sex-specific in the three species.

(2) Gonad mobility and accessory duct characteristics together reliably identify sex in dead hatchlings and posthatchlings of *C. caretta*, *C. mydas* and *D. coriacea*.

(3) Gonad characteristics used in previous studies such as size, color, and granularity were not reliable.

(4) The sex-specific characters we identify are consistent with several of those found to be reliable in laparoscopic examination of loggerhead posthatchlings[6].

This is the first demonstration of how these several morphological characteristics together may be used to identify sex in deceased neonate sea turtles.

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