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Evaluating Noninvasive Methods of Sex Identification in Green Sea Turtle (Chelonia mydas) Hatchlings

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ABSTRACT. — The goal of this study was to test the effectiveness of analyzing sex hormone profiles to sex green sea turtle (Chelonia mydas) hatchlings. To assess the efficacy of this method, 200 eggs of C. mydas were incubated at five different temperatures, and a chemiluminescent immunoassay system was used to determine the sex hormone profiles (estrogen and testosterone) in the amniotic fluid (from eggshells) and plasma of a subset of C. mydas hatchlings. Results were compared to a standard histological technique and revealed that evaluating sex hormone profiles from amniotic fluid is an effective, noninvasive technique for determining hatching gender.

KEY WORDS. — temperature sex determination; Estradiol; Testosterone; Guangdong Province; Sea Turtle Bay

A common practice for sea turtle conservation is captive rearing, which can include head-starting, ranching, and farming (Meylan and Ehrenfeld 2000). Because sea turtles exhibit temperature-dependent sex determination (TSD) (Merchant-Larios et al. 1997; Wibbels 2003), human manipulation of egg incubation has an effect on the sex of the hatching and furthermore the sex ratio. Sea turtles are long lived, slow to mature, and cryptic for most of their life histories; hence, the effect of sex ratios of hatchlings on future populations is unknown but is predicted to have a large influence (Casale et al. 2002; Kaska et al. 2006). Fieldwork on natural populations have shown a female bias in hatchlings, juveniles, and subadults that range from a 1:1 sex ratio (female: male) (Wibbels et al. 1993) up to 9:1 (Mrosovsky and Provancha 1992; Hanson et al. 1998). It is crucial to accurately determine the sex of hatchlings before release to track sex ratios, as well as replicate natural sex ratios in manipulated clutches (Hamann et al. 2010).

An ideal method to determine hatching sex would be simple and inexpensive and would minimize the stress on an individual. The sex of adult sea turtles can be easily differentiated through the sexually dimorphic tail length. However, the sex of juvenile and hatching individuals is difficult to determine based on external morphology (Zhang et al. 1995; Wibbels 2003). The most common methods to sex nonadults are gonadal biopsy histology (Yntema and Mrosovsky 1980) and laparoscopy (Blair 2005; Chaloupka and Limpus 2005). The gonads may be considered morphologically immature in all sea turtle species (Larios 1999), but these two methods can be heavily invasive and sometimes require euthanization (Wibbels 2003; Hamann et al. 2010), rendering the individual useless for head-start and release. A third method is analyzing sex hormones in blood (Owens et al. 1978; Wibbels et al. 1987) or amniotic fluid (Gross et al. 1995). This method is simple and minimally to not invasive, depending on the fluid collected. Gross et al. (1995) developed the amniotic fluid method for the loggerhead turtle (Caretta caretta) using radioactive tracers (Larios 1999). Braun-McNeill et al. (2007) recommend that radioimmunoassay (RIA) should be used in conjunction with laparoscopies and that environmental conditions (e.g., seasonal effects on testosterone concentrations) should be considered carefully. But with technological and methodological advances, sex hormone detection is often performed using chemiluminescent immunological methods. Chemiluminescent immunological methods are advantageous because they are sensitive, allow high throughput, are relatively affordable, do not require use of radioisotopes, and exhibit long-term stability (Woodhead and Weeks 1985; Zhang 2004; Qiu et al. 2010). However, this methodology has not been evaluated on any other turtle species.

In this study, we tested whether profiling sex hormones is an effective way to sex hatchlings of Chelonia mydas and compare these results to Gross et al. (1995). We evaluated efficacy by comparing sexing of hatchlings via hormone profiles of amniotic fluid to hormone profiles of blood, as well as gonadal histology. In addition, we provide a detailed guide to gonadal morphology/histology of C. mydas and comment on the pivotal temperature of C. mydas in southern China.

Study Site. — From June 2006 to January 2008, experiments were conducted at Huidong Gangkou Sea Turtle National Nature Reserve (HGSTNNR) in Guangdong Province, China (22°33’N, 114°54’E; also named China Sea Turtle Bay). HGSTNNR is located at the southernmost point of the Renping Peninsula, between Daya Bay and Honghai Bay. The nesting beach is
approximately 1 km in length, and 1–20 female *C. mydas* have nested here every year for the past few decades (Chan et al. 2007). Eggs were collected from this stretch of beach.

**Assessment Methods.** — A total of 200 eggs from four clutches laid by two female turtles were collected and transported back to the laboratory to be marked, measured, and weighed. Fifty eggs were collected from each clutch. For the experiments, eggs were divided into two sets of 100 (50 eggs from each female). Each set was further divided into 5 groups, 20 eggs for each temperature (10 eggs from each female) and incubated in microcomputer-controlled incubation boxes that maintained a constant temperature and humidity (Shanghai Permanent Science and Technology Limited Company, model LHS-150SC). Humidity was maintained at 85%–90%, whereas temperatures in five boxes were set to 28.0°C, 28.5°C, 29.0°C, 29.5°C, or 30.0°C (0.1°C resolution). Eggs were buried in sand and covered with sponges, with the animal pole position of embryos pointing upward. Incubation boxes were checked every day until the day of hatching. This protocol was repeated twice, once for each of the two sets of eggs.

From the 200 eggs, 161 successfully hatched. Thirty individuals were randomly chosen (6 from each temperature) from the pool of successfully hatched turtles. When these 30 hatchlings emerged from their eggshells, the remaining amniotic fluid was taken with a pipette for sex hormone analyses. The amniotic fluid was preserved by freezing at −240°C.

Sex hormones in both amniotic fluid and blood serum were analyzed with a U.S. Beckman Coulter ACCESS2 automatic microparticle chemiluminescence immunoassay system (Boever et al. 1986; Bidlingmaier et al. 2009; Xin et al. 2010). Student *t*-tests in SPSS 11.0 were used to determine significant differences (*p* < 0.05).

**Results.** — Based on histological work, the sex of the 30 euthanized hatchlings was determined to be 16 male and 14 female. Hatchling gonads were identified by a white strip located in the front of the ventral kidney surface. The male reproductive system consists of paired testes, epididymis, vas deferens, and a penis, whereas the female reproductive system is composed of paired ovaries, mesenteries, oviducts, and a clitoris. The morphology of male and female gonads (preserved in Bruin’s fluid) are shown as viewed by eye (Fig. 1), a dissecting microscope (Fig. 2), and an optical microscope (Fig. 3).

When trying to locate the position of hatchling gonads, it is best to first identify the bean-shaped kidney and find the white structures on ventral side of the kidney (Fig. 2). By eye, the thickness of the ovary appears uneven and has two wavy edges, and oviducts are apparent in the abdominal wall (Fig. 1). In contrast, males have a pair of smooth testes and degenerative
Muellerian Ducts. It should be noted that, because of their small size, ovaries and testes are sometimes difficult to distinguish in hatchlings. In these cases, histological preparations can be used as an additional tool for determining sex of hatchlings (Fig. 3).

Observed by using a dissecting microscope (Fig. 2), the ovary is white, crescent-shaped, and has tapered ends, and the mesovarium is partially visible. In males, the testis is long, white colored bars, whereas the epididymis is yellow, leaf-shaped, and is located outside of the testis.

Observed by using an optical microscope (Fig. 3), both male and female gonads consist of cortex and medulla. An ovary has a thick cortex, in which there are many immature oocytes of different sizes, and has relatively few cavities in the medulla. The testis, in contrast, has a thinner cortex and a medulla with more cavities (Fig. 3).

The 30 individuals sexed using histological methods were also sexed analyzing sex hormone profiles in both amniotic fluid and serum. These sex hormone results were correlated with histological results. The data from estradiol (E$_2$) and testosterone (T) content in both amniotic fluid and serum are shown in Table 1.

All biochemical values for respective sex hormones detected in serum and amniotic fluid were statistically indistinguishable ($p > 0.05$). Female hatchlings had significantly higher levels of estradiol compared to males ($p < 0.05$), while male hatchlings had significantly higher levels of testosterone ($p < 0.05$). In addition, E$_2$:T ratios for female hatchlings were significantly higher than that found in males ($p < 0.05$).

E$_2$, T levels, and E$_2$:T ratios from amniotic fluid and serum were plotted, and there seemed to be a critical value for each when differentiating between males and females (0.15, 0.15, 1.5, respectively; Figs. 4, 5). Using these critical values, accuracy rates of determining sex based on amniotic fluid E$_2$ levels, T levels, and E$_2$:T

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**Figure 2.** The location and morphology of *Chelonia mydas* hatchling gland; left: female, right: male. From edges of the ovary, the mesovarium attaches the ovary to the peritoneum that overlays the kidney (slides prepared by ZRX).

**Figure 3.** The cross-section of a 2-month-old *Chelonia mydas* hatchling’s gonad; left: ovary, right: testis (slides prepared by ZRX).
ratios were 86.7%, 83.3%, and 96.7%, respectively. Sexing of individuals based on serum hormone levels produced an accuracy of 76.7%, 83.3%, and 96.7%.

Based on our results, using E$_2$:T ratios to distinguish male and female produced the highest accuracy (96.7%), with amniotic fluid and serum showing the same results. When E$_2$:T ratio was greater than 1.5, the hatchling was female; if the ratio was less than 1.5, the hatchling was male.

Using the E$_2$:T ratio value from the previous analysis, we sexed the remaining 131 individuals by analyzing hormone levels in serum from the animals larger than 100 g. The results from these analyses are shown in Table 2. Based on our data on sex ratio, the pivotal temperature seems to be approximately 29.5°C (Table 2; Fig. 6). As expected, when incubation temperature was below 29.5°C, the proportion of male increased, whereas temperature above 29.5°C produced more females (Fig. 6).

**Discussion.** — When studying sea turtles, the ability to accurately determine the sex of individuals is important, especially when many conservation programs incubate eggs and head-start hatchlings for release. Wibbels (2003) and Hamann et al. (2010) stated that the most accurate method of sexing hatchlings currently available is histological examination of the gonads from hatchlings. *Chelonia mydas* has a long lifespan and can take more than 40 years before reaching sexual maturity (Goshe et al. 2010). As adults, sex can be determined by examining the length of the tail, but this method can be ambiguous (Wibbels 2003). At our study site (Sea Turtle Bay), male *C. mydas* have a long tail, equivalent to about 50% of the straight carapace length (SCL), whereas a

### Table 1. Content and ratio of estradiol (E$_2$) and testosterone (T) in amniotic fluid and serum.

<table>
<thead>
<tr>
<th>Gender</th>
<th>n</th>
<th>Sample</th>
<th>E$_2$ (ng/mL)</th>
<th>T (ng/mL)</th>
<th>E$_2$:T</th>
</tr>
</thead>
<tbody>
<tr>
<td>♂</td>
<td>16</td>
<td>Serum</td>
<td>0.132 ± 0.037</td>
<td>0.186 ± 0.058</td>
<td>0.788 ± 0.338</td>
</tr>
<tr>
<td>♀</td>
<td>14</td>
<td>Serum</td>
<td>0.205 ± 0.050</td>
<td>0.105 ± 0.030</td>
<td>2.000 ± 0.438</td>
</tr>
<tr>
<td>♂</td>
<td>16</td>
<td>Amniotic fluid</td>
<td>0.120 ± 0.035</td>
<td>0.194 ± 0.071</td>
<td>0.735 ± 0.425</td>
</tr>
<tr>
<td>♀</td>
<td>14</td>
<td>Amniotic fluid</td>
<td>0.207 ± 0.019</td>
<td>0.106 ± 0.020</td>
<td>2.005 ± 0.316</td>
</tr>
</tbody>
</table>

![Figure 4.](image) Content and ratio of estradiol (E$_2$) and testosterone (T) in amniotic fluid of *Chelonia mydas* hatchlings (n = 30).
female’s tail is only about 6% of the SCL (Xia, Z.R., 2008, unpubl. data). However, sexing juvenile turtles based on external morphology is difficult (Wibbels 2003). Zhang et al. (1995) reported that male and female hatchlings could be distinguished based on external morphology, such as body shape; a male hatchling is smaller and its carapace oval, whereas a female is larger and has a more circular carapace. However, it should be noted that body size of hatchlings depends heavily on nutritional condition and is unreliable. Traditional methods using histological analysis are effective in sexing juvenile turtles because it is easy to distinguish between testes and ovaries (Figs. 1–3) but require euthanizing individuals (Hamann et al. 2010).

Gross et al. (1995) distinguished loggerhead turtle hatchling sex according to $E_2:T$ ratios; when the ratio of $E_2:T \geq 1.25$, the loggerhead turtle was a female hatchling, and when the ratio of $E_2:T < 1.25$, the hatchling was male. The success rate of this technique was 96%. In our work, by using a critical value of $E_2:T = 1.5$, we had a similar success

**Figure 5.** Content and ratio of estradiol ($E_2$) and testosterone ($T$) in blood serum of *Chelonia mydas* hatchlings ($n = 30$).

**Table 2.** The hatchling turtles and the number of males and females under different incubation temperatures.

<table>
<thead>
<tr>
<th>Temperature</th>
<th>Hatchling</th>
<th>$\Omega$</th>
<th>$\Theta$</th>
<th>$\Omega/\Theta$</th>
<th>Percentage of female (%)</th>
<th>Percentage of male (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>28.0</td>
<td>30</td>
<td>4</td>
<td>26</td>
<td>1/6</td>
<td>13.33</td>
<td>86.67</td>
</tr>
<tr>
<td>28.5</td>
<td>34</td>
<td>7</td>
<td>27</td>
<td>1/4</td>
<td>20.59</td>
<td>79.41</td>
</tr>
<tr>
<td>29.0</td>
<td>39</td>
<td>15</td>
<td>24</td>
<td>5/8</td>
<td>38.46</td>
<td>61.54</td>
</tr>
<tr>
<td>29.5</td>
<td>27</td>
<td>14</td>
<td>13</td>
<td>1/1</td>
<td>51.85</td>
<td>48.15</td>
</tr>
<tr>
<td>30.0</td>
<td>31</td>
<td>20</td>
<td>11</td>
<td>2/1</td>
<td>64.52</td>
<td>35.48</td>
</tr>
<tr>
<td>Total</td>
<td>161</td>
<td>60</td>
<td>101</td>
<td>3/5</td>
<td>37.27</td>
<td>62.73</td>
</tr>
</tbody>
</table>
rate (96.7%). The differences in critical value may be an interspecific difference and should be investigated further.

With the exception of Gross et al. (1995), estradiol and testosterone levels are usually sampled via blood (Jessop et al. 2000; Al-Habsi et al. 2006). However, in our work, we found that results from amniotic fluid in hatched eggs produced almost identical results to blood. Sampling amniotic fluid is highly advantageous in that it is a simple and noninvasive method.

Pivotal temperature (PT) can differ interspecifically, intraspecifically, and between geographical populations (Mrosovsky 1988; Horacio et al. 1997; Gabriel et al. 1999). Specifically for *C. mydas*, estimates of PT have been reported between 29.4°C and 29.5°C in Suriname (Godfrey and Mrosovsky 2006) and 29.2°C in the eastern Mediterranean. Our results for turtles nesting at HGSTNNR are similar to previous findings, with a PT of 29.4°C–29.5°C in southern China.

Our work has shown that hormone profiles from amniotic fluid are an effective way to sex *C. mydas* individual, especially hatchlings. Also, to minimize the stress on individuals, turtles at different developmental stages should be sexed using different protocols. For hatchlings emerging from eggs, collecting amniotic fluid from the discarded eggshell for sex hormone assessment is recommended. For hatchlings where amniotic fluid is not available, collecting blood is a feasible alternative for sex hormone assessment, because approximately 1 mL of blood can be obtained from the carotid sinus without sacrificing the hatching. Chaloupka and Limpus (2005) recommend that endoscopy or blood samples for hormone analyses can be used to determine the sex of an individual older than three years. Furthermore, a recent advance has been the adaptation of surgical laparoscopy to determine sex of several months old posthatching turtles (Wyneken et al. 2007; Hamann et al. 2010). Finally, in the case of adult turtles, males and females can usually be distinguished by tail length, but when ambiguous (Wibbels 2003), sex hormone assessment can also be used. Using such methods should facilitate the study and conservation of sea turtles in China and around the world.

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