Histological validation of gonad gross morphology to sex juvenile loggerhead sea turtles (*Caretta caretta*)

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Sea turtles exhibit sexual dimorphism only as adults, hence diagnosing the sex of hatchlings and juveniles requires the employment of different techniques that vary in their level of accuracy and costs. In order to validate the observation of external gross morphology of gonads as a sexing method for juveniles, we compared results obtained in this way with those obtained through histology in 99 loggerhead turtles with curved carapace length (CCL) ranging from 24.0 to 69.0 cm, found in the Adriatic Sea and in the central Mediterranean. Sex was correctly diagnosed in 92.9% of the 99 cases. The highest error rate due to wrong or uncertain sexing was found in turtles with a CCL less than 30.0 cm (33.3%). In turtles with a CCL of 30.0–40.0 cm and 40.0–50.0 cm, the error rates were low (5.3% and 6.7%, respectively), while no errors occurred in larger individuals (CCL greater than 50.0 cm). The results show that gonadal morphology is a reliable sexing method for large juveniles, but for those of less than 30 cm CCL we recommend verification by histology.

Key words: gonads, histology, methods, sex determination

INTRODUCTION

The sex ratio is among the most important parameters for understanding the demography of a species and is essential information in assessing the reproductive potential of a population. Hence, knowledge of sex ratio values and their possible temporal changes is of fundamental interest, particularly for conservation of endangered species such as sea turtles. As with most reptiles, the sex of sea turtles is environmentally determined by the incubation temperature (temperature-dependent sex determination, TSD), with a 1:1 sex ratio being produced at a pivotal temperature, T_p (Janzen & Paukstis, 1991; Mrosovsky & Pieau, 1991). Although some level of interpopulation variation in T_p exists in some sea turtles, it still seems to be evolutionarily conservative, varying slightly around 29 °C across most species and populations. Higher incubation temperatures will result in a greater proportion of females, whilst temperatures below T_p will produce more males (see Wibbels, 2003 for a review). In the light of global climate changes and their effects on various ecological processes (see Stenseth et al., 2002 for a review), the impact of temperature fluctuations on sex ratios of animals with TSD has attracted the attention of researchers, raising conservation concerns (e.g. Janzen, 1994; Hays et al., 2003).

Sea turtles exhibit sexual dimorphism only as adults, which makes sex ratio studies on these animals even more difficult (Wibbels, 2003). For example, in the Mediterranean an average male loggerhead turtle (*Caretta caretta*) starts developing secondary sex characteristics (a longer tail) at a curved carapace length of 70 cm (Casale et al., 2005), so in general sex identification based upon external morphology is not possible in turtles below this size. Diagnosing the sex of hatchlings and juveniles therefore requires the employment of different techniques, which vary in their level of accuracy and costs (for review see Marchant-Larios, 1999; Wibbels, 1999, 2003; Wibbels et al., 2000). Sex identification of juveniles additionally requires sampling of turtles at sea or of stranded individuals, making juvenile sex ratio the most difficult to obtain. On the other hand, juveniles represent the greatest part of a population, so studies on juveniles may provide a valuable insight into the demographic structure of populations (Wibbels, 2003; Casale et al., 2006), even though determining the population of origin in shared developmental habitats may be a complicating factor in interpreting the results.

The methods mostly used for sexing live juvenile sea turtles are assay of blood hormones (Owens, 1978) and observation of gonads by laparoscopy (Wood et al., 1983; Wyneken et al., 2007), while in dead turtles sex is usually determined through observation of the external gross morphology of the gonads during necropsy (Work, 2000). Although laparoscopy and serum testosterone are considered the best methods for sexing juvenile sea turtles, both have constraints: testosterone assay requires validation of the accuracy of the analytical method with turtles of known sex, whilst laparoscopy requires specialized equipment and specially trained researchers (Wibbels et al., 2000). Therefore, determination of sex by visual examination of gonads and associated structures

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Table 1. Results of sex determination in loggerhead sea turtles by gross morphology of gonads (external morphology) validated by histological analysis (histology), and corresponding error rates (CCL = curved carapace length). See text for detailed explanation. *P* values are for Fisher's exact tests.

	External morphology				Histology					Error rate (%)		
CCL (cm)	Males	Females	?	% females	Males	Females	% females	Total number	*P* value	Wrongly sexed	Uncertain sexing	Total
20–30	3	5	1	62.5	6	3	33.3	9	0.347	22.2	11.1	33.3
30–40	16	20	2	55.6	18	20	52.6	38	0.820	0.0	5.3	5.3
40–50	13	17	0	56.7	15	15	50.0	30	0.617	6.7	0.0	6.7
50–60	6	8	0	57.1	6	8	57.1	14	1.000	0.0	0.0	0.0
60–70	5	3	0	37.5	5	3	37.5	8	1.000	0.0	0.0	0.0
Total	43	53	3	55.2	50	49	49.5	99	0.565	4.0	3.0	7.1

during dissection of dead stranded individuals is certainly the easiest and most affordable sexing method in juveniles and has been widely used in numerous studies (e.g. Cannon, 1998; Koga & Balazs, 1996; Stabenau et al., 1996; Casale et al., 2006; Lazar et al., 2006).

The gross morphology of male and female gonads is similar in all sea turtle species and it is well described in the literature (for reviews see Wyneken, 2001; Miller & Limpus, 2003). However, in hatchlings at least, the gross appearance of the gonads alone is not considered the best indicator of sex, and it is influenced by the experience of an observer (Whitmore et al., 1985). Thus, visual sex identification has routinely been verified by histology (for a review see Wibbels, 2003), which is considered the most accurate method for sexing hatchling sea turtles (Mrosovsky & Benabib, 1990).

So far, visual examination of gonads as a sexing method has been validated by histology in hatchlings and posthatchlings (for reviews see Wibbels, 2003; Wyneken et al., 2007), but not in larger juveniles, probably due to the assumed reliability of the method and higher levels of gonadal differentiation in larger turtles. However, in many cases necropsies and subsequent visual sex determination are performed on more or less decomposed carcasses and on animals that have been deep-frozen, which may alter some morphological structures of the gonads and gonadal ducts. The aim of this study was to verify the reliability of visual examination of gonadal gross morphology as a method for sexing dead loggerhead juveniles as available under common circumstances.

MATERIALS AND METHODS

We performed general necropsies on the carcasses of 99 juvenile loggerheads ranging from 24.0 to 69.0 cm curved carapace length (CCL), notch to tip (Bolten, 1999) (mean CCL: 41.8 cm; SD: 10.3), that had been captured dead in fisheries, stranded dead on the beach, or died in rescue centres, in the period 2000–2004. The turtles were found in two regions of the Mediterranean: the Adriatic Sea (Croatia and Slovenia; *n*=58) and the central Mediterranean waters around the island of Lampedusa, Italy (*n*=41).

Visual sex identification was based upon gross morphology of gonads (shape, surface and attachment) and gonadal ducts (presence of oviducts with ostium in females, and epididymis and vas deferens in males), following Wyneken (2001). For each of the two study regions sex identification was done independently, by two research groups. After visual sex determination, we sampled the gonads and fixed the tissue in 10% formaldehyde for a minimum of 48 h, after which we renewed the fixative and stored the samples at 4 °C until processing. Later, the tissue was dehydrated through a graded series of alcohol, embedded in paraffin, and sectioned at 8 μm. Sections were stained with hematoxylin and eosin, and examined with a light microscope by an expert histologist (G.L.). Sex determination based upon histological analysis of gonads followed Miller & Limpus (2003). Gonads composed of flat, monostratified surface epithelium and seminiferous tubules with more or less differentiated germ/spermatogenic cells were determined as testes; ovaries exhibited a membranous structure, folded, often partly transparent, enclosing spherical follicles.

All cases where the observer was unable to diagnose the sex from gross morphology of gonads (uncertain sexing), or where the sex determination differed between these two methods (wrongly sexed) were classified as an incorrect sex diagnosis. Based upon carapace length, we divided loggerheads into five groups: 1) CCL <30.0 cm (*n*=9), 2) CCL = 30.0–40.0 cm (*n*=38), 3) CCL = 40.0–50.0 cm (*n*=30), 4) CCL = 50.0–60.0 cm (*n*=14) and 5) CCL = 60.0–70.0 cm (*n*=8). We calculated the error rate (ER) as the percentage of turtles with an incorrect sex diagnosis due to wrong and/or uncertain sexing. We tested the impact of ER on sex ratio determination between the two methods with a two-tailed Fisher's exact test.

RESULTS AND DISCUSSION

Overall, the sex was correctly diagnosed in 92.9% of the 99 cases, and the error rate (7.1%) did not significantly affect the sex ratio as determined by morphology of gonads (Fisher's exact test, *P*=0.565). The highest error rate (33.3%) was found in turtles of <30.0 cm CCL (Table 1); in two cases (22.2%) it was due to sex misidentification, while in one case the observer was not able to diagnose the sex. However, due to the small sample size in this size-class (*n*=9), sex ratios did not differ significantly between these two methods (*P*=0.347). In turtles with CCL of 30.0–40.0 cm and 40.0–50.0 cm, the error rates were 5.3 and 6.7%, respectively, whereas in larger turtles (CCL

>50.0 cm), no error was observed (Table 1). All loggerheads that were sexed wrongly or with uncertainty were males, indicating that this is the sex with higher morphological ambiguity, and suggesting that gonadal gross morphology in small turtles may bias sexing results towards females.

Although we expected some discordance between the two methods, the error rate in small juveniles (CCL <30 cm) is high and is mostly attributable to sex misidentification (22.2%; Table 1). In a similar study on hatchlings, ER ranged between 5.0 and 25.0% (n=60), depending on the observer and characteristics used for sex determination (Whitmore et al., 1985). Likewise, in a sample of 244 biopsied posthatchlings (85–88 mm straight carapace length) originally sexed by laparoscopy, histology showed an overall accuracy of 97%, increasing from 86% in the first year of the study to 92% in the second and 100% in the third year (Wyneken et al., 2007).

There are three plausible explanations for such error rates in our study. First, our sample of turtles of less than 30 cm CCL is small and it is possible that error rates would decrease with increased sample size. Second, we found the level of gonad development to be surprisingly low in some specimens in this group. In some animals, smoothness of the surface and serration of the edges of the gonad did not differ markedly between ovaries and testes, unlike the descriptions of typical juvenile gonads (Wyneken, 2001). In the case of one specimen (CCL = 25.0 cm) we were unable to diagnose the sex by gross morphology of gonads, not even by the presence/absence of coiled vas deferens or oviducts with ostium (Wyneken, 2001). Nonetheless, we found that juvenile gonadal ducts are well developed in the majority of smaller juveniles, and are a good indicator of sex in addition to the gross appearance of gonads. Third, visual sex determination of (post-) hatchlings was done on live turtles (Wyneken et al., 2007), fresh material, or the samples of fresh gonads preserved in fixative (Whitmore et al., 1985). In our case, however, turtles were deep-frozen after recovery, and thawed prior to dissection for a period of one to three days, so the process of decomposition perhaps resulted in loss of some visual markers.

In larger loggerheads with a higher level of gonadal differentiation (30–50 cm CCL) we found a five- to six-fold decrease in ER, while no errors occurred in juveniles of greater than 50.0 cm CCL. As a result, there was no significant difference in sex determination between the methods applied for loggerheads of more than 30.0 cm CCL (P>0.05, Table 1). In all these turtles, male and female gonads and associated structures exhibited a typical gross morphology (Wyneken, 2001; Miller & Limpus, 2003).

Our study, like most other at-sea studies on juvenile sea turtles, was done in inshore waters, where larger, neritic-stage individuals constitute the majority of the population. Therefore, it is likely that such studies will always suffer from low sample sizes for small juveniles (CCL <30 cm) and, consequently, less experience for researchers in visual sexing during necropsies.

In conclusion, our results show that sex determination based upon visual examination of the gross morphology of gonads is a reliable sexing method in juvenile sea turtles with a CCL of greater than 30 cm. However, in smaller juveniles (<30 cm CCL), this method may lead to incorrect estimates of sex ratios, underestimating the number of males and biasing sex ratios towards females. Hence we recommend verification by histological examination of gonads.

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