

## Determining Hatchling Sex

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### Introduction

Sexual differentiation in mammals depends on the transformation of the undifferentiated gonad into a testicle. The gene that controls this initial event is SRY and is localized in the short arm of the Y chromosome (Koopman *et al.*, 1990). In non-mammalian vertebrates, SRY related genes of the SOX family are found in both sexes and are independent of the presence of sex chromosomes (Tiersh *et al.*, 1991). The identity of the gene or genes that control sexual differentiation in non-mammalian vertebrates is still unknown. However, as in placental mammals, morphological sexual differentiation in the embryo seems to initiate in the embryo's gonad. It is reasonable to postulate that the factor or factors required for sexual differentiation act primarily at the level of this organ, controlling its transformation into an ovary or a testicle.

Although the physiological mechanism by which temperature or other environmental factors influence sexual differentiation is unknown, vertebrates have been divided into two groups: (1) Organisms in which environmental factors have no influence over their sexual differentiation are classified as having Genotypic Sex Determination (GSD); (2) Organisms whose sex determination is influenced by the environment, undergo an Environmental Sex Determination (ESD) (Bull, 1983). In sea turtles, determination of sex by temperature has been found in *Caretta caretta* (Yntema and Mrosovsky, 1980), *Chelonia mydas* (Miller and Limpus, 1981), *Dermochelys coriacea* (Rimblot *et al.*, 1985), *Lepidochelys olivacea* (Morreale *et al.*, 1982), *Lepidochelys kempii* (Shaver *et al.*, 1988), and *Eretmochelys imbricata* (Dalrymple *et al.*, 1985).

### Identifying Sex in Hatchlings

There is a thermosensitive period (TSP) for sex determination during development placed around the second third of total time of incubation. TSP is defined as "that time span or developmental-stage span outside of which temperature manipulations do not exert any influence on sexual phenotype" (Mrosovsky and Pieau, 1991). Furthermore, in all species of marine turtles, there are no external morphological characters which may be used to determine the sex of organisms at hatching stage, and only through dissection and direct observation of the gonads is this possible.

### Invasive Methods (Dissection)

Three procedures, based on morphological observations, are available: (1) direct observations of the gonads in situ; (2) clearing technique of gonads in toto; (3) histological study of the gonads.

Criteria based on (1) are concentrated on gonadal morphological details observable immediately after the viscera that cover them (*e.g.*, intestines, liver, stomach) are removed. Gonads appear as two clear bands that extend along the length of the kidneys (mesonephros). McCoy *et al.* (1983) attempted to sex *L. olivacea* gonads based on the fact that ovaries tend to have a wrinkled surface and are larger in size than the testicles. As this criteria is questionable, van der Heiden *et al.* (1985) proposed method (2) which requires the dissection of the urogenital complex (gonad and kidneys) and fixation in 10% formalin. Afterward, the gonad is separated from the kidney and submerged in 100 ml of 4% formalin solution and 5 ml glycerol (a few drops of copper sulphate should be added to avoid fungal contamination). Using a dis-

section microscope, these authors sexed *L. olivacea* and *C. mydas* hatchlings. Besides the gross external morphology, from which the sexes can be clearly distinguished when the material is processed while still fresh (ovaries have a wrinkled surface and are larger than testicles), gonads in their interior also show clear differences. This is particularly true in the anterior and posterior ends (since they are narrower there) where more detailed observation is possible. According to the authors, testicles are distinguishable by a granular appearance that possibly corresponds to the presence of seminiferous tubules.

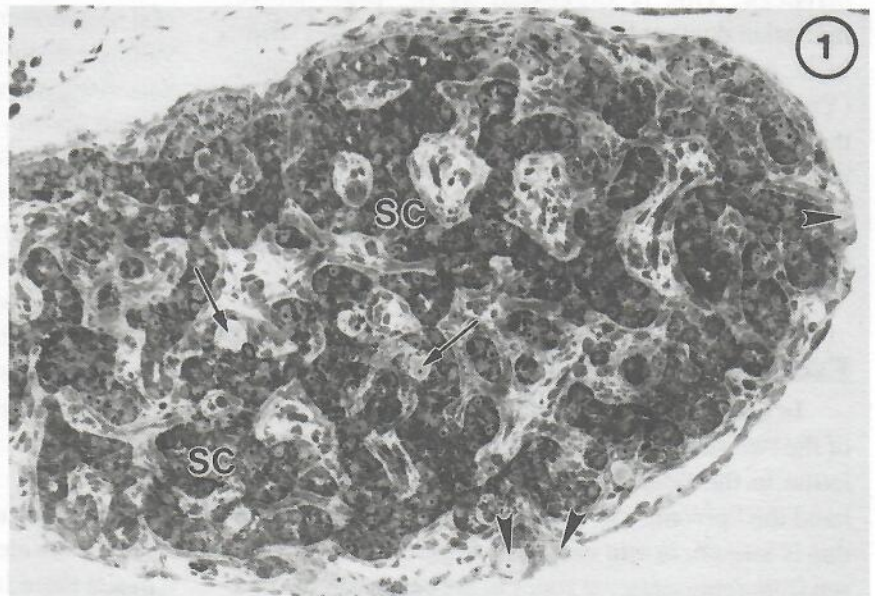
In spite of the ease with which the previous procedures can be performed to determine the sex of hatchlings, some authors have expressed serious concerns of their value, proposing that the only reliable criterion is provided by a histological study of the gonad (Mrosovsky and Benabib, 1990; Mrosovsky and Godfrey, 1995). In this case, gonads need to be fixed, dehydrated, embedded in wax or plastic from which stained sections can be obtained and observed under the microscope. An adequately equipped laboratory is required for this procedure.

For the purpose of exemplifying key features, a detailed interpretation of hatchling gonad histology, based on an embryological study of *L. olivacea* hatchling gonads (see Merchant-Larios *et al.*, 1989), is presented below:

In males (Figure 1), the surface epithelium is flat, monostratified and frequently contains various germinal cells. The medullary cords appear separated from the surface epithelium although some remain attached to it. The medullary cords, surrounded by a basal membrane, are formed by epithelial type cells with abundant lipid droplets. Germ cells are scarce and there is no lumen within the medullary cords that would justify the name of "seminiferous tubules", the correct term would be "seminiferous cords". Among these formations and bordering the surface epithelium there is a basal membrane and abundant stromal tissue, formed largely by extracel-

lular matrix, fibroblasts cells and blood vessels. Ovaries are distinguished by a conspicuous thickening of the surface epithelium (Figure 2). It appears as a columnar epithelium, one or more cells in thickness. It contains a thick basal membrane that separates the surface epithelium from the medullary region of the gonad. The medullary cords are vestigial and appear as small groups of epithelial cells surrounded by a basal membrane. Stromatic tissue is abundant in the medullary region.

In our experience it is possible to combine procedures (2) and (3) sequentially, taking advantage of the practical advantages of the first and the precision of the second. In cleared gonads, the sex can be easily identified but only if they are well differentiated and the preservation is satisfactory. However, in difficult cases of inter-sexes (alternating regions along the gonad with well developed cortex and medullary cords) or indifferentiation of the gonads (when both medullary cords and surface epithelium remain poorly developed), the same cleared gonads can be dehydrated in ethanol and embedded in paraffin or plastic for a further histological analysis. Preservation is excellent as can be seen in Figure 3.



**Figure 1.** *Lepidochelys olivacea* testicle fixed 3 days after hatching. One may clearly appreciate the seminiferous cords (Sc) formed by epithelial cells with dense cytoplasm due to numerous lipid granules. Some germ cells are situated in the cords (arrows) and others in the epithelial surface (arrowheads). Semithin section (2mm) fixed with paraformaldehyde-glutaraldehyde (Karnovsky, 1965), post-fixed with OsO<sub>4</sub> and embedded in Epon. 200X magnification.

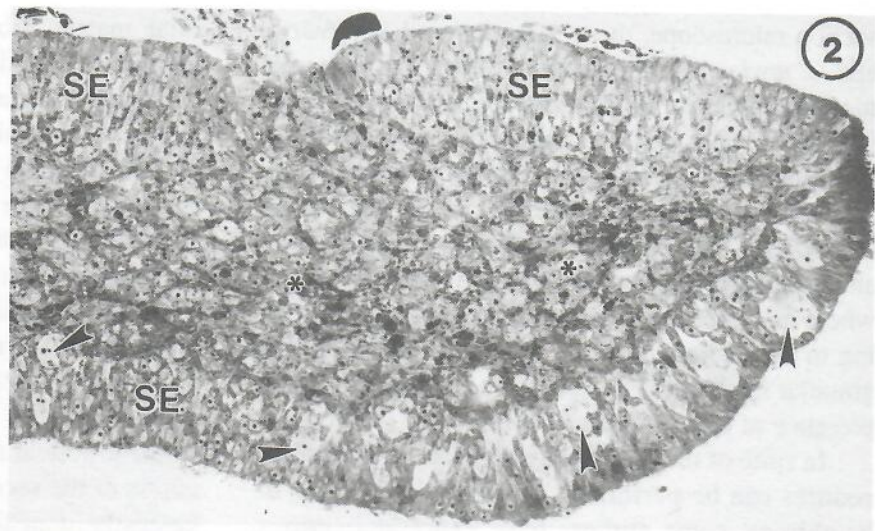
## Non-invasive Methods (Radioimmunoanalysis)

A non-invasive method for the diagnosis of sex in recently hatched organisms has been attempted. Gross *et al.* (1995) reported the possibility of sexing *C. caretta* hatchlings using radioimmunoanalysis (RIA) of the chorioallantoic and amniotic fluids (CAFs). They found that in males the ratio of estradiol (E) to testosterone (T) concentrations is significantly lower than in females, allowing them to predict sex with acceptable precision. The same authors found a similar E:T ratio in plasma from hatchlings of the same species. In olive ridley turtles we have carried out RIA of serum prior to and post-hatching of various steroid metabolites including E and T (Merchant-Larios and Salame-Méndez, unpubl. observ.). Unfortunately, no significant differences could be found in any of the metabolites which would permit a distinction of sex in this species. It is possible that in *C. caretta*, the endocrine activity of the gonads is more advanced than in *L. olivacea*. This is suggested by the presence of mullerian ducts at hatching in the latter species while in *C. caretta* they have disappeared almost totally (Yntema and Mrosovsky, 1980). Bearing in mind these significant differences in developmental timing between species, it is recommended that hatchling hormonal patterns for the species under study and its correlation with sex be established before RIA is used as a method for sex identification.

## Estimating Sex Ratios in Hatchlings

In order to make an estimation of the proportion of the two sexes (sex ratio) present in a natural population in the field, it is convenient to know beforehand the "pivotal" or "threshold" temperature. Once this is known, *in situ* nest temperatures of nests chosen from representative zones in the beach can be used to extrapolate the overall temperature range and, from these, derive the sex ratio for the rookery during the particular season nesting season.

The pivotal temperature is defined as the incubation temperature at which the resulting sex ratio in the clutch is 1:1. Experimentally, this value is obtained



**Figure 2.** *Lepidochelys olivacea* ovary fixed two days after hatching. The surface epithelium (SE), formed by a columnar, multistratified epithelium, contains numerous germ cells (arrowheads). In the medullary region, there are remnants of fragmented medullary cords (\*). Sample processed as in Figure 1 200x.

from incubating groups of eggs at various constant temperatures and determining the resulting proportion of male and female hatchlings. At a range of temperatures still allowing normal development (around 24-34 C), one can determine the masculinizing and feminizing temperature range (which produce 100% males or females, respectively) and estimate the pivotal temperature (50% of each sex). Precise, constant values are difficult to determine because of genetic variations of the individual specimens in each experimental group (see Mrosovsky and Pieau, 1991, for a major discussion on this point). For sea turtles species which have been studied, the pivotal temperature reported is around 30°C. Studies of *C. caretta* (Mrosovsky, 1988) specimens have shown variations of pivotal temperature as high as one degree centigrade, depending on nest size and genetic factors.

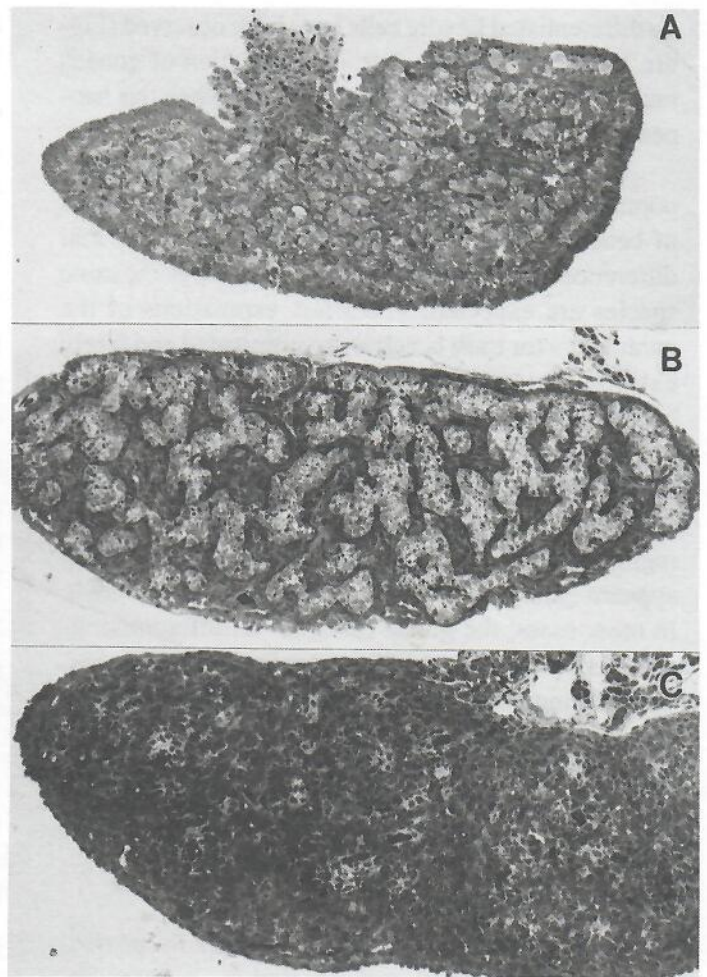
It has been suggested that temperature possibly counteracts a genetic control of gender. If this is true, then the pivotal temperature could be taken as the condition under which the genetic sex is expressed without external alterations (Mrosovsky and Pieau, 1991). Considering that in a nest or in a population at a beach there is a variable ratio of genotypical males to females and that response to temperature varies according to genetic sex, the pivotal temperature may vary as much as one degree centigrade (Mrosovsky, 1988). Thus, an estimation of pivotal temperature among the different turtle populations that nest in different beaches is recommended. A minimum of 5-6

nests per beach per season, following up for at least three consecutive years, would render values in close approximation of the true range of the pivotal temperature for the population being studied.

Although pivotal temperature is a reliable indicator when estimating sex ratios under natural conditions, a knowledge of "transition ranges of temperature" (TRT) is also convenient. This parameter refers to the difference in values between the low temperature producing 100% males and the high that results in 100% females (Mrosovsky and Pieau, 1991). As with pivotal temperature, TRT does not have a fixed value. No doubt there will be some variations depending on the sample size. Hence, as with pivotal temperature, estimation of standard deviation in a particular population will be optimized by measuring temperatures in as many nests as possible in different places on the beach in question and repeating the study over several years.

The equipment necessary for measuring temperature on the beach has been fully discussed by Godfrey and Mrosovsky (1994). They designed a module that memorizes maximum and minimum temperatures. Apart from the equipment being economical and resistant, it withstands burial, reducing the possibility of theft. The core sensor is a commercial memory thermometer (Radio Shack 277-302 or 630/1020) protected inside a Plexiglas box.

Converting sand temperatures into hatchling sex ratio is not as straightforward as it may seem. Pivotal temperatures of turtles are generally derived from incubation of the eggs at constant temperatures in the laboratory, unlike field incubation conditions (Bull, 1985). Microenvironmental factors, such as metabolic warming of the eggs, can cause nest temperatures to differ from sand temperatures (Mrosovsky and Yntema, 1980; Godfrey *et al.*, 1997). This factor should be taken into account when measuring temperatures on the beach, and sensors should be positioned as close as possible to the nest or among the eggs in each nest in order to derive the sex ratio. Also, inter-clutch variation in pivotal temperatures can complicate conversions of beach temperature to sex ratio; hence, adequate sample sizes are important (see above). Nevertheless, sand temperature profiles are useful, particularly in assessing the impact of management techniques on sex ratio (see Godfrey and Mrosovsky, this volume).



**Figure 3.** *Dermochelys coriacea* ovary (A), testicle (B) and undifferentiated gonad C previously treated with the clarifying technique (see text). Glycerol was eliminated with a phosphate buffer and the samples were treated as for Figure 1 and 2 material.

## Final Considerations

The gonads of different species of sea turtle reveal variations in the degree of differentiation at hatching. The most and least differentiated gonads are those of *C. caretta* and *D. coriacea* respectively, while an intermediate differentiation may be observed in *L. olivacea* and *C. mydas*. However, the gonads may be considered morphologically and physiologically immature in all sea turtle species. In vertebrates, differentiated ovaries contain oocytes surrounded by follicular cells and differentiated testes have seminiferous tubules and Leydig cells. In sea turtle hatchlings, as in other species, the onset of meiosis is delayed and there is no follicle formation in the ovaries. In the testes, no differentiated seminiferous tubules are present and only medullary cords, with few germ cells, are found. In most species, some germ cells remain in the surface epithelium and

no differentiated Leydig cells have been observed (Figure 1). Therefore, complete differentiation of gonads must occur sometime after hatching and when this happens still has to be established.

Genetic variations among different sea turtle populations and the varying environmental conditions of beaches located at different latitudes means that differences in pivotal temperature in turtles of the same species are expected. Therefore, estimations of the parameters for each beach is recommended and freely extrapolating from the results obtained in other beaches should be avoided.

Finally, it is important to consider the relative frequency of gonads referred to as "inter-sex". In these samples, the medullary cords are conserved in some regions, as in the testicles, and the surface epithelium appears enlarged, in other regions, as in the ovaries. In other cases, the gonad remains "undifferentiated" and there is no clear development towards either sex. Considering the gonad's vulnerability to temperature and its immaturity at the hatchling stage, it is not surprising to find variations in its development, probably in response to abrupt changes in temperature during the sensitive period.

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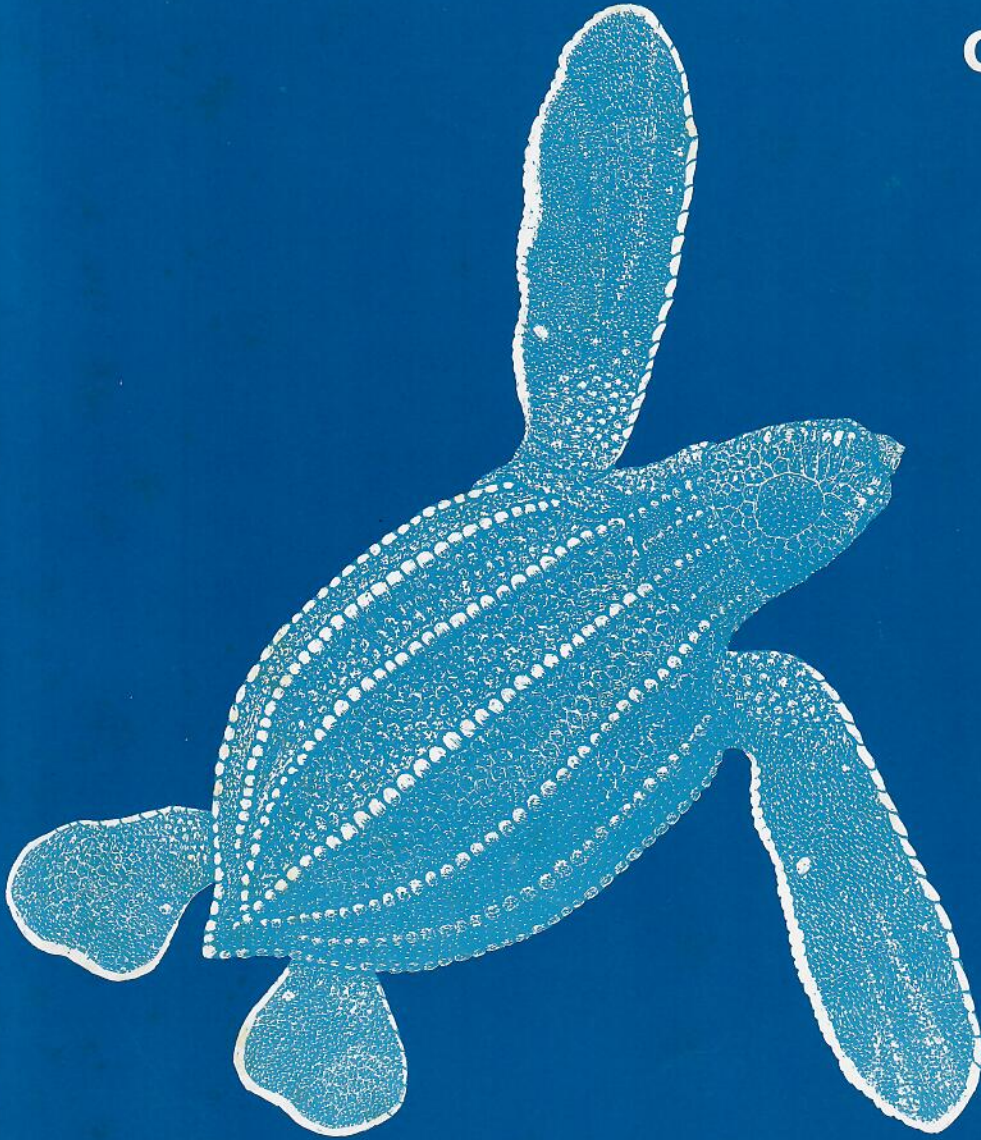
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# Research and Management Techniques for the Conservation of Sea Turtles



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