Sexual differentiation as a function of the incubation temperature of eggs in the sea-turtle *Dermochelys coriacea* (Vandelli, 1761)

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Abstract. Eggs of *Dermochelys coriacea* were incubated in the laboratory at different constant temperatures between 27 and 32°C up to hatching. The genital system of 72 neonates and 11 embryos was dissected and processed for histological study. All the individuals from the 27, 28 and 28.75°C incubations were phenotypic males. In the testes, the medulla was voluminous and very dense, composed of many epithelial cords, some enclosing germ cells, whereas the surface was covered by a thin layer of epithelial cells between which germ cells were still visible. The Müllerian ducts were in the process of regression. In all the individuals from eggs incubated at 29.75, 30.5 and 32°C, the gonads were somewhat longer than testes but were considerably reduced in width and thickness. The medulla was strongly inhibited, showing similar epithelial cords but much less numerous than in testes. The epithelium of the gonadal surface was pseudo-stratified and enclosed germ cells which had not enter meiotic prophase as in typical ovarian cortex. The Müllerian ducts were complete. These individuals were classified as potential females. These results show that temperature influences sexual differentiation of the gonads of *Dermochelys coriacea* and indicate that the threshold temperature (or pivotal temperature) lies between 28.75 and 29.75°C. They also reinforce fears about masculinising turtle populations by incubating eggs at cool temperatures in artificial hatcheries.

Résumé. Des oeufs de Tortue Luth (*Dermochelys coriacea*) ont été incubés au laboratoire à différentes températures constantes, comprises entre 27 et 32°C. Le phénotype sexuel de l'appareil génital de 72 nouveau-nées et de 11 embryons a été déterminé à partir de la dissection et de la structure histologique des gonades et des canaux de Müller.

Tous les individus issus d'oeufs incubés à 27, 28 et 28.75°C présentent un phénotype mâle. Dans les testicules, la médulla, volumineuse et très dense, est constituée par de nombreux cordons épithéliaux; certains de ces cordons renferment des cellules germinales; l'épithélium germinatif est aminci. Les canaux de Müller sont en cours de régression.

Chez tous les individus issus d'oeufs incubés à 29.75, 30.5 et 32°C, les gonades sont légèrement plus longues et beaucoup plus fines que les testicules. La médulla présente des cordons épithéliaux semblables aux cordons testiculaires mais beaucoup moins nombreux, ce qui indique une inhibition de leur proliféra-

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tion. L'épithélium germinatif est épais, pseudostratifié et contient des cellules germinales, mais celles-ci sont relativement peu nombreuses et ne sont pas entrées en prophase méiotique comme dans un cortex ovarien typique. Les canaux de Müller sont complets. Les individus présentant ces caractères sont considérés comme des femelles potentielles.

Ces résultats montrent que la température influence la différenciation sexuelle des gonades de la Tortue Luth et que, chez cette espèce, la température critique est comprise entre 28.75 et 29.75°C. Ils attirent de nouveau l'attention sur la nécessité de contrôler la température dans les écloseries, afin d'éviter un surnombre de mâles parmi les nouveau-nées destinées à l'enrichissement des populations naturelles.

Introduction

Sexual differentiation of the gonads has been shown to be sensitive to the incubation temperature of eggs in three orders of reptiles: lizards (Charnier, 1966; Wagner in Bull, 1980), turtles (Pieau, 1971, 1972, 1974; Yntema, 1976; review by Bull, 1980), crocodilians (Ferguson & Joanen, 1982). In turtles, higher temperatures (30-33°C) generally lead to 100% females while lower temperatures (23-27°C) lead to 100% males (review by Bull, 1980). This phenomenon may have important consequences for conservation measures on behalf of marine turtles. For instance, in order to protect eggs from predators, they are sometimes collected immediately after laying and then placed in styrofoam boxes. Incubation proceeds in artificial hatcheries and emerging neonates are released on the beach. Such practices run the risk of masculinising the turtle populations, since the temperature in the hatcheries is often lower than the temperature in the natural nests (Mrosovsky, 1978; Mrosovsky & Yntema, 1980). Sex ratio biases among hatchlings from eggs incubated at different temperatures have already been observed in three marine turtle species: *Caretta caretta* (Yntema & Mrosovsky, 1979, 1980), *Chelonia mydas* (Miller and Limpus, 1980; Mrosovsky, 1982; Wood & Wood, 1982; Morreale et al., 1982) and *Lepidochelys olivacea* (Ruiz et al., 1981; Morreale et al., 1982). Leatherback turtles (*Dermochelys coriacea*) are very different from other sea turtles, and are presently placed either in a separate family or in a superfamily. Here, we show that temperature also affects gonadal sexual differentiation in leatherbacks.

Material and Methods

The conditions of collection, incubation and development of eggs of *Dermochelys coriacea* are given in Table 1. Samples of 12 to 51 eggs from 8 different clutches were randomly collected immediately after being laid, either in Suriname (clutch II) or in French Guiana (clutches I and III to VIII), put into styrofoam boxes and transported to one of three laboratories. The duration of transport was 1 to 4 days. Eggs from clutches I, and III to VIII were placed in one layer in styrofoam boxes with moist sand as substrate. Boxes containing clutches I, III, and IV were put in "Bibel-Jouan" incubators which were placed in a constant temperature room (25°C) at the J. Monod Institute, Paris. Under these conditions, the temperature fluctuation was ± 0.2°C for each incubation (27, 30.5 and 32°C). Boxes containing clutches V to VIII were placed in thermostat control incubators ("Facis") at 28 ± 0.5°C, 28.75 ± 0.5°C, and 30.5 ± 0.5°C at

the Muséum National d'Histoire Naturelle, Paris. Eggs from clutch II were incubated at the University of Toronto. They were placed individually in plastic containers filled with moist vermiculite, with the top of the eggs exposed and dusted with sulphadiazine powder. The containers were put randomly in incubators (Freas/Precision Scientific Co.) running at the following temperatures: $27.25 \pm 0.3°C$, $28.25 \pm 0.3°C$, $29.75 \pm 0.3°C$, $32 \pm 1.8°C$. Eggs were left in the incubators until hatching occurred.

Table 1 shows that in no case did 100% of the eggs hatch. The best figure for hatching was 56% (eggs of clutch VI incubated at $28.75 \pm 0.5°C$). The rate of development was accelerated at higher temperatures. However, it differed from one clutch to another at the same temperature (compare, for example, incubation at 30.5°C for clutches V and VII). Many eggs were rotten when they were opened. Since the number of spotted eggs had not been systematically counted early in incubation, it is not possible to say how many eggs were infertile and how many embryos died. Perhaps the percentage of fertilized eggs changes during the breeding season. For embryo mortality there are various possible causes such as the conditions of transport (duration, temperature fluctuations, shaking of the eggs, exposure to X-rays at airports), the suboptimal incubation temperatures for normal development (for example, in clutch I, only 4% of the eggs hatched at 32°C, whereas 50% hatched at 27°C, Table 1) and the properties of the substrate (cleaness of sand, humidity, air circulation). Of a total of 290 eggs, 72 (24.8%) hatched. The animals were killed at hatching. In addition, 11 embryos were near term, 5 of these were alive and were only slightly delayed in their development, and 6 were dead but could be sexed. The genital system was examined under a dissecting microscope just before and after fixation in Bouin's fluid. For each individual, one gonad and the corresponding mesonephros and gonoducts were embedded in paraffin, serially sectioned at 7.5-10.0 μm and stained with hematoxylin-eosin or PAS. Identification of sexual phenotype was based on the histological structure of gonads and of Müllerian ducts (for clutch II only the gonads were examined).

Results

Table 2 groups the result from different clutches by incubation temperature. Incubations at the lower temperatures (27, 27.25, 28, 28.25 and 28.75°C) resulted in 100% of the hatchlings being phenotypic males. In these animals, the gonads were relatively big, measuring after fixation 10-12 mm in length, 1.2-1.4 mm in width and 0.5-0.7 mm in thickness (dorso-ventral distance) in their middle part. Their surface was not regular but displayed transverse grooves of varying depth (Fig. 1).

Histological sections of these gonads (Figs. 6a and b) show that the medulla is voluminous and very dense, composed of many epithelial cords and sometimes tubes, some of which enclose germ cells. The surface is covered by a thin layer of epithelial cells in which germ cells are still visible. Since the germinal epithelium has regressed, these gonads are testes. The Müllerian ducts are thin and irregular in diameter. In

Table 1. Collection, incubation and development of eggs of *Dermochelys coriacea*. The duration of incubation lies from egg collection to hatching; the ranges of temperature fluctuations are given in Materials and Methods (FG: French Guiana; S: Suriname).

|	I		II			III	IV	V	VI	VII		VIII	
Designation of the clutches	I		II			III	IV	V	VI	VII		VIII	
Date of collection	7/20/81		6/10/82			7/6/83	8/3/82	5/7/83	5/22/83	6/5/83		6/24/83	
Country of collection	FG		S			FG	FG	FG	FG	FG		FG	
Duration of transport (days)	3		1			4	3	3	2	2		4	
Substrate of incubation	sand		vermiculite			sand	sand	sand	sand	sand		sand	
Temperature of incubation (°C)	27	32	27.25	28.25	29.75	30.5	27	30.5	28.75	28	30.5	28	28.75
Duration of incubation (days)	73-75	56	74-76	63-65	?	60	74-76	64	68-70	68	54-56	73	61
Number of incubated eggs	24	25	11	10	11	12	51	19	25	12	28	26	26
Number of hatchlings	12	1	2	1	0	3	18	5	14	2	8	2	1
Number of living embryos near term	0	0	1	0	0	0	3	0	0	0	0	0	0
Number of dead embryos (sex recognizable)	0	2	2	0	0	2	0	0	0	0	0	0	0
% hatchlings + living embryos	50.0	4.0	27.2	10.0	36.3	25.0	41.1	26.3	56.0	16.6	28.5	7.6	3.8

Table 2. Sex ratio as a function of the incubation temperature of eggs in *Dermochelys coriacea*. The sexual phenotype of the gonads and of the Müllerian ducts was established among 72 hatchlings and 11 embryos (5 alive and 6 dead) which were near the term (see Table 1).

Temperature of incubation (°C)	27	27.25	28	28.25	28.75	29.75	30.5	32
Number of individuals sexed	33	5	4	1	15	4	18	3
Number of males	33	5	4	1	15	0	0	0
Number of potential females	0	0	0	0	0	4	18	3

transverse sections, the epithelial canals are absent (Fig. 3a) or are severely reduced in diameter and are composed of degenerating cells in the thinner parts of the ducts. In the wider parts of the ducts, the canal is less regressed (Fig. 3b). All along the canals the surrounding connective tissue is scarce, loose, and disorganized. In one case, the Müllerian ducts were found to be complete and almost intact (Fig. 4), although the gonads were testes.
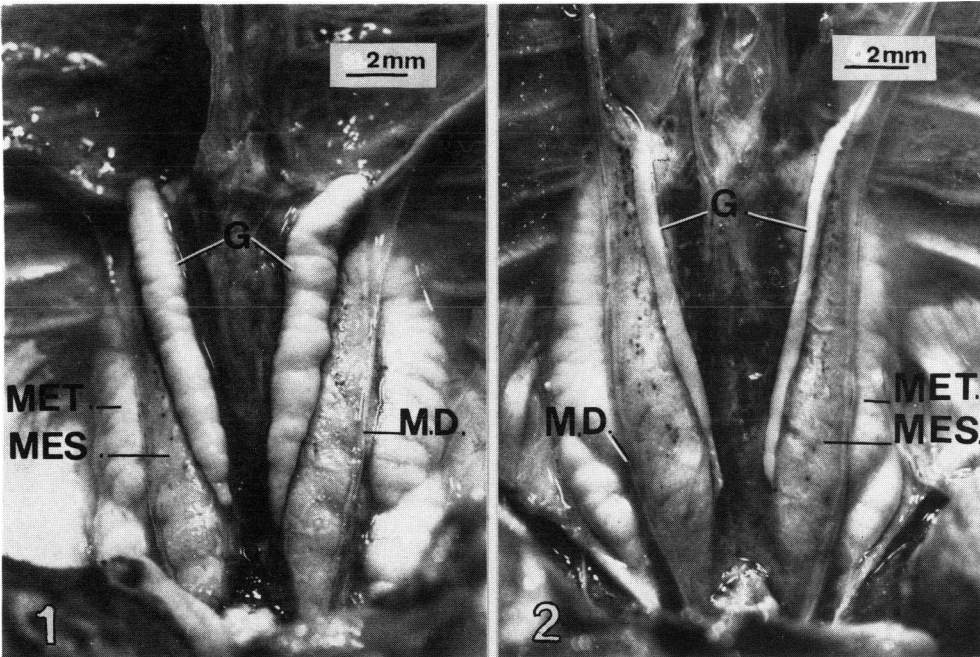
Figs. 1 and 2. Genital system of leatherback hatchlings from eggs incubated at different temperatures.
Fig. 1 - 28.75°C. Phenotypic male: the gonads (G) are relatively big and display transverse grooves, the Müllerian ducts (M.D.) are very thin and irregular in diameter.
Fig. 2 - 30.5°C. The gonads (G) are thin and the Müllerian ducts (M.D.) are complete and regular in diameter (MES: mesonephros; MET: metanephros).
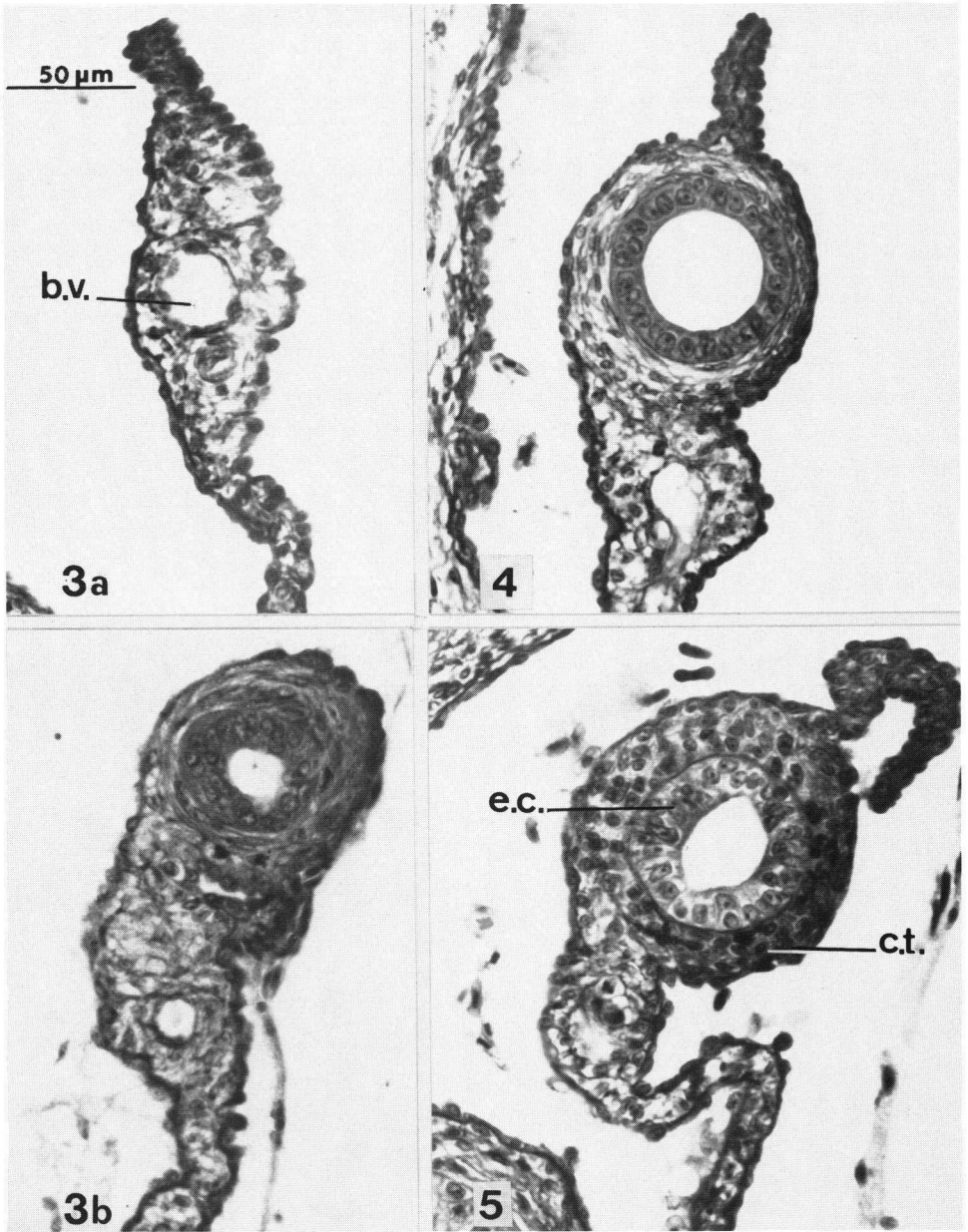
Figs. 3a, 3b, 4 and 5. Transverse sections through Müllerian ducts of leatherback hatchlings from eggs incubated at different temperatures

Fig. 3a - 27.°C. Male: the epithelial canal has regressed (b.v.: blood vessel).

Fig. 3b - 27°C. Male: the epithelial canal begins to be disorganized and the surrounding connective tissue is degenerating.

Fig. 4 - 27°C. Male: the epithelial canal is still intact, the surrounding connective tissue is relatively scarce and loose.

Fig. 5 - 32°C. Potential female: the epithelial canal (e.c.) is pseudostratified, the surrounding connective tissue (c.t.) is dense.

At the higher temperatures (29.75, 30.5 and 32°C), the gonads and Müllerian ducts of all individuals were feminized. The gonads were somewhat longer (12-13 mm mean) than testes but were considerably reduced in width (0.6-0.75 mm) and thickness (0.25-0.30 mm) (Fig. 2). Although the number of animals is not great, it seems that the higher temperature, thinner the gonad.

On histological sections (Figs. 7a and b), the medulla of gonads from the higher temperatures appears to be formed of epithelial cords fairly similar to those of the testes from the lower temperatures; however, these cords are much less abundant and some of them tend to become lacunae. The gonadal surface is covered by a pseudostratified epithelium containing germ cells. The latter are more frequent than in the testes, sometimes forming bundles, but not numerous and do not enter meiotic prophase. In all the cases, the Müllerian ducts were complete. Under the dissecting microscope, they appear to be regular in diameter, somewhat more consistent but not evidently wider than in males (Fig. 2). Histological study shows that the epithelium of the canals is intact, often pseudostratified and surrounded by dense connective tissue (Fig. 5).

Discussion

The fact that incubation of eggs below 29°C results in 100% phenotypic males is enough to establish that sexual differentiation of the gonads is sensitive to temperature in *Dermochelys coriacea*, as it is the case in the majority of turtles (review by Bull, 1980) and in the three other marine turtles previously studied (Yntema & Mrosovsky, 1979, 1980; Miller & Limpus, 1980; Ruiz et al., 1980; Mrosovsky, 1982; Wood & Wood, 1982; Morreale et al., 1982). The results obtained at the higher temperatures (around 30°C and above) reinforce this conclusion and are of particular interest. At these temperatures, the gonads are neither typical testes nor typical ovaries. Indeed, testicular-like cords are present in the medulla but the germinal epithelium has begun to thicken. Some medullary cords become lacunae but the germ cells have not enter meiotic prophase and there is not a typical ovarian cortex. The most striking character is the considerable reduction of the number of medullary cords and the associated reduction of the size (width and thickness) of the gonads. This indicates a strong inhibition of the medullary component. Similar action of the high temperatures has already been suggested in *Emys orbicularis* and *Testudo graeca* (Pieau, 1972, 1982). It is also comparable to the action of estradiol in these species (Pieau, 1970, 1974). The inhibition of the medulla seems to be a perequisite for the development of an ovarian cortex. In *Dermochelys coriacea*, the cortex is not as well differentiated at hatching as it is in *Caretta caretta* (Yntema & Mrosovsky, 1980) and *Chelonia mydas* (Miller & Limpus, 1980). Probably further differentiation occurs later. If this interpretation is correct, then the gonads of hatchlings from eggs incubated at high temperatures should be classified as potential ovaries and the animals as potential females (Table 2). However, since medullary cords are still present, it remains conceivable that some of these gonads might develop into testes. Rearing of young for several weeks or several months after eclosion is required to verify our hypothesis. If it is confirmed, the threshold temperature (or pivotal
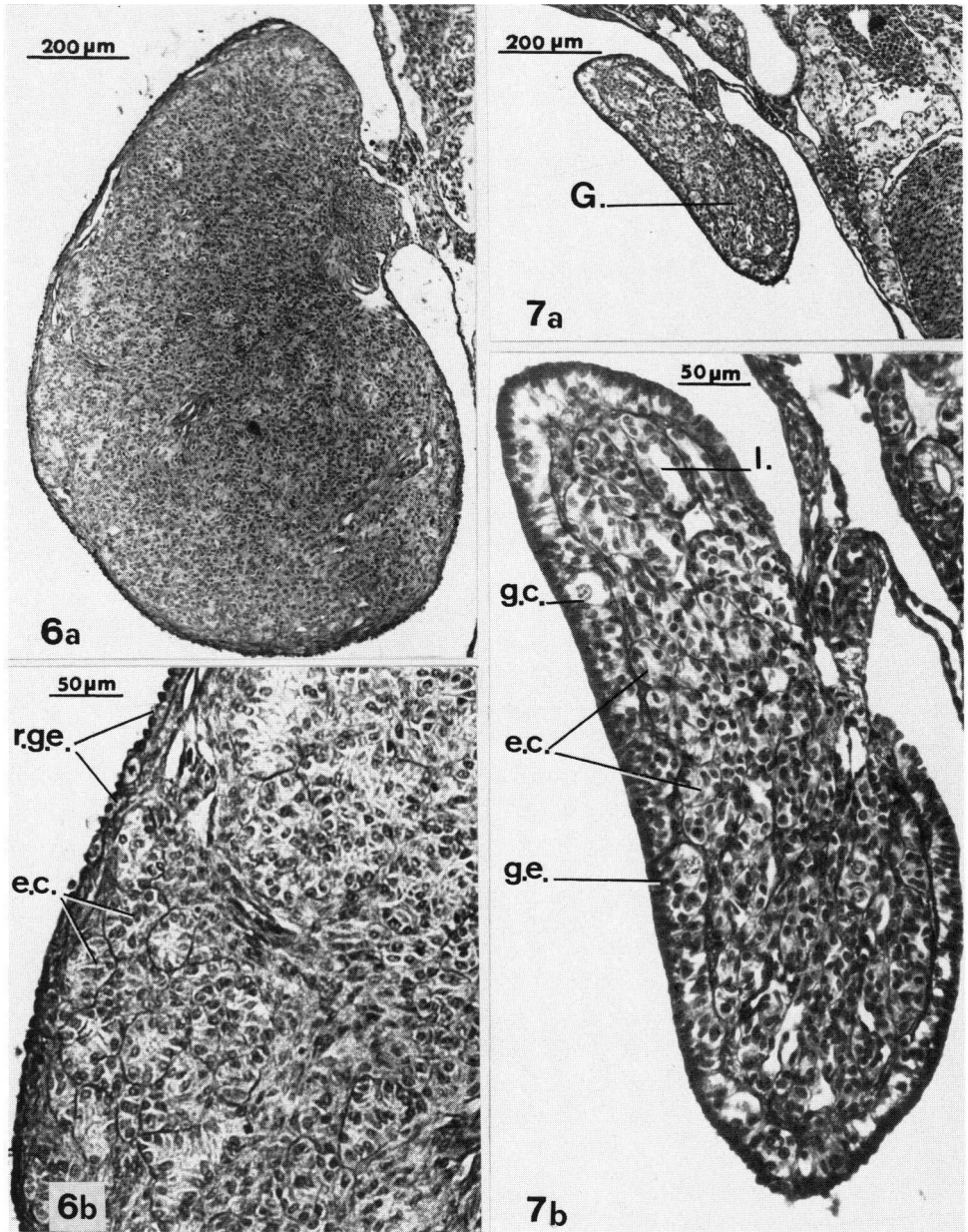
Figs. 6a, 6b, 7a and 7b. Transverse sections through gonads of leatherback hatchlings from eggs incubated at different temperatures.

Fig. 6a - 27°C. Testis showing a dense medulla, composed of numerous epithelial cords; the epithelium of the surface is thin.

Fig. 6b - Enlarged part of the testis seen in fig. 6a (e.c.: epithelial cords; r.g.e.: remnant of the germinal epithelium).

Fig. 7a - 32°C. The gonad (G) is very reduced when compared with the testis (Fig. 6a).

Fig. 7b - Enlargment of the gonad seen in fig. 7a. Epithelial cords (e.c.) are present in the medulla and begin to form lacunae (l.). The germinal epithelium (g.e.) is thickened and encloses germ cells (g.c.).

temperature, i.e. the temperature at which both males and females can be obtained from eggs of the same clutch) lies between 28.75 and 29.75°C in *Dermochelys coriacea*. This temperature is similar to that for *Chelonia mydas* (Miller and Limpus, 1980; Morreale et al., 1982). In *Caretta caretta*, the threshold temperature is 30°C (Yntema & Mrosovsky, 1982).

The demonstration that leatherback turtles also have their sexual differentiation influenced by temperature is of especial relevance to management programmes because this species so frequently lays its eggs below the high tide line (Dutton & Whitmore, 1983; Mrosovsky, 1983). These doomed eggs offer an extra opportunity for conservationists to intervene on behalf on the species (Schulz, 1975), but at the same time it becomes correspondingly more important to incubate any eggs saved from the seas at an appropriate temperature. Styrofoam boxes (Schulz, 1975) have been widely used but since these can be 1.4°C cooler than the sand (Mrosovsky, 1982), they probably introduce a considerable masculinising bias. This is being currently investigated for leatherbacks in Suriname (Dutton et al., in preparation) and in French Guiana.

Other points also require further study. Suppose styrofoam boxes do produce a high proportion of males, then what should be substituted instead? In places such as French Guiana where predation is high (Fretey & Frenay, 1980) reburying doomed eggs higher up the beach is not an attractive alternative. Our delineation of the pivotal temperature for this species should prove helpful in the design of thermally appropriate hatcheries. But it is also necessary to have information about the sex ratios that are produced in natural circumstances. In general, the more that is understood about temperature effects on sexual differentiation, the clearer the practical options should become. For instance, it is not yet known whether average temperature over the critical period or particularly large excursions are the most important. With *Emys orbicularis* eggs incubated in nature, female differentiation was favored when exposure to high temperatures (reaching 35 to 40°C) alternated daily with longer exposure below the threshold temperature during the first half of the thermosensitive period (Pieau, 1982). If, in *Dermochelys coriacea*, a short period of relatively intense warming is more potent than a longer period of mild warming, then perhaps a brief thermal pulse could protect against the masculinising effects of generally cooler temperatures. Information on such matters might also help in discovering the physiological basis of the phenomenon. The nature of the mechanisms whereby temperature can influence gonadal differentiation in turtles to follow male or female directions is still unknown. However, preliminary data in *Emys orbicularis* indicate that temperature acts on synthesis of enzymes involved in gonadal steroidogenesis (Pieau, 1974; Pieau et al., 1982). Since the gonads of *Dermochelys coriacea* are much larger than those of *Emys orbicularis*, they might provide plentiful material with which to investigate steroidogenesis and other physiological changes which occur with various temperature changes.

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