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Environmental Regulation of Sex Determination in Reptiles [and Discussion]

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Environmental regulation of sex determination in reptiles

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[Plates 1 and 2]

The various patterns of environmental sex determination in squamates, chelonians and crocodilians are described. High temperatures produce males in lizards and crocodiles but females in chelonians. Original experiments on the effects of incubation at 30 °C (100% females) or 33 °C (100% males) on development in *Alligator mississippiensis* are described. These include an investigation of the effect of exposing embryos briefly to a different incubation temperature on the sex ratio at hatching, and a study of the effects of 30 °C and 33 °C on growth and development of alligator embryos and gonads. A 7-day pulse of one temperature on the background of another was insufficient to alter the sex ratio dramatically. Incubation at 33 °C increased the rate of growth and development of alligator embryos. In particular, differentiation of the gonad at 33 °C was enhanced compared with 30 °C. A hypothesis is developed to explain the mechanism of temperature-dependent sex determination (TSD) in crocodilians. The processes of primary sex differentiation are considered to involve exposure to a dose of some male-determining factor during a specific quantum of developmental time during early incubation. The gene that encodes for the male-determining factor is considered to have an optimum temperature (33 °C). Any change in the temperature affects the expression of this gene and affects the dose or quantum embryos are exposed to. In these cases there is production of females by default. The phylogenetic implications of TSD for crocodilians, and reptiles in particular, are related to the life history of the animal from conception to sexual maturity. Those animals that develop under optimal conditions grow fastest and largest and become male. A general association between the size of an animal and its sex is proposed for several types of vertebrate.

Introduction

The determination of vertebrate sex is controlled by genetic or environmental factors or both. Mammals and birds exhibit genetic sex determination (GSD), which is characterized by the gender of the individual being fixed at conception. Environmental sex determination (ESD) is common in other vertebrates and some invertebrates (Bull 1980, 1983), and is characterized by the establishment of sex only after the embryonic or larval stage of the animal has been exposed to various environmental factors during development. Temperature-dependent sex determination was first observed (Charnier 1966) in an agamid lizard and has subsequently been observed in other reptiles. The temperature of egg incubation is the major environmental factor determining the sex of the individual and sex appears to be fixed at hatching (Pieau 1971, 1972; Yntema 1976, 1979; Ferguson & Joanen 1983: Webb & Smith 1984). Temperature also influences primary sex determination of some fish, whereas in some hermaphroditic fish (see Harrington 1967, 1968) and amphibians (see Witschi 1929; Gallien 1974; Houillon & Dournon 1978) temperature induces a change in the sexual phenotype of the adult.

Environmental regulation of reptilian sex determination is described and reviewed here. First, the incidence and patterns of temperature-dependent sex determination in reptiles are reviewed. Second, original experiments that examine the effects of temperature on sex determination and development in eggs of *Alligator mississippiensis* are described. Third, we speculate on possible mechanisms of reptilian sex determination and their phylogenetic implications.

Temperature-dependent sex determination in reptiles

Many studies of the phenomenon of temperature-dependent sex determination (TSD) in reptiles have observed the relation between temperature and sex ratio in natural nests (Ferguson & Joanen 1982, 1983; Bull 1985; Schwarzkopf & Brooks 1987), whereas others have been laboratory investigations examining the effects of constant incubation temperature on sex determination (Pieau 1971, 1972; Yntema 1976, 1979; Bull & Vogt 1979, 1981; Bull 1987*a*, *b*). Laboratory experiments involving a switch in the incubation temperature of eggs from one that induces 100% males to one that induces 100% females and vice versa have defined the temperature-sensitive period (TSP) for sex determination. The TSP is the earliest period of incubation when the sex ratio can be significantly switched. In many cases the TSP for a switch from a high to a low temperature is later than the TSP for a low to high switch (Ferguson & Joanen 1983; Webb *et al.* 1987; Bull 1987*a*), contrary to what one would intuitively predict given that higher temperatures accelerate external development of the embryo.

Squamata

The phenomenon of temperature-dependent sex determination (TSD) is poorly reported in the lizards and snakes despite the fact that it was first described for the lizard *Agama agama* (see Charnier 1966): an incubation temperature of 29 °C produced 100% males compared with 98% females at 26–27 °C. The gekkonid lizard *Eublepharis macularius* shows a similar pattern to *Agama* (see Warner 1980; Bull 1987*a*, 1987*b*). A long TSP has been described for *Eublepharis*, occurring between morphological stages 32–37 during the first half of incubation (Bull 1987*a*). A different pattern of sex determination has been observed in *Gekko japonicus* (Tokunaga 1985). Females are induced at low and high temperatures, with males occurring at intermediate temperatures. Temperature-dependent sex determination is absent in the lacertid *Lacerta viridis* (Raynaud & Pieau 1972). The iguanid lizard *Dipsosaurus dorsalis* has GSD but incubation temperature has a small influence on the sex ratio (Muth & Bull 1981).

Most snakes exhibit genetic sex determination and possess heteromorphic sex chromosomes (Baker *et al.* 1972; Bull 1980). Skewed sex ratios do, however, occur in some populations of snakes (Shine & Bull 1977; Gutzke *et al.* 1985). Incubation temperature does not affect the sex ratio of the snake *Nerodia fasciata* (Osgood 1980) and TSD comparable to that observed in lizards has not been reported. A form of TSD occurs in *Pituophis melanoleucus*, which has differential mortality of the eggs: low temperatures kill male embryos whereas higher temperatures kill females (see Burger & Zappalorti 1988).

Chelonia

In contrast to squamates (and crocodilians), incubation of chelonian eggs at high temperatures induces female hatchlings; low temperatures induce males in both field and laboratory investigations of most species studied (table 1). In at least four species, however,

TABLE 1. THE DOCUMENTED INCIDENCE OF TSD IN THE ORDER CHELONIA

suborder: Cryptodira	
family: Carettochelyidae	
Carettochelys insculpta	Webb *et al.* (1986).
family: Chelydridae	
Chelydra serpentina	Yntema (1976, 1979, 1981); Wilhoft *et al.* (1983); Packard *et al.* (1984, 1987).
Macroclemys temmincki	Bull (1980).
family: Chelonidae	
Caretta caretta	Yntema & Mrosovsky (1980, 1982); Stoneburner & Richardson (1981); Mrosovsky *et al.* (1984); Standora & Spotila (1985); Limpus *et al.* (1985).
Chelonia mydas	Mrosovsky & Yntema (1980); Miller & Limpus (1981); Wood & Wood (1982); Morreale *et al.* (1982); Mrosovsky *et al.* (1984); Standora & Spotila (1985); Spotila *et al.* (1987).
Eretmochelys imbricata	Dalrymple *et al.* (1985).
Lepidochelys olivacea	Dimond & Mohanty-Hejmadi (1983); McCoy *et al.* (1983); Standora & Spotila (1985).
family: Dermochelyidae	
Dermochelys coriacea	Mrosovsky *et al.* (1984); Standora & Spotila (1985); Rimblot *et al.* (1985); Rimblot-Baly *et al.* (1986–1987).
family: Emydidae	
Chinemys reevesii	Hou Ling (1985).
Chrysemys picta	Bull & Vogt (1979, 1981); Bull *et al.* (1982); Gutzke & Paukstis (1983, 1984); Paukstis *et al.* (1984); Vogt & Bull (1984); Schwarzkopf & Brooks (1985, 1987).
Emys orbicularis	Pieau (1971, 1972, 1973, 1974, 1982); Pieau & Dorizzi (1981); Pieau *et al.* (1982).
Graptemys ouachitensis	Bull & Vogt (1979, 1981); Bull *et al.* (1982); Vogt & Bull (1984); Bull (1985).
G. geographica	Bull & Vogt (1979); Bull *et al.* (1982); Vogt & Bull (1984); Bull (1985).
G. pseudogeographica	Bull & Vogt (1979); Bull *et al.* (1982); Vogt & Bull (1984); Bull (1985).
Pseudemys scripta	Bull *et al.* (1982).
family: Kinosternidae	
Sternotherus odoratus	Bull & Vogt (1979); Vogt *et al.* (1982).
Kinosternon flavescens	Bull (1980); Vogt *et al.* (1982).
family: Testudinidae	
Testudo graeca	Pieau (1971, 1972).
suborder: Pleurodira	
family: Pelomedusidae	
Podocnemis expansa	Alho *et al.* (1985).

temperature has little effect on sex determination: *Trionyx spiniferus* (Trionychidae), *Emydura macquarii*, *E. signata* (suborder Pleurodira: Chelidae) and *Clemmys inscupta* which as yet is the only emydid turtle not to exhibit TSD (Vogt & Bull 1982; Thompson 1983, 1988; Bull *et al.* 1985). Thus both TSD and GSD exist among turtles, even among closely related species.

A second pattern of TSD is characterized by the induction of females at high and low incubation temperatures, with males at intermediate temperatures. This occurs in at least two

families of turtle (Chelydridae and Kinosternidae) and is well documented for *Chelydra serpentina* (Yntema 1976, 1979, 1981; Wilhoft *et al.* 1983) and *Sternotherus odoratus* (Vogt *et al.* 1982). In *C. serpentina* incubation at 22 °C, 24 °C or 26 °C induced male development but at higher temperatures (28 °C and 30 °C) females were produced. Eggs incubated at 20 °C were not viable but transfer to 26 °C (male-producing) after 83–88 days allowed the eggs to hatch and produced 100% females (Yntema 1976). Low-temperature females have not been observed in wild nests of *C. serpentina* (Wilhoft *et al.* 1983). By contrast, eggs of *S. odoratus* can produce 81% females at 23.5 °C with males predominating at 25 °C and females predominating again at 28.0–30.5 °C (Vogt *et al.* 1982). This pattern also occurs in *Macroclemmys temincki* and *Kinosternon flavescens* (Bull 1980) although more data are necessary (Vogt *et al.* 1982).

Temperature-sensitive periods have been established for *C. serpentina* and they vary according to the direction of the temperature shifts (Yntema 1979; Wilhoft *et al.* 1983). For shifts from 20 °C to 26 °C the TSP is between Yntema (1968) stages 13–17; for 26 °C to 30 °C stages 16–20; for 30 °C to 26 °C stages 12–15 and for shifts from 26 °C to 20 °C stages 12–18. Less time at 30 °C is required to feminize a clutch of eggs than is required at 26 °C to masculinize it (Yntema 1979). In the laboratory, exposure to 30 °C for at least 4 h per day was sufficient to induce females (Wilhoft *et al.* 1983).

Laboratory studies using eggs of *Chrysemys picta* have shown that females can be induced at temperatures that normally produce males. Extremely dry nest environments (not more than −1100 kPa) at a temperature of 25 °C induce females despite the masculinizing effect of the temperature (Gutzke & Paukstis 1983; Paukstis *et al.* 1984). Nest humidity may therefore affect sex determination.

Crocodylia

Heteromorphic sex chromosomes are absent in all crocodilian species (Cohen & Gans 1970) and TSD is prevalent (Ferguson 1985; Deeming & Ferguson 1988). Macroscopic and histological examinations of the gonads of crocodilian hatchlings have shown that sex is fully determined at the time of hatching and no hermaphrodites, sex reversals or intersexes occur (Ferguson & Joanen 1983; Webb & Smith 1984).

One pattern of TSD is present in *Alligator mississippiensis* (Ferguson & Joanen 1982, 1983), *Caiman crocodilius* (Lang *et al.* 1988), *Crocodylus niloticus* and *Crocodylus siamensis* (Hutton 1987; Lang 1987). Artificial incubation of eggs at low temperatures (not more than 30 °C in *A. mississippiensis*; not more than 31 °C in *C. niloticus*; not more than 31.5 °C in *C. crocodilus*) induces 100% females; high temperatures (not less than 33 °C) induce 100% males. At intermediate temperatures both sexes are produced although there is a strong female bias; the incidence of males increases with increasing incubation temperature (Ferguson & Joanen 1983; Joanen *et al.* 1987). In temperature shifts from 30 °C to 33 °C the TSP occurs between days 14 and 21 of a total incubation period of 65 days, but in shifts from 33 °C to 30 °C it occurs between days 28 and 35 (Ferguson & Joanen 1983).

A second pattern of TSD is present in *Crocodylus johnstoni* (Webb *et al.* 1983, 1987; Webb & Smith 1984), *Crocodylus porosus* (Webb *et al.* 1987; Webb 1988) and *Crocodylus palustris* (Lang *et al.* 1988). In *C. johnstoni*, female hatchlings can be induced at any viable incubation temperature (Webb *et al.* 1983; Webb & Smith 1984). Males could only be produced within a narrow range of temperatures (31.0 °C–32.5 °C) but the highest sex ratio that could be achieved in the laboratory was 36% males (Webb & Smith 1984). In contrast to

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A. mississippiensis, high temperatures (33 °C and 34 °C) induced females that had large oviducts, ovaries with a distinct cortical region and a small clitiropenis (Webb & Smith 1984). High-temperature females have also been reported in *C. porosus* although the pattern of TSD in this species has similarities with that in *A. mississippiensis*: high temperatures (32 °C and 33 °C) induce 100% males (Webb *et al.* 1987). Similarly, in *C. palustris* incubation of eggs below 31.5 °C induces 100% females and at 32.5 °C, 100% males. Males occur in varying proportions at intermediate temperatures but incubation at 33 °C produces only 47% males (Lang *et al.* 1988). Temperature-shift experiments on eggs of *C. johnstoni* have been done despite the difficulties in interpretation associated with the absence of any temperature that induces 100% males (Webb *et al.* 1987). Switches from low temperatures (29 °C, 30 °C, and 31 °C) to 32 °C were found to be effective in producing males despite the fact that at constant temperatures these are female-inducing conditions. Over half of the incubation period was temperature sensitive though the morphological age at which femaleness could be maintained increased with greater incubation temperatures (Webb *et al.* 1987). Shift experiments in *C. porosus* and *C. palustris* showed similar results to *A. mississippiensis* (Webb *et al.* 1987; Lang *et al.* 1988). Possibly the primitive pattern of TSD is high- and low-temperature females, with intermediate-temperature males; in some species selection for viable incubation temperatures has merely eliminated either the high (e.g. crocodilians) or low (e.g. chelonians) temperature category (Deeming & Ferguson 1988).

Temperature-sensitive sex determination is not a phenomenon restricted to laboratory experiments. In the natural habitat of *A. mississippiensis* in Louisiana, U.S.A., three basic types of nest mound were described (Ferguson & Joanen 1982, 1983). Wet marsh nests were the coolest and most humid and produced 100% female hatchlings. Levée nests were the warmest and driest and produced 100% males. The dry marsh nest was intermediate in temperature and humidity, and produced both sexes. Nest maps revealed that males only developed in the warmest parts of the nest. Nests of *C. palustris* that attain 31.5–32.0 °C by 30 to 40 days of incubation contain more males than cooler nests (Lang *et al.* 1988). In *C. johnstoni* the hatchling sex ratio is biased towards females, and only 33% of immature animals and 17% of adults are male (Webb *et al.* 1983). Natural nests that produce 100% males have been observed in *C. johnstoni* (Smith 1987), despite the fact that this is not attainable under laboratory conditions (Webb *et al.* 1987). Induction of males is correlated to the total length of incubation, with males emerging after 72–82 days (Smith 1987). The adult population structure of all wild crocodilians is heavily biased towards females (Ferguson & Joanen 1982, 1983; Webb *et al.* 1983, 1987; Ferguson 1985; Hutton 1987).

Temperature not only influences the sex of crocodilian hatchlings but also the rate of embryonic development (Ferguson 1985; Webb *et al.* 1987; Webb 1988). The difference in the incubation period for eggs incubated at 30 °C (93 days) and 33 °C (78 days) is 15 days in *C. porosus* (Webb 1988) but only 8 days in *A. mississippiensis* (74 and 66 days respectively) (Joanen *et al.* 1987). Hatchling size in crocodilians is related to incubation temperature (Ferguson & Joanen 1982, 1983; Webb *et al.* 1987; Joanen *et al.* 1987). Eggs of *A. mississippiensis* incubated at extremes of temperature (29.4 °C and 32.8 °C) produced larger hatchlings than those incubated at intermediate temperatures (30.6 °C and 31.7 °C). Hatchlings from high incubation temperatures also have more residual abdominal yolk compared with hatchlings from lower temperatures (Ferguson & Joanen 1982, 1983; Webb *et al.* 1987). Post-hatching growth rates, under constant conditions, were also related to

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incubation temperature. Hatchlings from intermediate incubation temperatures grew faster than those from the extremes. Males always grew faster than females from the same incubation temperature (Joanen *et al.* 1987). The length, but not the mass, of hatchlings of *C. niloticus* and *C. porosus* was influenced by incubation temperature: male hatchlings were shorter, but by three months of age they were significantly larger than females (Hutton 1987; Webb 1988). Incubation temperature also affects post-hatching thermoregulation, at least in *C. siamensis*, with males consistently selecting higher temperatures for thermoregulation (Lang 1987). In addition, the pigmentation pattern of *A. mississippiensis* hatchlings is affected by incubation temperature. Males from eggs incubated at 33 °C are darker than females from eggs incubated at 30 °C (Deeming & Ferguson 1988).

Original experiments on TSD in *Alligator mississippiensis*

Surprisingly, despite the wealth of data on the patterns and incidence of TSD in reptiles, no-one previously has investigated its mechanism. We have examined three aspects of the effect of incubation temperature on general development, and in particular sex determination, in embryos of *Alligator mississippiensis*: (1) the effects of a pulsed change in incubation temperature; (2) the effects of a male- (33 °C) and a female- (30 °C) inducing temperature on the growth of embryos in terms of mass and morphometric measurements; (3) the effects of male (33 °C) and female (30 °C) temperatures on gonadal growth and differentiation. These new findings are used with other data to develop a hypothesis concerning the mechanisms of TSD in crocodilians.

'Shift twice' pulsed temperature experiments

Previous experiments to determine the time of sex determination have concentrated upon defining temperature-sensitive periods (Yntema 1979; Bull & Vogt 1981; Ferguson & Joanen 1982, 1983; Bull 1987*a*), though some shift-twice experiments have also been done (Yntema 1981; Bull & Vogt 1981; Pieau & Dorizzi 1981), in which embryos are exposed to short pulses of one incubation temperature on the background of another. The effects on sex determination of exposing embryos of *A. mississippiensis* to this type of experimental treatment have been examined. The experimental design and results are illustrated in figure 1.

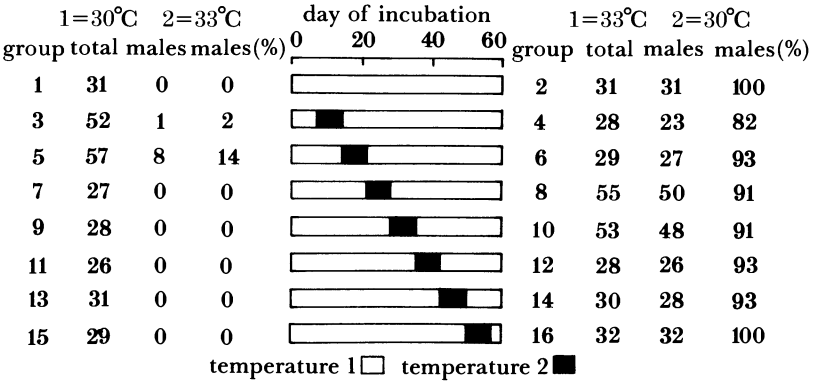

Figure 1. Details of a 'shift-twice' experiment done on eggs of *Alligator mississippiensis*. The eggs in each group were incubated either at 30 °C (exclusively female-producing) or 33 °C (exclusively male-producing) and exposed to 7-day periods of the other temperature. Groups 1 and 2 are controls. Macroscopic sexing was done on day 60 of incubation and confirmed in several animals by histological examination (Ferguson & Joanen 1983). The number and percentage of males in each group was recorded.

A 7-day pulse of one incubation temperature on the background of a second temperature was insufficient to alter radically the sex ratio associated with the original temperature (figure 1). A shift from 30 °C to 33 °C was very poor at producing male embryos; only in the third week of incubation (14–21 days) did 33 °C induce any males to develop. A 7-day pulsed reduction in temperature from 33 °C to 30 °C during the second to the seventh weeks of incubation could induce a few females only. The highest percentage of females was induced during the second week (7–14 days) pulse (figure 1).

Temperature and growth

Unlike avian eggs, which require a specific incubation temperature for normal development, reptilian eggs can successfully develop at a range of temperatures (28–34 °C in *A. mississippiensis* (Ferguson 1985)). It is not clear what effect temperature has on the growth and development of many reptiles although it is assumed that higher temperatures accelerate development. Indeed, higher temperatures do produce higher growth rates in *Python molurus*, two species of crocodile and many turtles (Vinegar 1973; Webb *et al.* 1987; Ewert 1985). This section describes an experiment that studied the non-sexual effects of temperature on embryonic development in *A. mississippiensis*.

Eggs of *A. mississippiensis* were incubated at 30 °C or 33 °C ($\pm$0.01 °C) in 100% humidity. At particular times during incubation eggs were removed from the incubator, opened and the embryos removed and fixed in formal saline (100 g l^{-1}) or Karnovsky's fixative until they were measured. Embryos were staged by using the criteria of Ferguson (1985) and wet mass was determined after fixation. Various morphometric parameters of the embryos were measured with a Wild M8 dissecting microscope eye-piece graticule or Vernier scale calipers. Parameters measured included total length of the animal, head length, eye length and the length of the trunk (measured as the distance between the limbs).

Incubation at 33 °C accelerated embryonic development and growth as assessed by all parameters measured. Embryos incubated at 33 °C reached morphological stage 20 approximately 5 days earlier than at 30 °C (figure 2*a*). Embryonic growth was also accelerated by the higher temperature (figure 2*b*). The total length of the animal and the length of the trunk were greater at 33 °C from early in incubation (figure 3*a*, *b*). For any morphological stage (particularly later in incubation), however, embryo mass was smaller at 33 °C than at 30 °C. Head length increased more rapidly during incubation at 33 °C than at 30 °C (figure 4*a*). Eye length showed a similar pattern although its rate of growth declined later in incubation (figure 4*b*). The ratio of eye length to head length was similar at the two temperatures (figure 4*c*), indicating that although increased incubation temperature accelerates external development it does not stimulate asynchronous growth of different parts of the embryo.

The effect of temperature on gonad development

Three distinct phases in the development of the gonad–kidney complex have been described in *A. mississippiensis*: the period of genital ridge formation, the period of bisexuality and the period of visible sex differentiation (Forbes 1940). The origin and route of migration of the germ cells is unknown in any crocodilian (Ferguson 1985). The genital ridge arises from a thickening of the ventromedial coelomic epithelium surrounding the mesonephros, which proliferates and develops rete cords. This phase persists in embryos up to 40 mm crown–rump

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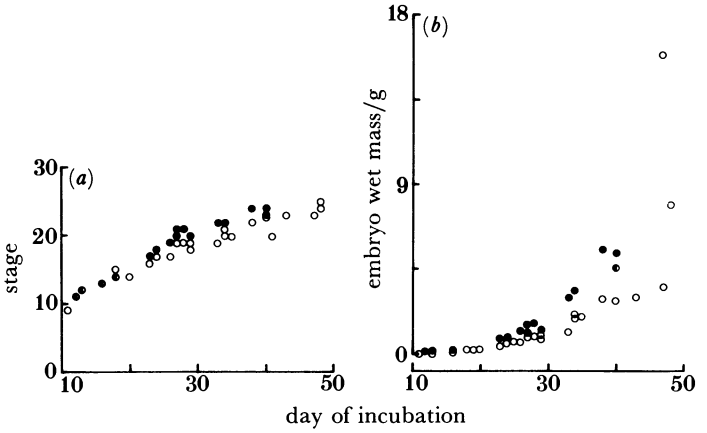

FIGURE 2. The development and growth of embryos of *A. mississippiensis* from eggs incubated at two incubation temperatures. (*a*) Relationship between morphological stage (Ferguson 1985) and days of incubation. (*b*) Growth of embryos measured as wet mass (in grams). Open circles, 30 °C; closed circles, 33 °C.

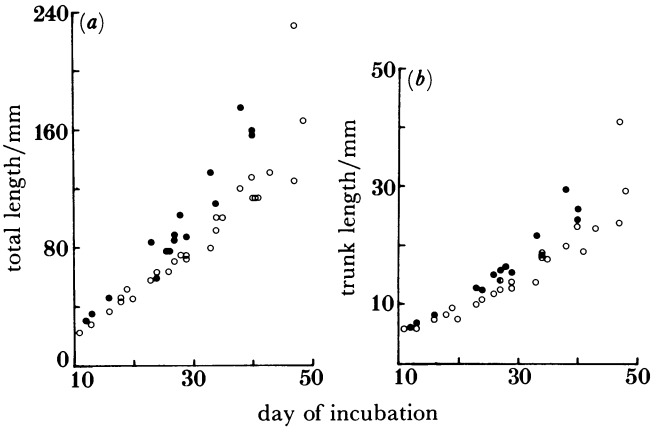

FIGURE 3. Two plots showing the effect of incubation temperature on the total length of the embryos of *A. mississippiensis* (*a*) and the length of the trunk, measured as distance between the limbs (*b*). Open circles 30 °C; closed circles, 33 °C.

length but no precise stages or time of incubation were recorded (Forbes 1940). The period of bisexuality is characterized by gonads that have distinct cortical and medullary parts but the sex of the individual is indeterminate. This phase persists in embryos up to a crown–rump length of 85 mm. Sexual differentiation then begins. Contrary to the report of Gutzke (1987), cortical tissues of the presumptive gonad differentiate into ovarian tissue compared with differentiation of the medulla to form testicular tissue (Forbes 1940).

There are several problems associated with the report by Forbes (1940). The eggs and embryos used in the study were from several sources and precise data on their incubation age or temperature were not available. The present study examines the effect of temperature on the development of the gonad in embryos of *A. mississippiensis* incubated at 30 °C and 33 °C ($\pm$0.01 °C). The gonad–kidney complex was dissected from the embryos that had been fixed in formal saline and measured, as described above. The gonad–kidney complex was prepared for histology, serially sectioned (5 μm), stained with haematoxylin and eosin, and examined by

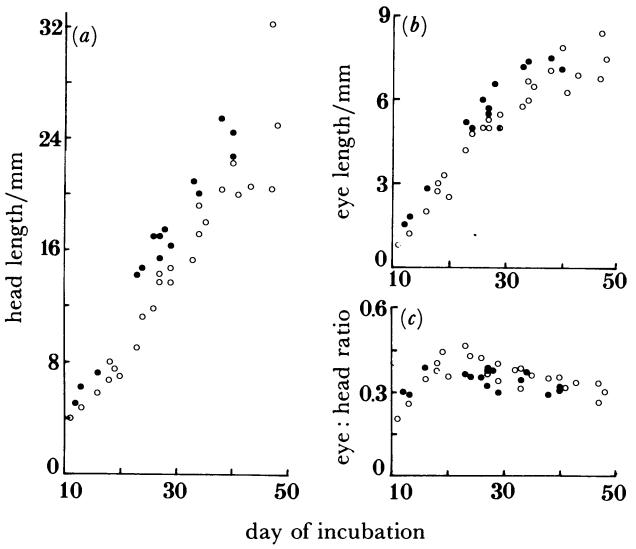

FIGURE 4. The effect of temperature on the growth of the head (*a*) and the eye (*b*) of embryos of *A. mississippiensis*. The relation between the eye:head ratio and time is plotted (*c*). Open circles, 30 °C; closed circles, 33 °C.

light microscopy. The length and thickness of the whole gonad and of the medulla were determined from the sections.

Incubation at 30 °C

At sixteen days of incubation (stage 13) the gonad was undetermined, with a thickened epithelium (continuous with the coelomic epithelium) covering a loosely arranged medulla. The gonad continued to grow and on day 24 (stage 17) there was a distinct change in the structure of the epithelium. The cells had a high nuclear:cytoplasmic ratio and a characteristic stalk-like appearance (figure 9, plate 1). Germ cells were sparse, and randomly arranged within the gonad. The medulla was densely cellular and not organized (figure 9). This general gonadal description remained similar up to day 40 (stage 23), when the cortical epithelium showed a larger number of germ cells (figure 9). By day 47 (stage 25) the gonad was differentiating into an ovary. The anterior portion of the ovary appeared to differentiate first, with posterior tissues beginning to form the medullary rest (Forbes 1940).

Incubation at 33 °C

Up to day 24 of incubation (stage 18) the appearance of the gonad was the same as at 30 °C, with the characteristic stalked epithelium and dense medulla (figure 9). Thereafter, there was a distinct organization of the medullary cells to form clumps and rounded masses (figure 9). By day 34 of incubation (stage 22) the gonad was well organized and differentiating into a testis (figure 9). On day 40 (stage 23) the gonad had a well-organized medullary region, the epithelium had lost its stalked appearance and was much flatter (figure 9). Germ cells were present in the medulla from the earliest stages, although they were difficult to observe as they bore a greater resemblance to medullary cells than to epithelial cells.

The position of the gonad on the mesonephros was predictable (figure 5). At the cloacal end of the complex, the gonad lies on the mediolateral ventral surface of the mesonephros. Further

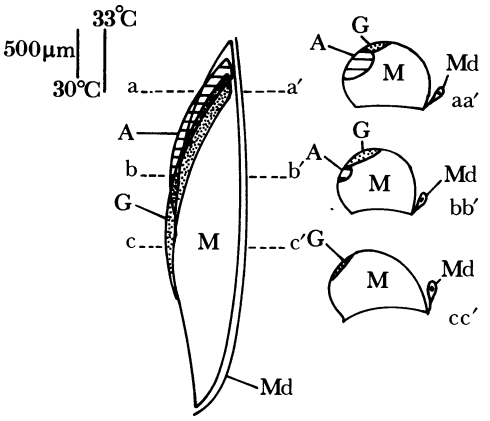

FIGURE 5. A diagrammatic representation of the spatial relation between the gonad, adrenal and mesonephros in embryos of *A. mississippiensis* at stage 19 of development. The ventral aspect of the gonad–kidney complex is shown; anterior is towards the top of the figure. Three transverse sections are shown and their positions on the gonad–kidney complex are indicated. A, adrenal gland; G, gonad; M, mesonephros; Md, Müllerian duct. Two scale bars (approximate) are included, owing to the differing effects of temperature on growth of the gonad at any stage of development.

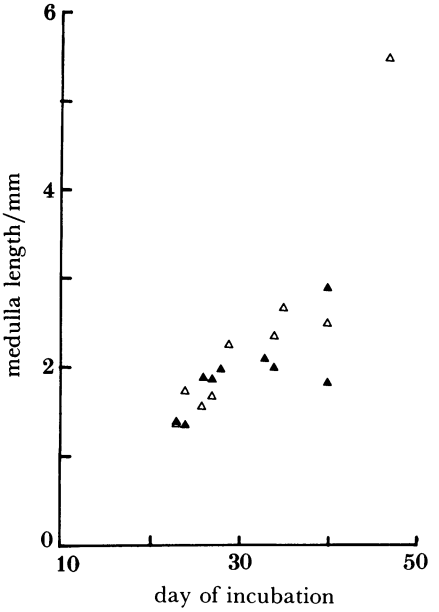

FIGURE 6. The relation between the length of the medullary region of the developing gonad and time. Open triangles, 30 °C; closed triangles, 33 °C.

DESCRIPTION OF PLATES 1 AND 2

FIGURE 9. Light micrographs of histological sections of gonads of *Alligator mississippiensis* embryos incubated at 30 °C (100% female-producing) and 33 °C (100% male-producing). Sections are from the centre of the gonad and are stained with haematoxylin and eosin. (*a*) Day 24, 30 °C, Ferguson (1985) stage 17. The medullary cells are loose and the epithelial cells have a characteristic 'stalked' appearance. (*b*) Day 34, 30 °C, stage 20. Similar to (*a*) but larger. (*c*) Day 40, 30 °C stage 23. The medulla is dense but does not show any signs of organization. (*d*) Day 24, 33 °C, stage 18. Note the stalked appearance of the epithelium. (*e*) Day 34, 33 °C, stage 22. The medulla is beginning to show signs of organization. (*f*) Day 40, 33 °C, stage 23. Note the flattened epithelium and the highly organized medulla. Scale bar 25 μm. A, adrenal gland; G, gonad; M, mesonephros.

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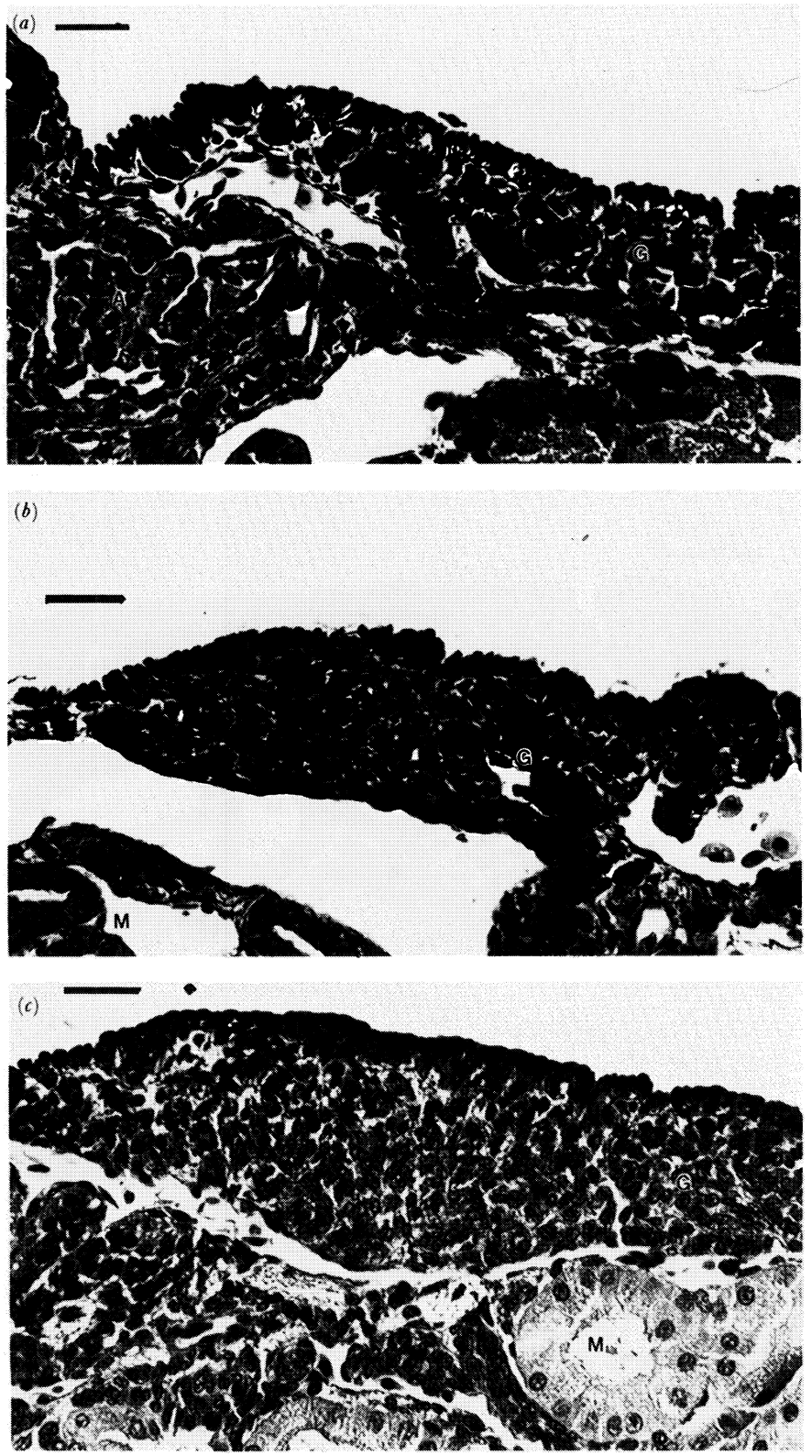

FIGURE 9 *a–c*. For description see opposite.

(*Facing p.* 28)

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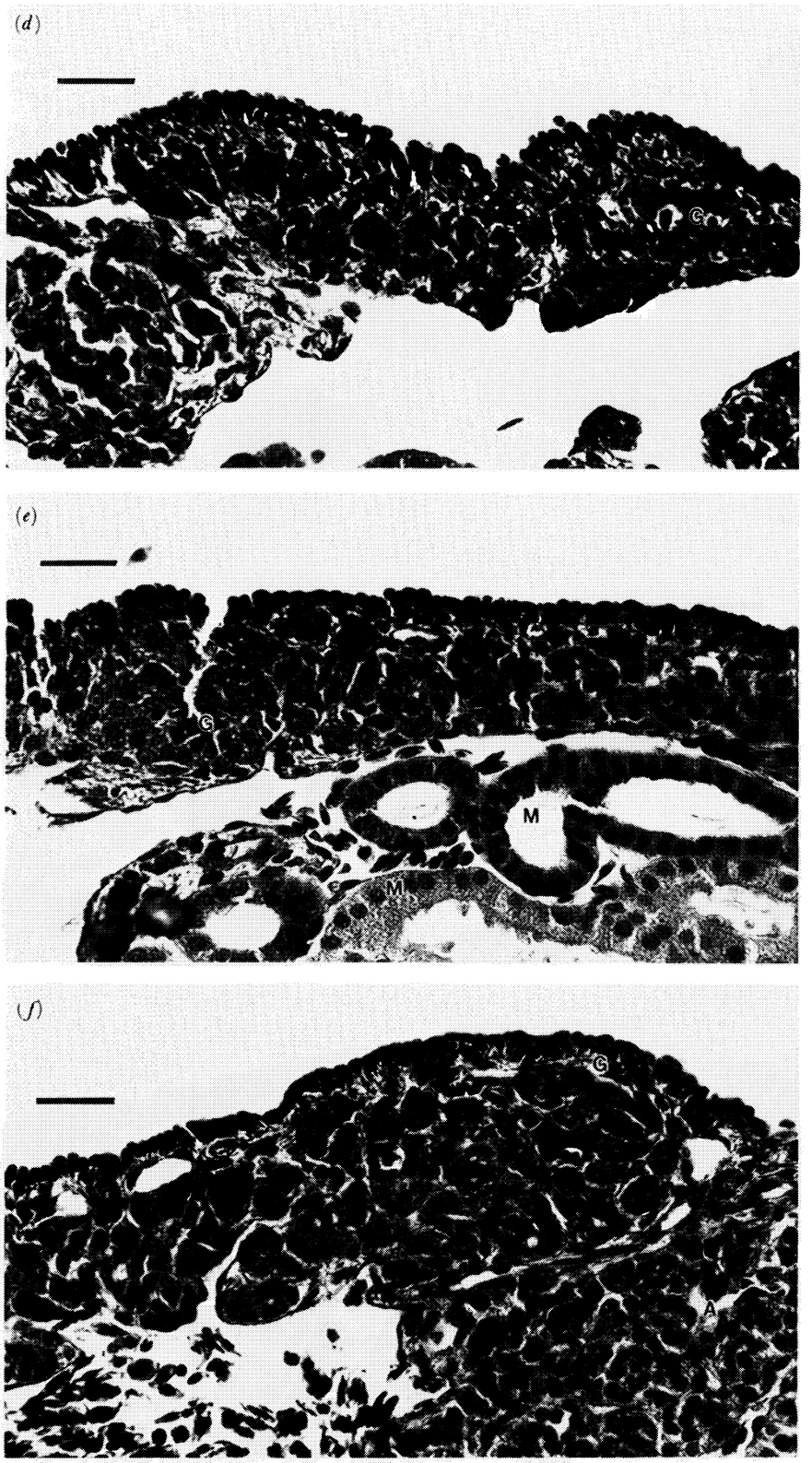

FIGURE 9 *d–f*. For description see p. 28.

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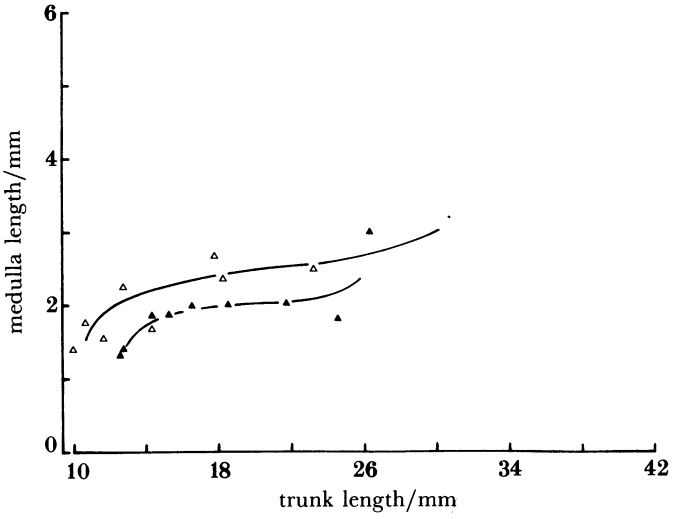

FIGURE 7. The relation between gonad size, measured as the length of the medulla, and the size of the embryo, measured as length of trunk. The two lines are fitted by eye. Open triangles, 30 °C; closed triangles, 33 °C.

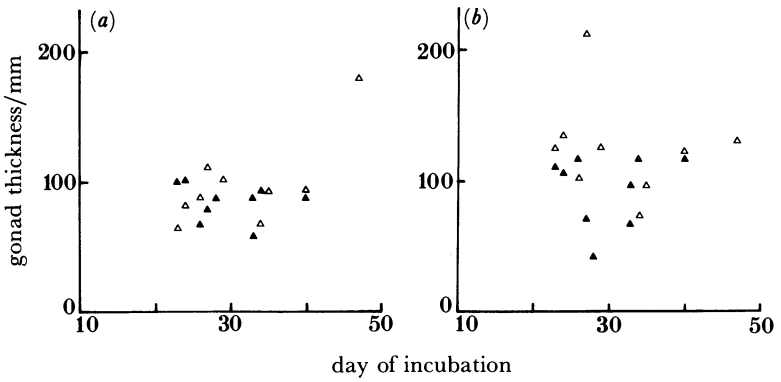

FIGURE 8. The relation between the thickness of the gonad and time of incubation. (*a*) Thickness of the gonad measured one third from the cephalic end of the medullary region. (*b*) Thickness two thirds from the cephalic end of the medullary region. Open triangles, 30 °C; closed triangles, 33 °C.

towards the cephalic end, the gonad lies on the ventral surface of the mesonephros (figure 5). This change is associated with the development of the adrenal gland, which occupies a similar position in relation to the mesonephros as do posterior portions of the gonad. Nearer the anterior tip of the mesonephros the gonad is absent but the adrenal extends further in a cephalic direction (figure 5.)

The point at which the germinal epithelium of the gonad arose from the coelomic epithelium was difficult to define. By contrast, the point at which the medulla arose was easily recognized and its overall length could be measured precisely. For this reason the length of the medulla was used as an assessment of gonadal growth. As incubation proceeded the length of the medullary region of the presumptive gonad increased (figure 6). When gonad length was compared with the length of the trunk, the rate of increase was very slow compared with the rapid elongation of the body (figure 7). For any given length of trunk the gonad was longer

in embryos incubated at 30 °C than in those at 33 °C. This is associated with the fact that growth and development of the embryo is accelerated at 33 °C. An embryo mass of 5 g is attained by 33 days at 33 °C but is only reached by 45 days at 30 °C (figure 2). The gonad appears to have a longer time to grow at 30 °C and is able to maintain a better ratio to body length than at 33 °C. The thickness of the gonad was unaffected by temperature (figure 8*a*, *b*) or by time for a significant period of incubation. The majority of variation in thickness of the gonad was associated with the medullary region as the germinal epithelium only contributed one or two layers of cells on the outer surface of the gonad.

Possible mechanisms of TSD

A hypothesis to explain TSD in crocodilians

It has long been assumed that the temperature-sensitive period in reptiles is the time of sex determination (Bull 1987*a*; Gutzke 1987). This assumption may be unjustified in light of the present findings. The shift-twice experiment showed that the change in sex that is associated with a change in temperature is not simply a switch event at one particular embryonic age or stage. One week would appear to be insufficient to affect the processes of sex determination significantly. Conventional, single temperature-shift experiments on eggs of *A. mississippiensis* show that the TSP is between 14 and 21 days for shifts from 30 °C to 33 °C, and 28 to 35 days for shifts from 33 °C to 30 °C (Ferguson & Joanen 1983). The former period corresponds to the maximum number of male embryos that could be induced after a 7-day pulse of 33 °C on a background of 30 °C (figure 1). In the reverse experiment (30 °C pulse on a 33 °C background), during this period there was a reduction in the number of males produced. Indeed, a pulsed reduction in temperature appears to be more effective at allowing development of females than is an increase in temperature effective at inducing males.

Our hypothesis is that single-shift experiments do not define the period of sex determination but rather define the population end-points of a period during which primary sex is being determined. The definite sex of an animal is established after this primary period, when a secondary event occurs. A more precise assessment of the primary sex-determining period can only be achieved by shift experiments to and from the lowest and highest incubation temperatures. During the primary sex-determining period, of 14 to 35 days in *A. mississippiensis* embryos, the ability to develop into a male is established. The embryo must be exposed to a 'dose' of some male-determining factor for a specific quantum of time. Any disruption of the length of the quantum period or of the dose causes the embryo to develop into the default sex, which in alligators is female. Natural variation will exist within the population, both in terms of the required dose of male-determining factor and in the length of the quantum period necessary to induce male embryos. Thus in some individual embryos the conditions required to be a male are minimal, so 1-week pulses early in the quantum period allow these to develop into males, but the majority of embryos develop into females by default (figure 1). Starting off as 'male' during this period, however, is no guarantee that the embryo will remain male. Exposure to 30 °C on a background of 33 °C reduces both the dose and the quantum period, so that those individuals in the population requiring long exposure to the male-determining factor do not receive it, and so develop into females by default.

The nature of the factor that is active during the quantum period is not known. In *A. mississippiensis*, development and differentiation of the gonad is rapid at 33 °C relative to that

at 30 °C. This may be a direct effect of temperature or an indirect effect of a male-determining factor that is inducing primary medullary organization. The size and relative development of the gonad in relation to the overall size of the embryo may also play some role in the process of secondary sex determination.

Although primary sex determination may occur during the quantum period, it is not the only phase of development concerned with sex determination. Embryos incubated at 33 °C can be converted into females even after a long period at the male-determining temperature (figure 1). In adult mammals and birds luteinizing hormone (LH) has an important role in maturation of both testes and ovaries: it is present during development of chick embryos (Woods 1987). If it is postulated that this single hormone is influencing gonadal differentiation during the development of two sexes in embryos of *A. mississippiensis*, then the second stage of sex determination may be associated with the presence and concentration of this hormone or its receptors during development. Incubation at different temperatures may affect hypothalamic control of LH secretion via luteinizing-hormone releasing hormone (LHRH), or may influence the presence of LH receptors on the two parts of the gonad, or both. The various non-sexual effects of temperature on development of crocodilians also suggests that incubation temperature is affecting the activity of the embryonic hypothalamus. The hypothalamus can detect temperature, pO_2, pCO_2, blood osmolality and pH in the adult and this ability presumably develops *in ovo*. Moreover, the hypothalamus not only controls LH secretion via LHRH but also growth (via growth-hormone releasing hormone), pigmentation (via melanocyte-stimulating hormone) and thermoregulation (via thyroid-hormone releasing hormone), which are all parameters influenced by the temperature of egg incubation in crocodilians. The implications of this hypothesis are dealt with by Deeming & Ferguson (1988).

Speculation on the cellular and molecular basis of TSD *and its phylogenetic significance*

The cellular basis of gonadal differentiation, particularly in reptiles, is poorly documented, yet our understanding of the mechanism of TSD, and sex determination in general, is reliant on such data. Several reports have shown the morphological and histological differences between the gonads of reptiles at hatching (Pieau 1971, 1972; Yntema 1979; Pieau & Dorizzi 1981; Ferguson & Joanen 1983; Webb & Smith 1984; Tokuanga 1985; Rimblot-Baly *et al.* 1986–1987), yet the present study is the first to describe the effects of two different temperatures on the gonadal development of any reptile. This highlights the problems associated with defining the mechanism of TSD in reptiles, yet despite the obvious lack of information several hypotheses have been advanced to explain the phenomenon.

One hypothesis is the presence of steroid hormones during development (Gutzke 1987). Exogenous testosterone and oestradiol applied to reptilian embryos that exhibit TSD can induce sex reversal (Gutzke & Bull 1986; Bull *et al.* 1988). Testosterone can be aromatized into oestrogen by embryos, inducing female development (Gutzke & Bull 1986). A theory of sex determination based on the relative proportions of steroid hormones has been developed to explain sex determination (Gutzke 1987). Similarly, the differing quantities of the enzyme aromatase during development at two temperatures has also been suggested to explain TSD (Bogart 1987).

The presence of H-Y antigen in the heterogametic sex in birds and mammals has led to suggestions that this may be important in TSD in turtles (Engel *et al.* 1981; Zaborski *et al.* 1982), although it is now considered to be less important (Gutzke 1987). Heat-shock proteins have

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been isolated in vertebrate tissues (Pelham 1985; Schlesinger 1986) and may have some role to play in the molecular basis of TSD. However, molecular analyses of different heat-shock proteins expressed during gonadal development in turtles have been disappointing (J. M. P. Joss, personal communication). Likewise, a hypothesis of sex determination based on differential gonadal growth as has been advanced for birds and mammals (Mittwoch 1971, 1973, 1983) clearly does not apply in alligators on the basis of the data presented in this paper. The idea that TSD induces differential mortality of eggs at different temperatures is contradicted by numerous studies (Bull 1980, 1983; Ferguson & Joanen 1983).

Recent work has isolated the gene for a mammalian testis-determining factor (TDF) on the Y chromosome and identified a homologue on the X chromosome (Page *et al.* 1987). These genes encode a DNA-binding protein or proteins regulating the expression of other genes. It may be that maleness in mammals and birds is associated with the presence of two doses of a single gene product, so that sex determination may be a dose-dependent phenomenon (Page *et al.* 1987). Using a cDNA probe for the human *TDF* gene, we have demonstrated hybridization to both male and female alligator DNA under conditions of high stringency, indicating the alligator *TDF* gene is very similar to that in man (figure 10).

Significantly, the temperatures that in reptiles cause the embryos to differentiate and develop fastest are also the ones that cause them to be male. It is easy to postulate that this is the optimal temperature for transcription, translation and enzyme activities, and that at this

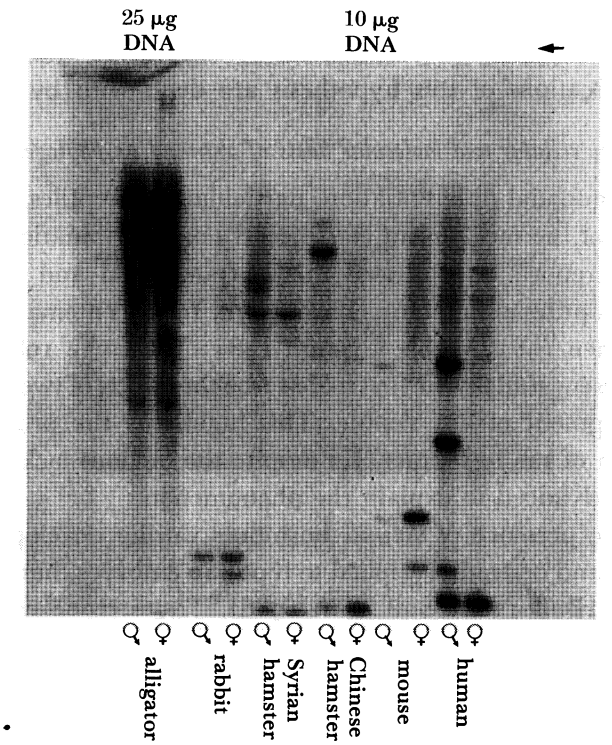

FIGURE 10. 'Ark blot' of DNA from male and female alligator, rabbit, Syrian hamster, Chinese hamster, mouse and man, hybridized with the PMF1 insert, 18 h at 55 °C, 1 M NaCl, 10% dextran sulphate and 1% SDS; washed 3 × 20 min, 65 °C, 0.2 × SSC (1 × SSC = 0.15 M sodium chloride + 0.015 M sodium citrate), 0.2% SDS and exposed for 3 days at −70 °C. Note that 25 μg of alligator DNA was loaded as opposed to 10 μg of mammalian DNA. Hybridization with the *TDF* probe to male and female alligator DNA is evident. This analysis was done by Dr. P. N. Goodfellow, Imperial Cancer Research Fund Laboratories, London.

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temperature the dose of the sex-determinating gene product will be maximal, resulting in male differentiation. Temperatures above and below this maximum are sub-optimal and will result in a lower dose of the sex gene product, causing a higher percentage of females by default. Such a dose-dependent hypothesis would explain data from constant-temperature incubation, shift and pulse experiments from a variety of species, each with its own optimal temperature. Moreover, the DNA-binding protein encoded by the sex-determining gene may regulate gene expression at both gonadal and extra-gonadal sites. Thus it may regulate the release of various regulatory hormones from the hypothalamus. This would consolidate the male gender and ensure that animals that were incubated at the optimal temperature retained into adulthood optimal values for characteristics such as thermoregulation and growth. Additionally, factors that affect the processes of gene expression (e.g. pO_2, pCO_2, pH) would also affect the dose of the sex-gene product and so the sex of the animal, particularly at pivotal temperatures (i.e. those producing a 50% sex ratio due to variation in the dose and quantum period in a population of embryos). To the extent that external factors influence embryonic growth, and gonadal and sexual differentiation, reptiles are 'nature's sexual transgenics'.

The phylogenetic advantage of TSD in crocodilians may be in the association of sex with size. Embryos incubated in optimal conditions exhibit maximal pre- and postnatal growth, and more rapid differentiation (this paper; Joanen *et al.* 1987; Webb 1988). Large adult alligators are at a selective advantage if they are male: the largest males control the largest harems, produce sperm for longer periods and mate more frequently than smaller males (Ferguson 1985), thereby passing on more genes to the next generation. Incubation conditions vary, however, according to nest site and climate. As a consequence embryos with GSD would develop randomly into large or small males depending upon local incubation conditions. However, in reptiles sex is not determined until late in incubation, and those embryos that are exposed to an optimal incubation environment, and that therefore develop and differentiate fastest, become males. Thus TSD allows association of maleness with the most rapidly developing embryos and the greatest adult growth potential. This phylogenetic advantage is clearly within the confines of the theory of Charnov & Bull (1977). Indeed, association of size and sex appear to be universal in the animal kingdom. Most chelonians show sexual dimorphism and the females are the larger, a fact presumably associated with the limitations of the shell on egg production (Head *et al.* 1987). Whether the silverside fish (*Menidia menidia*) exhibits TSD or GSD is related to its north–south location and the length of its growing period (Conover & Heins 1986, 1987): high temperatures and short growing periods produce small males. In this species it is advantageous to be large and female, as potential fecundity is thereby increased. In certain coral-reef fish, social contact with smaller conspecifics induces large females to change sex into males (Ross *et al.* 1983; Ross 1987). High temperatures during larval development of amphibians also produce phenotypic males from genetic females (Gallien 1974; Houllion & Dournon 1978).

In birds and mammals, size and sex are also related. If reptilian TSD is a dose-dependent mechanism, then it is easy to envisage how genetic sex determination could have evolved. More uniform incubation conditions (brooding in birds, viviparity in mammals) would tend to equilibrate the dose in all embryos. The response to this could be inactivation of one set of homologous genes in females (X-chromosome inactivation) but persistence of two sets of sex-determining genes in the male (Y chromosome). This leads to male heterogamety in mammals and subsequent evolution of the sex gene on the Y chromosome to control testis differentiation

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more closely (Page *et al.* 1987). Another response, seen in birds, could be the loss of the sex-determining and other genes in the female (W chromosome) but the persistence of homologues in the male (Z chromosome), leading to female heterogamety. Birds and mammals may also have some non-sexual genes, e.g. secretion of hypothalamic releasing hormones, which are regulated by the DNA-binding protein transcribed by the sex-determining gene.

It is clear, therefore, that reptilian systems offer a unique opportunity to investigate the cellular and molecular mechanisms of sex determination and to manipulate them experimentally. Reptiles also hold the key to understanding the evolution of genetic sex-determining systems in birds and mammals.

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Discussion

Ursula Mittwoch (*University College London, U.K.*). Could Professor Ferguson please explain the sex dosage hypothesis, which he mentioned, in a little more detail? If male and female reptiles with environmentally controlled sex determination have the same genotype, gene dosage must likewise be the same for both sexes. The different effects of temperature on the development of alligators seem to be due to differences in the developmental rate, a fast rate leading to male sex differentiation, whereas a slower rate of development results in females.

M. W. J. Ferguson. It is true that male and female alligator embryos develop at different rates as a consequence of being incubated at 33 °C for males and 30 °C for females. However, this difference in developmental rate primarily reflects differentiation rather than growth. Moreover, our hypothesis is not one of sex-gene dosage but rather the dosage of a product transcribed from a sex gene. We would postulate that this product may be a regulatory element such as that described recently for TDF namely a zinc finger protein. Our hypothesis is that

at the male-determining temperature of 33 °C, gene transcription, translation and enzyme activity are optimal. At higher or lower temperatures, transcription, translation and enzyme activity are less than optimal but compatible with embryonic survival. Males result when there is a high dosage of the gene product above a certain threshold. Contrarywise, females develop when the gene product falls below a certain threshold. This hypothesis is similar to one put forward by Page concerning sex determination in mammals whereby males would get two doses of a sex-gene product, one from the X-chromosome and the other from the Y, whereas females would only get one dose of the sex-gene product, namely from one X-chromosome, the other gene being inactivated as part of the X inactivation mechanism.

U. Wolf (*Institute of Human Genetics, University of Freiburg, F.R.G.*). What about experimental sex inversion by steroid hormones in reptiles?

M. W. J. Ferguson. As far as I am aware, only two experiments on reversal of temperature-dependent sex determination in reptiles by steroid hormones have been done. The first was by Bull and is cited in our paper. However, it must be emphasized that this study used a small number of eggs and had a very large mortality. Although sex reversal was demonstrated, the sex of all the dead embryos would need to be known to come to a firm conclusion. Second, there is an as yet unpublished study by Dr Dorizzi which she refers to in her comment on this paper. It would appear that steroids are capable of sex reversal in reptiles with temperature-dependent sex determination. However, I believe that this tells you little about the primary sex-determining mechanism.

Mireille Dorizzi (*Institut Jacques Monod, Paris, France*). I just want to add some information concerning the involvement of oestrogens on the differentiation of gonads – which are sensitive to temperature – in the turtle *Emys orbicularis*. If, during the thermosensitive period, oestrogens are injected into eggs incubated at 25 °C, a temperature yielding 100% phenotypic males, gonads differentiate into ovaries instead of testes. If antioestrogens or aromatase inhibitors are injected into eggs incubated at 30 °C, a temperature leading to 100% phenotypic females, testicular tubes differentiate. Therefore oestrogens inhibit the development of sex cords. When the production or the action of these steroids is prevented, testicular cords or tubes may differentiate.

P. Zaborski (*CNRS, Ivry-sur-Seine, France*). Would Professor Ferguson please add a comment concerning the dosage effect at the threshold (pivotal) temperature that produces both males and females with a sex ratio of 1:1.

M. W. J. Ferguson. It has always been a puzzle as to how under temperature-dependent sex determination mechanisms, one can get a 50:50 sex ratio at one temperature. We hypothesize that the population of embryos exhibits variation in the threshold levels of the sex-gene product that they require to specify maleness. We also hypothesize that the population of embryos varies in the length of the quantum period of time that they need to be exposed to this factor to ensure male sexual determination. Thus at the incubation temperature that produces a 50:50 sex ratio, we would hypothesize that those embryos that become male have a lower threshold for the sex-gene product or require exposure to this product for a shorter period of time, or both,

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than do the embryos that develop into females. In this way, variation in the population of embryos can explain the 50:50 sex ratio. We are certain that this 50:50 sex ratio cannot be explained by minor fluctuations in incubator temperatures. All of the experiments we describe were done in incubators with 16 temperature recording probes (accurate to 0.001 °C) at different locations throughout the incubator. We are confident that there are no gradient effects of temperature influencing any of our data.

H. Sharma (71 *Barrack Road*, *Hounslow*, *U.K.*). Can Professor Ferguson set up a temperature gradient across the egg?

In the natural environment temperatures fluctuate by night and day. Has Professor Ferguson done experiments of changing temperatures at shorter intervals?

M. W. J. Ferguson. First, it is unknown how long it takes the egg contents to equilibrate to the temperature of the incubator and how much of a buffering capacity exists within the egg. It is also unknown to what extent the shell and egg-shell membranes buffer the embryo from fluctuations in ambient temperature. Experiments involving the placing of microthermocouples within different locations of the alligator egg, followed by different external thermal régimes, would be required to address such questions. Second, nest temperatures do fluctuate between day and night and also at different times during the season. However, these effects are not of critical importance for our hypothesis of sex determination. We believe that embryos require exposure to a threshold dose of a sex-gene product during a quantum time of embryonic development. If they exceed this threshold, the embryos become male; conversely, if they fall below it, they become female. We also hypothesize that transcription, translation and enzyme activity, all of which affect this sex-gene product, are different at different incubation temperatures. Therefore fluctuating temperatures will produce males when gene transcription, translation and enzyme activity are optimal and females at other times when they are not. Clearly the relative contributions of optimal to suboptimal during the fluctuating critical period will determine whether the embryos become male or female. This mechanism allows the embryo to integrate temperature changes over a period of developmental time. These fluctuating temperatures may also be important in later sexual differentiation, particularly in the release of various hormones and the expression of receptors for such hormones on the gonadal tissues. It is conceivable that the optimal temperature for hormone release and the optimal temperature for receptor expression may not be the same, in which case fluctuating temperatures could be advantageous. However, in the absence of data this is wild speculation.

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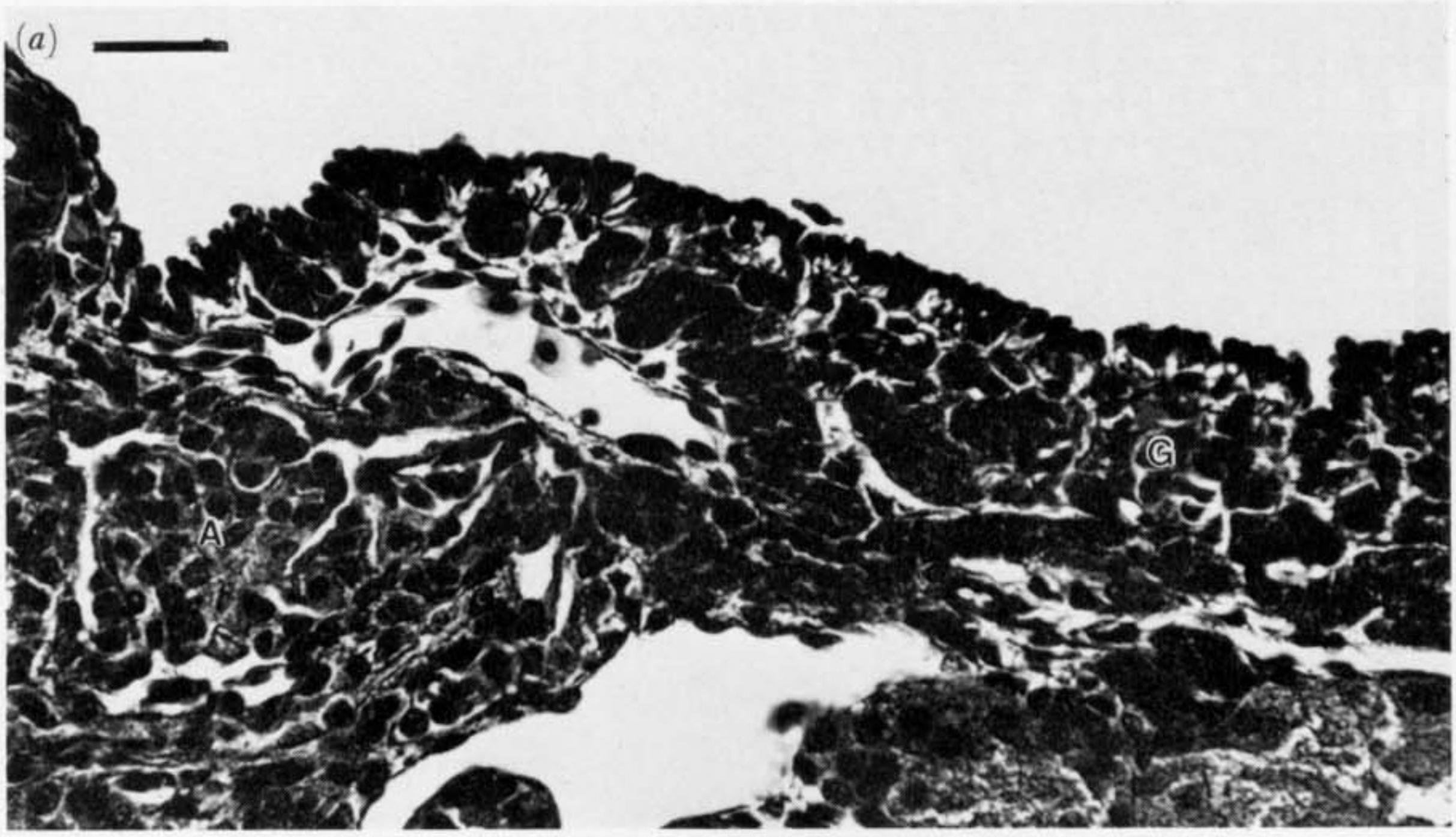


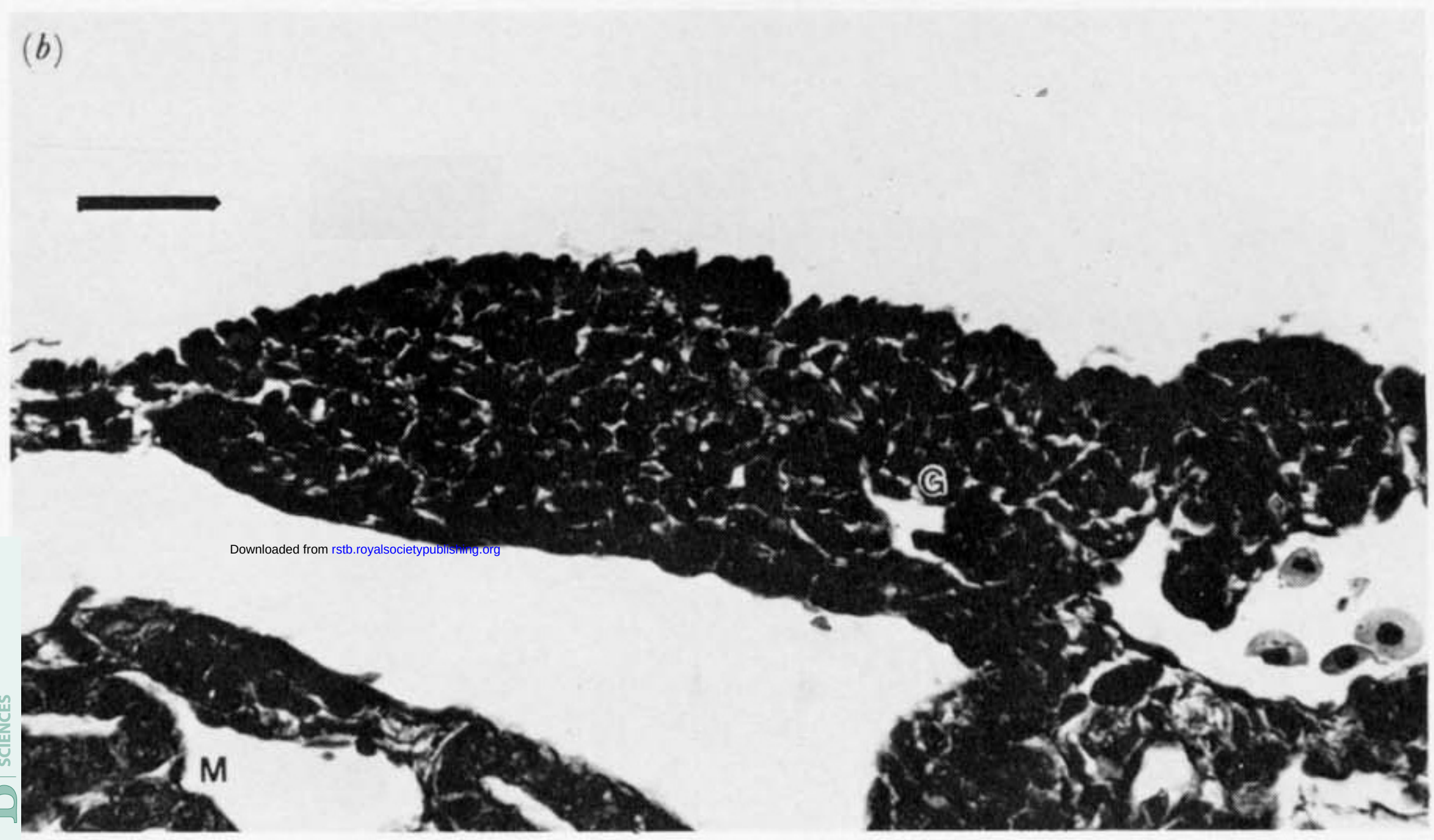
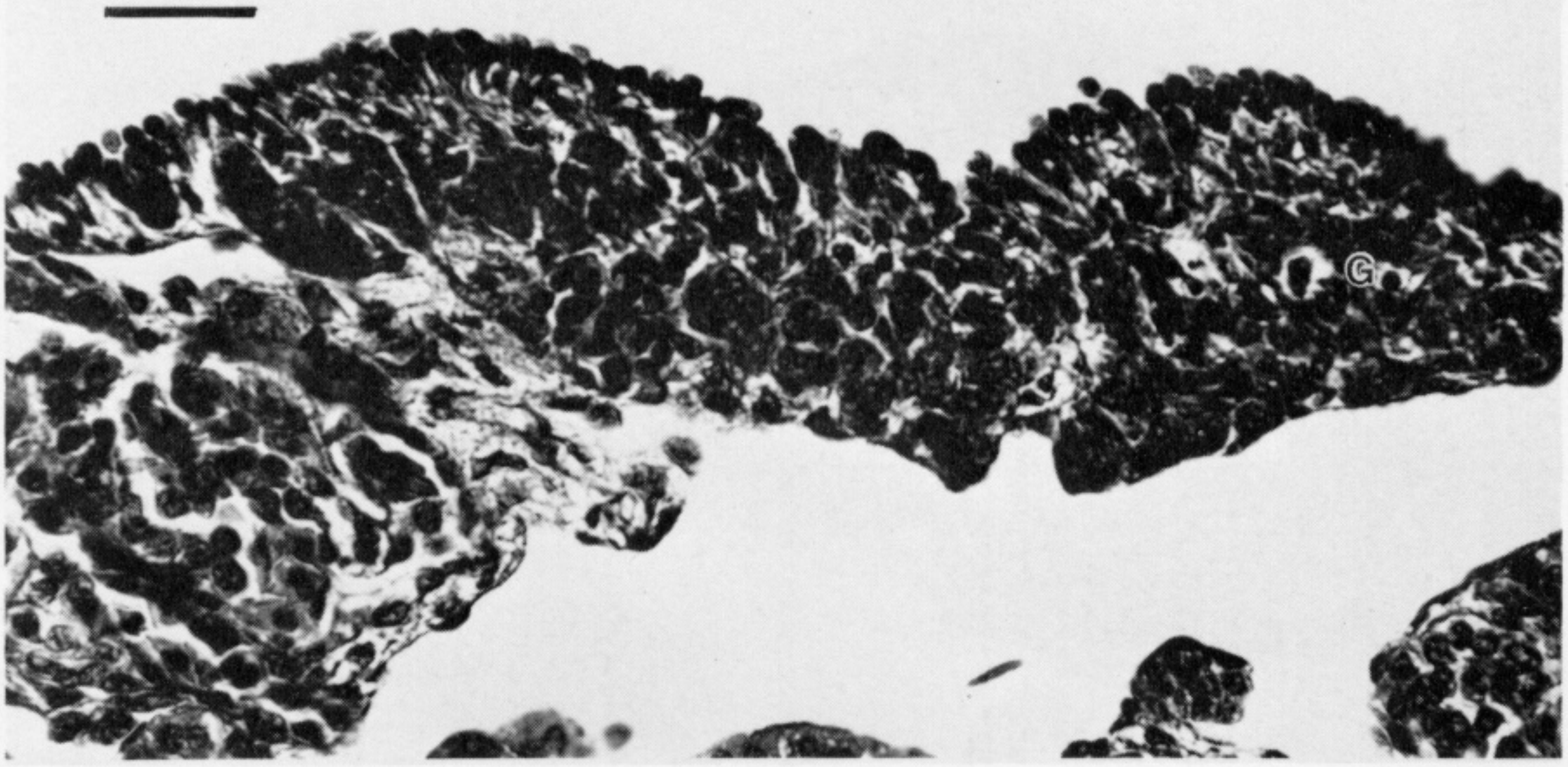


FIGURE 9 *a–c*. For description see opposite.

(d)



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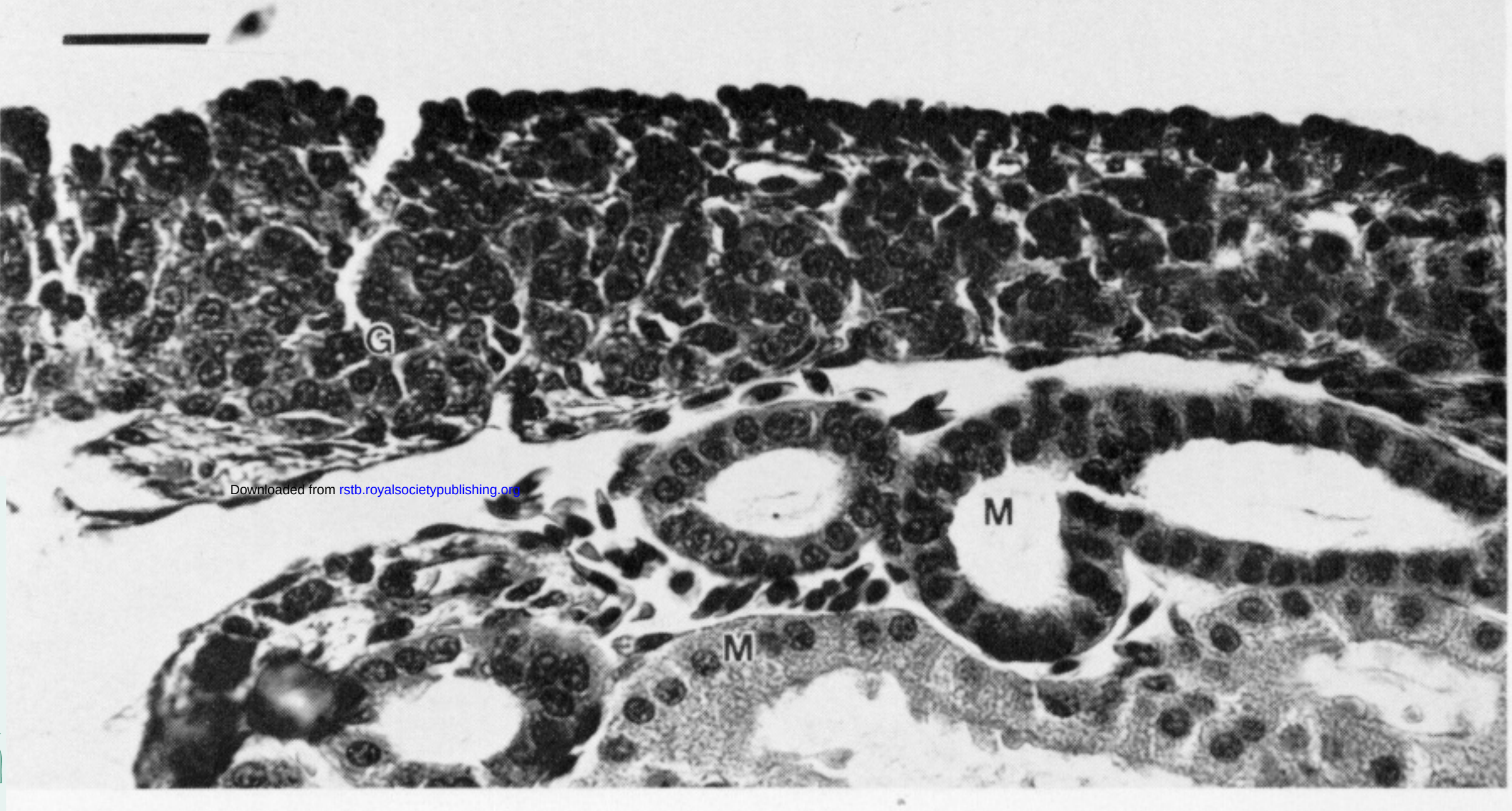

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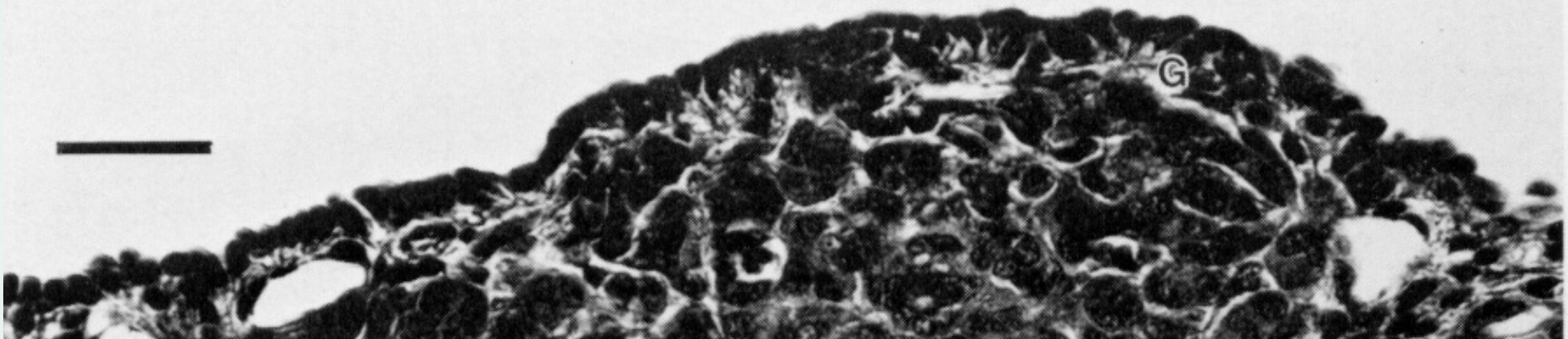

FIGURE 9 *d–f*. For description see p. 28.

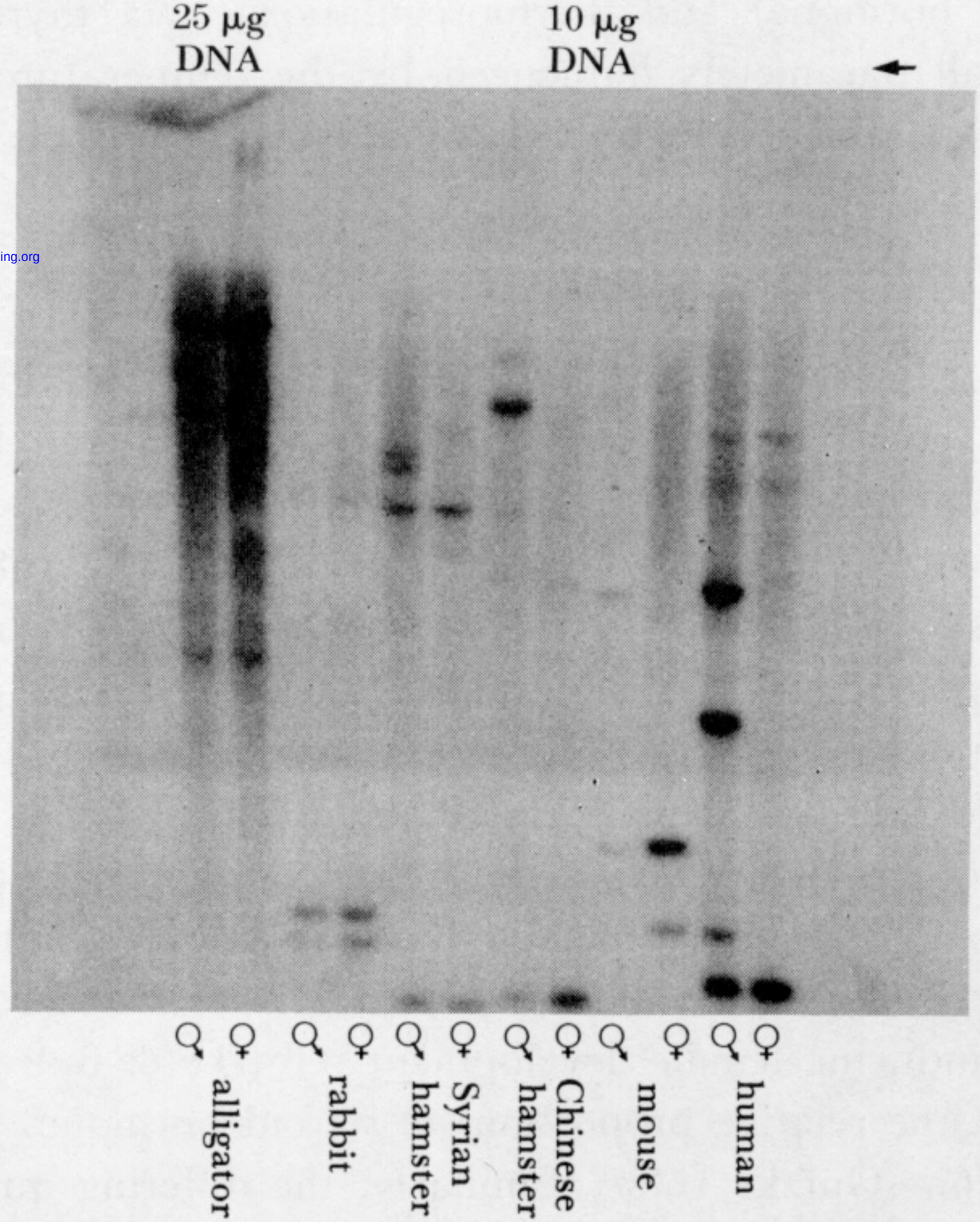

FIGURE 10. 'Ark blot' of DNA from male and female alligator, rabbit, Syrian hamster, Chinese hamster, mouse and man, hybridized with the PMF1 insert, 18 h at 55 °C, 1 M NaCl, 10% dextran sulphate and 1% SDS; washed 3 × 20 min, 65 °C, 0.2 × SSC (1 × SSC = 0.15 M sodium chloride + 0.015 M sodium citrate), 0.2% SDS and exposed for 3 days at −70 °C. Note that 25 µg of alligator DNA was loaded as opposed to 10 µg of mammalian DNA. Hybridization with the *TDF* probe to male and female alligator DNA is evident. This analysis was done by Dr. P. N. Goodfellow, Imperial Cancer Research Fund Laboratories, London.

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