

## Foetal testes control the prenatal growth and differentiation of the gubernacular cones in rabbits - a tribute to the late Professor Alfred Jost

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### SUMMARY

Gubernacular cones develop during foetal life in males of various species, including many of the common small laboratory animals. Postnatally these papilla-like organs invert and develop into the muscular cremaster sacs, providing space for testis descent. The mechanism governing male-specific development of these structures during foetal and postnatal life is unknown but foetal testicular androgens or anti-Müllerian hormone are unlikely to be involved. The present study of gubernacular cone development in 28-day-old rabbit foetuses castrated 5-9 days before questions whether foetal testis hormones play any role in these developmental processes. The study comprised an analysis of the microscopic slides in the legacy of the late Professor Alfred Jost in Paris.

Castration at an earlier (19 days) or later (23/24 days) day of foetal life interfered with gubernacular cone growth and differentiation. Unilateral castration partially inhibited ipsilateral gubernacular cone growth. Implantation of a foetal testis close to the ovary could induce male-type gubernacular cone growth in females. Together the data unequivocally support the concept of foetal testicular hormonal control of male-specific gubernacular cone development. Further study is required to unravel the nature of the active foetal testicular agent.

Key words: foetal castration, rabbits, gubernacular cone, cremaster sac, sexual differentiation

### INTRODUCTION

Gubernacular cones are male-specific, papilla-like structures growing in the abdominal inguinal region towards the end of prenatal life in various common small laboratory animals such as mice, rats, hamsters and rabbits (van der Schoot and Elger, 1993). The development of these organs is an important, albeit by itself insufficient, prerequisite for postnatal testis descent (Bergh et al., 1978). After birth, the organs differentiate further to become the muscular cremaster sacs and thus the space into which descent of the testes occurs. The gubernacular cones are to be judged specialized parts of the inguinal abdomen wall and they receive their innervation (N. genitofemoralis, ramus genitalis) and vasculature (A. and V. cremasterica; Pernkopf, 1989) from the caudoposterior abdomen wall and not from the foetal genital ridges.

The prenatal gubernacular cone development is part of the male body differentiation process. As a general rule (Jost et al., 1973), prenatal male bodily sexual differentiation occurs in response to the male-specific exposure of sex-shared anlagen to testicular androgens. The Müllerian ducts are an exception to this general rule as their failure to development in males is dependent on exposure to foetal testicular Anti-Müllerian Hormone (AMH). The gubernacular cones and the cremaster sacs, developing postnatally from them, seem a further exception to the above general rule of androgen action on masculine somatic sexual differentiation. The

organs do not show a definite growth response to androgen either during foetal life, or during adult life (Selye, 1943; Almquist and Andrews, 1944; van der Schoot, 1992a). Prenatal exposure of female rats to androgen does not result in female gubernacular cone growth (Greene et al., 1938; Schultz and Wilson, 1974). Prenatal exposure of male rats to potent anti-androgens such as cyproterone acetate (Elger, 1966) and flutamide allows normal gubernacular cone growth prenatally and cremaster sac growth postnatally (van der Schoot, 1992b; van der Schoot and Elger, 1993). It has further been amply demonstrated that the human and other mammalian syndrome of androgen insensitivity (also called the testicular feminization syndrome) is characterized by unopposed development of muscular cremaster sacs and inguinal hernia sacs (Polani, 1970; Brook et al., 1973; Quigley et al., 1992). Together the data demonstrate unusual properties of both the foetal cremaster sac anlagen and the adult organs as androgen-independent male bodily sex characters, but the agent responsible for their growth has not been investigated.

Various authors have proposed that AMH could be the foetal testicular agent responsible for the male-specific gubernacular cone growth. This hypothesis is difficult to reconcile with various kinds of data. (1) Recent studies with transgenic male and female mice showing increased expression of AMH do not reveal features indicative of abnormal prenatal gubernacular cone growth or their postnatal transformation to cremaster sacs (Behringer et al.,

1990). (2) In the human syndrome of AMH deficiency, affected males show retained Müllerian duct derivatives together with inguinal hernia sacs occupied or not by testes and other genital structures (e.g. Guerrier et al., 1989; Hutson and Watts, 1990). (3) An *in vitro* study of the factors potentially involved in gubernaculum cell proliferation revealed that AMH did not show such action while another testicular factor, suggestively called descendin, did (Fentener van Vlissingen et al., 1988). Together these data do not support a role for AMH in prenatal gubernacular cone growth or postnatal cremaster sac development.

Studies with marsupials suggest an entirely different explanation for the unusual behaviour of the gubernacular cones and cremaster sacs as male bodily sex characters. In addition to early observations by Burns (1961), investigators have proposed that bodily sexual differentiation in marsupials occurs partially before any gonadal differentiation has taken place. The initial appearance of the scrotum in males and pouch and mammary glands in females is judged to occur without a specific role of gonadal hormones (O et al., 1988; Renfree and Short, 1988; Shaw et al., 1988). The male-specific growth of the scrotum is, accordingly, suggested to reflect actions of Y-specific genes on these organs' anlagen rather than actions of gonadal hormones. The proposed 'direct' genetic influence of marsupial scrotum development could be conserved in eutherian mammals resulting in the cremaster sacs, a normal component of the adult scrotum, developing from actions on their anlagen of Y-specific gene products. The idea that Y-chromosome genes rather than testis hormones are responsible for prenatal growth of gubernacular cones and postnatal growth of cremaster muscles would be new to theories of eutherian male bodily sexual differentiation and would obviate the search for testis hormones involved in growth and differentiation of these organs (Hutson et al., 1990).

No conclusive evidence has been published yet to support unequivocally the involvement of testis hormones in prenatal gubernacular cone development. Present methodologies do not easily allow an experimental design to answer this question specifically. In the past, a potentially fruitful approach has been designed by the late Professor Alfred Jost using castration of rabbit foetuses before the time of sexual differentiation. Analysis of the histology of the genitalia allowed him to establish unequivocally, and in a unique fashion, the key role of testis hormones in male genital tract differentiation (Jost, 1947 I, II, III). Throughout his analysis no attention was paid to gubernacular cone development. Obviously, however, his microscopic preparations could be appropriate to determine whether or not the foetal rabbit testes are responsible for male-specific gubernacular cone growth.

## MATERIALS AND METHODS

The latter conclusion was substantiated during an exchange of ideas with Professor Alfred Jost who confirmed that the microscopic slides from the experiments during the nineteen forties had been carefully preserved and were available for research. His sudden death in February 1991 delayed examination of the slides.

However, the generous cooperation of Dr S. Magre and Mrs S. Perlman in the Laboratoire de Physiologie du Développement (Collège de France, Paris) has recently allowed the reexamination of the collection of slides in Jost's legacy.

All preparations that are included in the presented analysis were obtained from animals in the breeding colony of the Ménagerie in the Jardin des Plantes. Normal male and female rabbit preparations were available between the ages of 18 and 28 days post-coitum. There were further preparations of males castrated unilaterally or bilaterally on day 19 to 24 post-coitum and killed on day 28. Castration before day 19 had been incompatible with foetal survival (Jost, 1947 I). The earliest castrations had taken place in animals not bearing signs of genital ducts' sexual differentiation (Jost, 1947 III). Finally, slides were also available from female foetuses that had received one or more testicular transplants in the mesoalpinx on day 20 post-coitum and had been killed on day 28.

The animals had all been fixed in Bouin's solution. The lower body halves, excluding the extremities, had been sectioned transversely at 10 µm and every 25th section mounted. From selected parts of some animals, all sections had been mounted serially. The sections had been stained with haematoxylin and eosin (Jost, 1947 I). The preparations were undamaged and of an excellent quality. Much effort had been made, both during the nineteen forties and more recently, to keep all relevant data from all preparations together. The sex of all intact animals from 18 days and more p.c. could be identified from the study of the gonads: at day 18 post-coitum and later, testes and ovaries are unequivocally distinguishable from each other.

The sections were examined for the presence of the gubernacular cones recognizable through their specific appearance in the inguinal abdominal area (van der Schoot and Elger, 1993). The cones' lengths (in craniocaudal direction) was estimated from a count of the number of sections in which they were visible. A continuity correction was included by adding half of the distance between successive slides (=125 µm) at both ends. An estimate of their size further included the largest cross-sectional size. Wherever possible a distinction was made between the different components in the cones' tissue: vasculature, nerves, muscle cells and mesenchymatous cells.

## Statistics

Statistical analysis of the results comprised one-way ANOVA followed by analysis of significant ( $P < 0.05$ ) overall differences with the Tukey's honestly significant difference test (Kirk, 1968).

## RESULTS

### Gubernacular cones in normal males and females (Figs 1, 2; Table 1)

At 18/19 days post-coitum (p.c.), sexual differentiation of the genital ducts had not yet commenced. Both males and females showed well-developed mesonephric (Wolffian) ducts and minor paramesonephric (Müllerian) ducts. In the lower abdomen, the gubernacular cones were clearly distinguishable and there was no sex difference in either their general appearance (Fig. 1A-L) or their length (Table 1). The concentric arrangement of cells in the area close to the inguinal abdomen base was obvious and the peritoneal fold connecting the cones with the posterior body wall was of a simple and straight appearance. There was a length of 'empty' peritoneal folds between the tip of the gubernacular cones and the caudal end of mesonephros remnants.

At 23 days post-coitum, sexual differentiation of the genital ducts resulted in the presence of Müllerian ducts in

**Table 1. The length\* of the gubernacular cones in male and female foetal rabbits: effect of male castration on day 19 or 24 post-coitum**

Sex (days post coitum)	No. cones	Length (microns±s.e.m.)
male (18)	10	725±55***
male (23)	6	2083±76
male (28)	8	3844±187
female (18)	7	786±60
female (23)	6	1042±70
female (28)	7	1714±185
male (28) after castration (19)	10	2675±191
male (28) after castration (22)	11	2932±204
male (28) after castration (24)	10	3675±158
male (28) after unilateral castration on day 19		
side of castration	6	3083±127**
side with intact testis	6	4042±181**

\*Length estimated from counting the numbers of sections taking into consideration the distance between sections (250 µm) and a continuity correction (2× half the distance between successive sections)

\*\*Paired data - Student's *t*-test  $P<0.01$

\*\*\*Honestly significant difference test: all group means differing 280 µm are significantly different at the level  $P<0.01$ .

females and Wolffian ducts in males. The growth of gubernacular cones in females was slight compared to 18 days, but, in males, growth had occurred extensively both in size and length (Table 1) of these organs. Further sex differences had developed: the cones' tip in males touched the lower end of the developing Wolffian duct while, in females, a considerable 'empty' length of peritoneal fold was present between the tip of the cones and the lower end of the developing gonads. In males, differentiation had occurred in the cones between outer darkly staining layers of myoblasts and inner layers of mesenchymatous cells while the cones in females remained histologically homogeneous. The peritoneal fold between cones and posterior body wall remained 'simple' in females but developed to a large size and structural complexity in males.

At 28 days post-coitum, female gubernacular cones were not obviously different from those at day 23. Their connection to the posterior body wall consisted of two flat layers of peritoneal epithelium and 'empty' folds were present between the tip of the cones and the lower end of the gonads and developing Müllerian ducts (Fig. 2A-D). Below the cones, minor inguinal canals were recognizable carrying the blood vessels (A. and V. ligamenti teretis uteri) and nerves (N. genitofemoralis, pars genitalis) from the posterior body wall to the area in front of the pubic bones and further below (including the now easily recognizable anlagen of the preputial glands). Male gubernacular cones had become much enlarged and differentiation had further progressed between muscular outer layers and mesenchymatous inner layers. The peritoneal fold between the cones and the posterior body wall had further increased in size and structural complexity (Fig. 2E-H): obvious growth and differentiation in the tissues making up the parenchyma between the two peritoneal epithelial layers had occurred. Below the cones, wide inguinal canals developed carrying the blood vessels (A. and V. cremasterica) and genitofemoral nerves.

Further easily identifiable muscular development of the inguinal canal walls had taken place.

### Gubernacular cones in bilaterally castrated males (Fig. 3; Table 1)

Males castrated on day 19 showed easily distinguishable gubernacular cones on day 28 (Fig. 3A-F). Their cross-sectional size was of the same order as on day 18. However, their length was significantly more than that on day 18 and that in females of age 18 to 28 days (Table 1). The peritoneal fold between the gubernacular cones and the posterior body wall had a flat female-like appearance. No structural differentiation had taken place in the cone tissue between muscular outer layers and mesenchymatous inner layers.

Males castrated on day 23/24 showed advanced growth of the gubernacular cones on day 28. Their maximum cross-sectional diameter and length were significantly above those on the day of castration (Table 1). There was failure of differentiation between muscular outer and mesenchymatous inner layers of the cones. Also, further structural differentiation of the peritoneal fold between the cones and the posterior body wall had remained absent.

### Gubernacular cones in unilaterally castrated males (Table 1)

Unilateral castration on day 19 p.c. allowed seemingly normal gubernacular cone growth on the side where the testis remained in place. On the side of castration, however, gubernacular cone length was shorter in all 6 animals examined ( $P<0.05$ ).

### Gubernacular cones in females bearing transplanted testes (Fig. 4A-D)

Testicular transplantation had resulted in functional testicular activity in at least three females as persistence of the Wolffian ducts demonstrated. In these foetuses, considerable growth of the gubernacular cones in a male-like fashion occurred. At its best (Fig. 4A-D), the size and differentiation of the cones, but not of their dorsal peritoneal folds, developed to an extent similar to that of normal 28-day-old male foetuses.

## DISCUSSION

The present results demonstrate that foetal rabbit testes control the male-specific growth of the gubernacular cone anlagen. Castration at a time before emergence of an obvious sexual dimorphism in gubernacular cone development results in reduction of the further growth of these organs and in failure of structural differentiation. It is clear, however, that their development at day 28 p.c., after castration on day 19 p.c., is more than that of normal female foetuses at that time. Castration on day 23/24, after emergence of the sexual dimorphism in the organs' development, decreased further growth. Unilateral castration on day 19 p.c. allowed further growth and structural differentiation although the final size on day 28 was less at the operated side than at the unoperated side in all foetuses examined.

The further development at day 28 after castration on day 19 may indicate that the male-specific development is

partially independent of testicular hormones. However, another explanation may be valid as well. The further development after castration suggests that histologically visible

effects of testis hormones on the organs' anlagen may take time to develop. The mere absence of a structural sexual dimorphism at the time of castration (day 19) does not

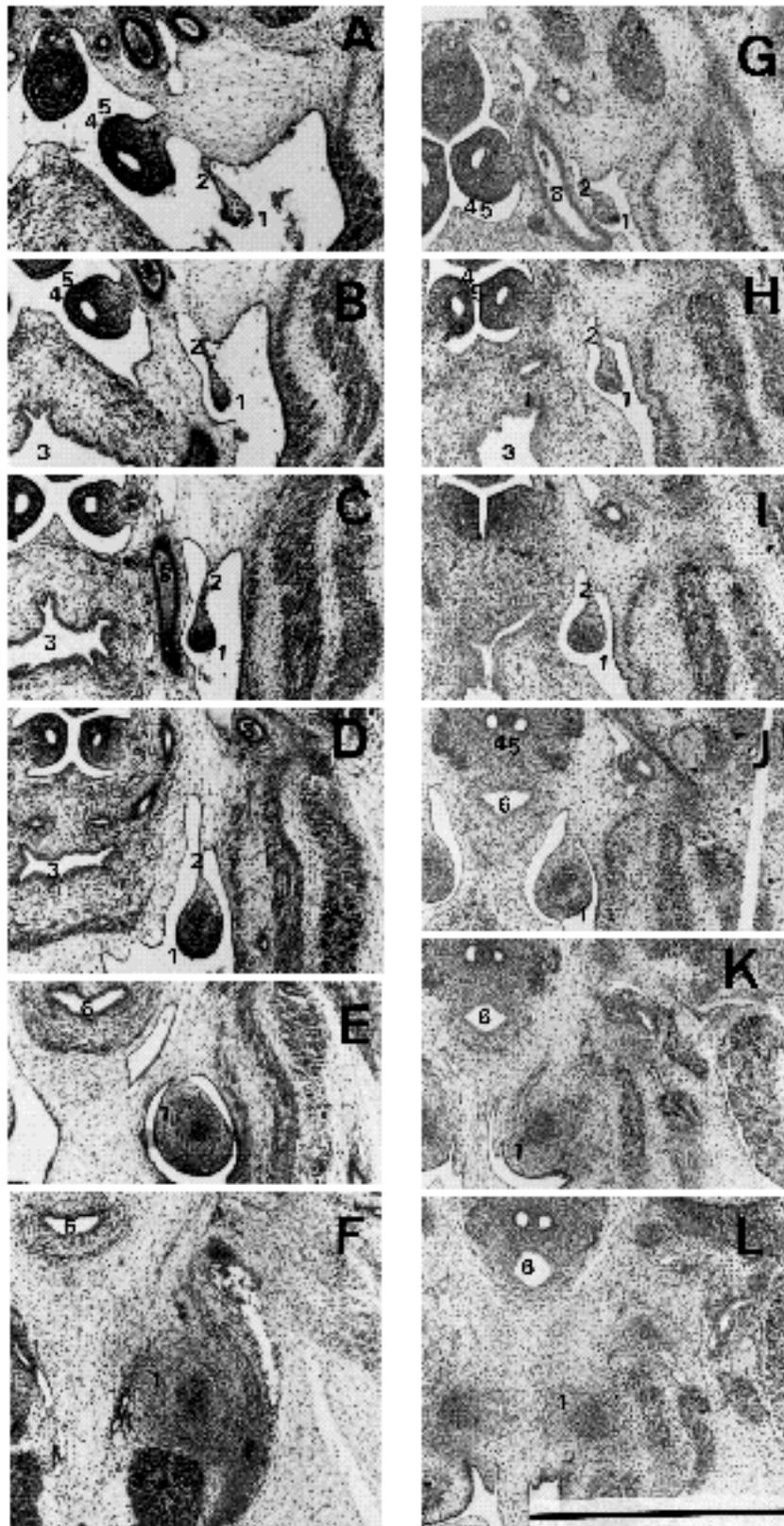


Fig. 1. For legend see p. 1333.

preclude that processes of sex-specific growth and differentiation have already started but have not yet resulted in histologically visible distinctions. Delayed appearance of androgen-dependent effects on prostate growth has been reported in mice after neonatal castration (Donjacour and Cunha, 1988). In fact, Jost (1947 III) showed that histologically recognizable prostate development could continue after foetal castration, thus in the absence of continued testicular hormonal support. It may well be that foetal testes have started male-specific hormonal activities before day 19 as sexual differentiation of the gonads starts as early day 15 p.c. (Jost, 1953). Castration before day 19 p.c. would seem

a logical next step to obtain supportive evidence. The painstaking efforts by the late A. Jost (1947-III) have indicated, however, that castration at an earlier age is to be judged incompatible with survival.

Earlier data indicated that androgens play no role in gubernacular cone growth. By day 19 p.c., there is no evidence to support androgen secretion by fetal testes to any extent (Wilson and Lasnitzki, 1971; Wilson and Siiteri, 1973). Large amounts of the anti-androgen cyproterone acetate in foetal rabbits and of the anti-androgen flutamide in foetal rats allowed their quantitatively normal growth and foetal exposure to androgen does not enhance gubernacular

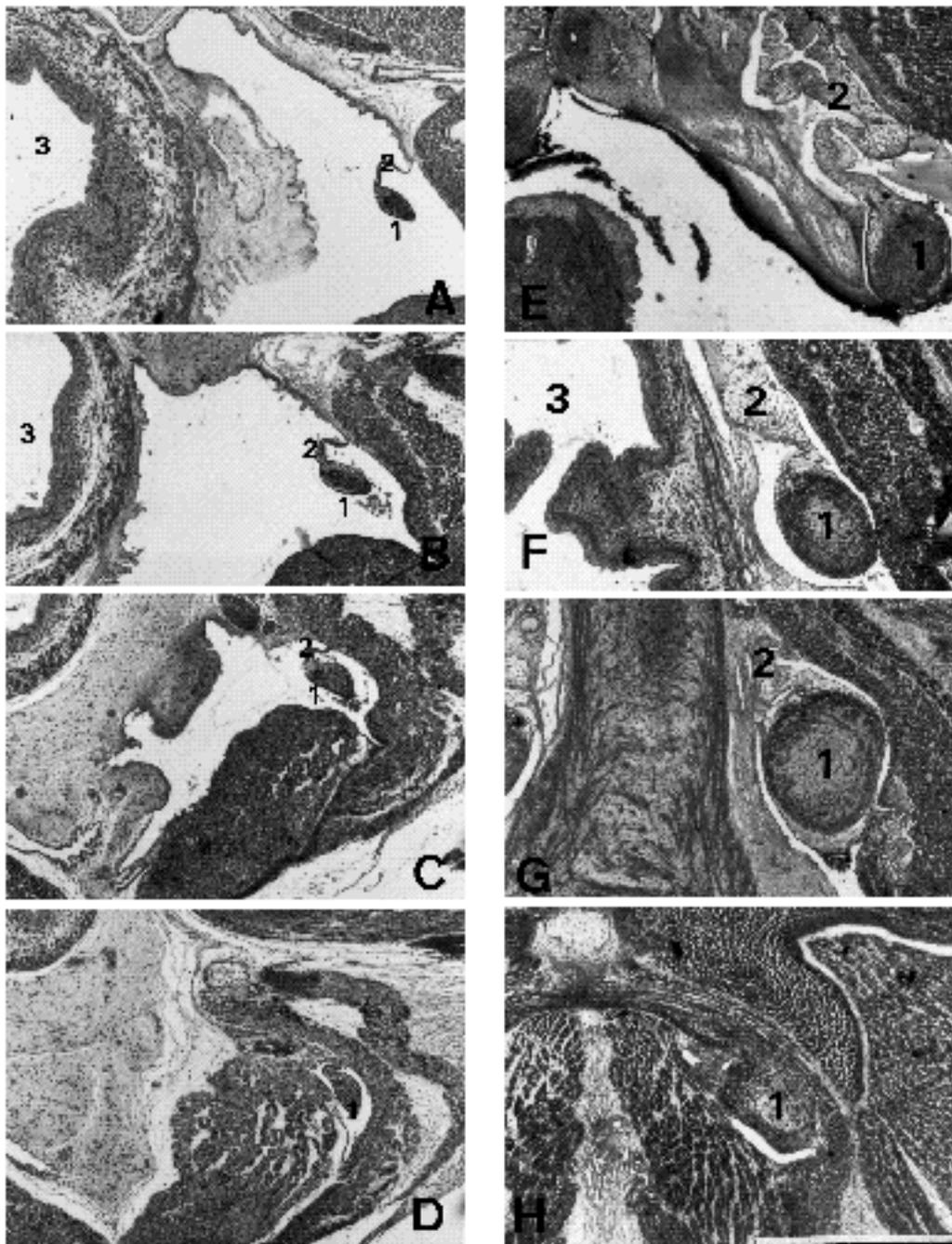


Fig. 2. For legend see p. 1333.

cone growth in female foetuses (Schultz and Wilson, 1974; van der Schoot and Elger, 1993). As argued before, AMH is unlikely to play a role in gubernacular cone development (Fentener van Vlissingen et al., 1988; Behringer et al., 1990; Guerrier et al., 1989; Hutson and Watts, 1990). To account for the hereby demonstrated testicular influence on the cones' anlagen, a further testis hormone should be postulated. In fact, it could be the hormone proposed recently to account for gubernacular mesenchyme growth in vitro by Fentener van Vlissingen et al. (1988). These authors characterized a foetal testicular substance in pigs with stimulatory action on growth of cells obtained from the pig's homologue of the mesenchyme in the core of the gubernacular cones.

Further work is required to determine the nature of the hereby postulated rabbit testicular substance involved in prenatal gubernacular cone growth and its possible relationship with the proposed 'descendin' (Fentener van Vlissingen et al., 1988). Alternatively, the testicular substance could be related to (or identical to) the proposed neurotransmitter calcitonin gene-related peptide which occurs, in a sexually dimorphic fashion, in the sexually dimorphic genitofemoral nerve and its spinal motor nucleus (Larkins et al., 1991). The peptide is proposed to be involved in the development, perinatally, of the cremaster muscle sacs from their gubernacular cone anlagen (Yamanaka et al., 1993).

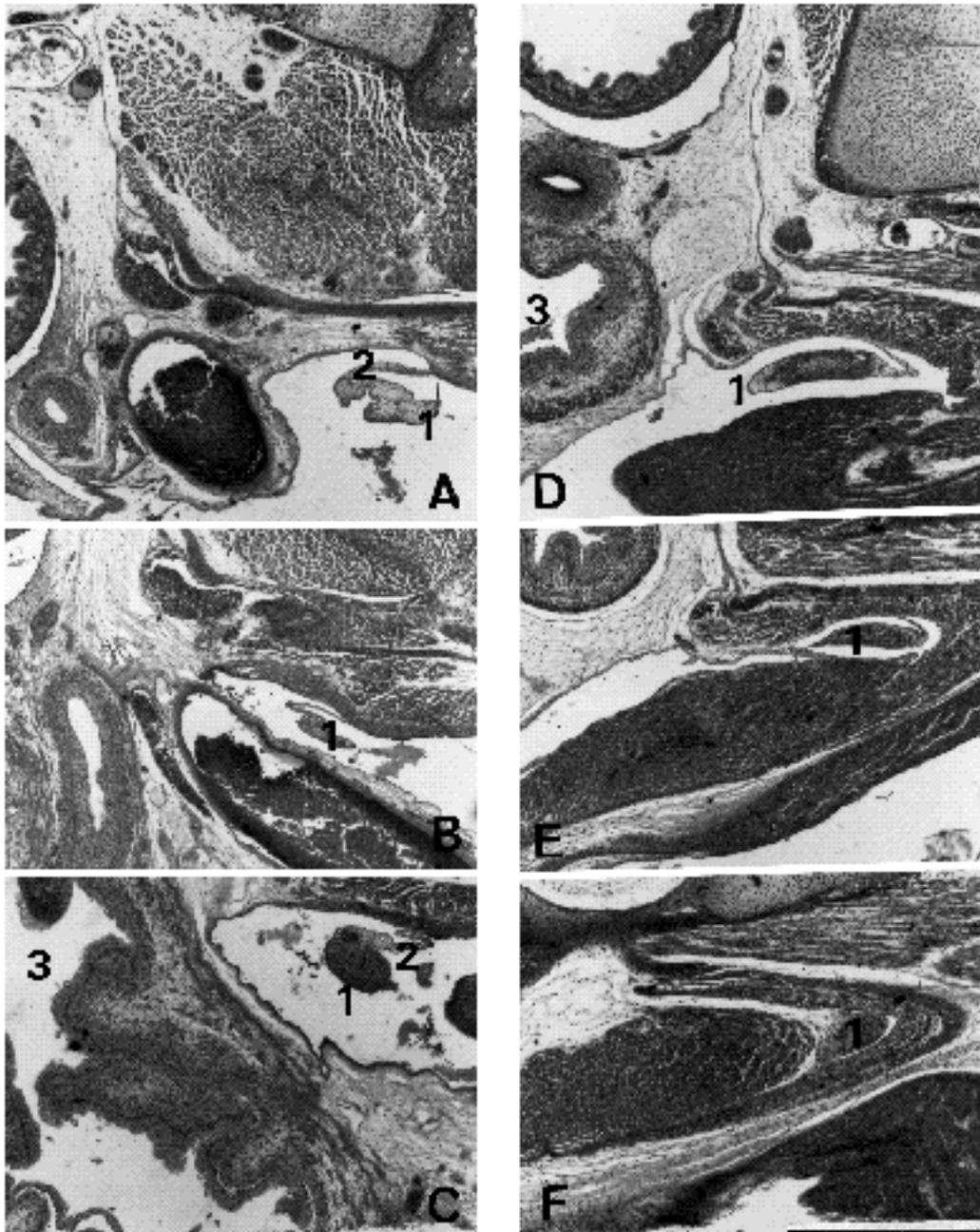


Fig. 3. For legend see p. 1333.

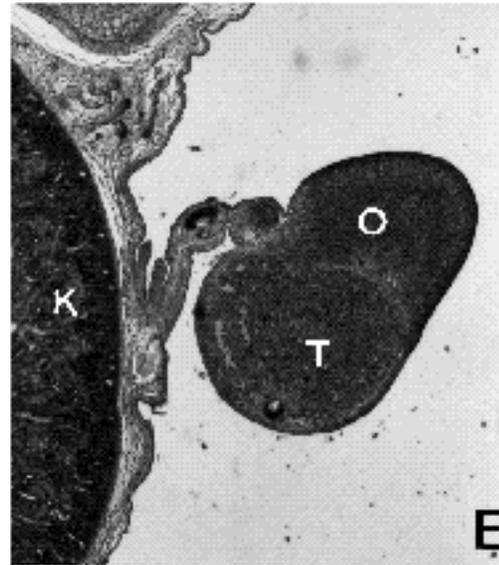
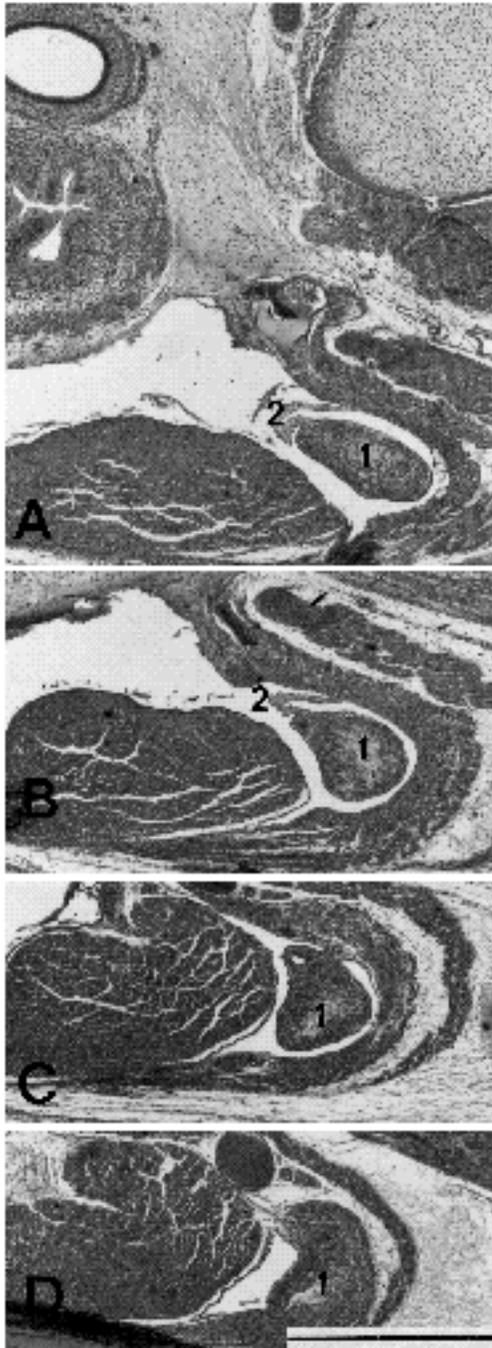


Fig. 4A-E

**Figs 1-4.** The gubernacular cones in 18- and 28-day-old male and female rabbits and the effect of castration on day 19 (males) and testis implantation on day 20 (females). All pictures represent series of transverse sections of one animal from cranial to caudal between the tip of the inguinal cones and their base in the lower inguinal abdomen; intestines have been removed. The upper side of all pictures represents the dorsal, and the lower part represents the ventral side of the body. The cones (1) emerge from the lower abdomen and their part protruding from the base is connected, via a peritoneal fold (2), with the posterior body wall. Further organs and structures have been indicated to facilitate understanding of the body site from which the section were taken: 3, bladder; 4 / 5, Müllerian / Wolffian duct; 6, urogenital sinus; 7, lateral body wall; 8, umbilical artery. The bars (lower right) indicate 1 mm.

**Fig. 1.** The gubernacular cone of a 18-day-old female (A-F) and male (G-L) rabbit. Distance between successive sections: 0.5 mm.

**Fig. 2.** The gubernacular cone of a 28-day-old female (A-D) and male (E-H) rabbit. Distance between successive sections: 1.0 mm.

**Fig. 3.** The gubernacular cone of a 28-day-old male rabbit that had been castrated on day 19 post-coitum. Distance between successive sections: 1.0 mm.

**Fig. 4.** (A-D) The gubernacular cone and (E) the transplanted testis (T) adjacent to the ovary (O) and kidney (K) of a 28-day-old female rabbit that had been implanted with a foetal testis 8 days before. Distance between successive sections A-D, 1.0 mm

The present data do not, of course, allow speculation on the nature of the testicular hormone involved. However, the ipsilateral reduction of gubernacular cone growth after unilateral castration shows a similarity to the unilateral action of AMH on the developing Müllerian ducts. If unilateral AMH action is to be explained through local tissue diffusion of a protein hormone rather than transport through the circulation, it can be presumed that the hereby postulated hormone for gubernacular cone growth is also a protein. Further work should be carried out to investigate this further. The difficulties met by the late A. Jost in collecting the material upon which this study is based make research following his methods unattractive. A more promising route

might be the analysis of testicular effects on the developing gubernacular cones *in vitro*. It will depend, however, on the nature of testicular action on the developing gubernacular cones whether such approach will yield fruitful results. The gubernacular cones are far more complex structures than has hitherto been appreciated (Wensing and Colenbrander, 1986). The studies performed by Fentener van Vlissingen et al. (1988) used dispersed cells of an undefined nature. As shown here, gubernacular cone development includes several components: cell proliferation but also cell differentiation. Moreover, the last stages of perinatal growth require morphogenetic events as the cones need to invert in order for development of cremaster sacs to take place (van der

Schoot and Elger, 1993). The sexually dimorphic growth and development further include growth of motor innervations of the developing cremaster muscle cells. Gubernacular cones prenatally and muscular cremaster sacs postnatally receive their motoric innervation via the genitofemoral nerves: the motoneurons innervating the cremaster muscle cells belong to a sexually dimorphic cohort of spinal motoneurons (Nagy and Senba, 1985). If intact connections between those motoneurons and the developing gubernacular cone myoblasts are a prerequisite for testicular effects on the conus differentiation (Hutson et al., 1988), it would almost seem impossible to design an appropriate model for in vitro analysis. Firstly, it should be determined whether or not in vitro foetal testicular effects on growth and differentiation of the gubernacular cones can be obtained and, if so, secondly, what is the nature of the active testicular agent.

Thanks are due to the board of the Collège de France for granting access to the legacy of Prof. Jost, to Dr. S. Magre and Mrs. S. Perlman (Laboratoire de Physiologie du Développement, Collège de France, Paris) for their enthusiastic cooperation during the collection and further working out of the data upon which this paper is based. The results of this study may hopefully encourage the creation of conditions for saving the collection of microscopic slides of the late Professor Alfred Jost for later generations of students: the efforts made to collect these unique materials during the nineteen forties justify that proper actions take place in the near future.

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