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Gonadal sex differentiation in embryos and neonates of the marsupial, *Monodelphis domestica*: arrest of testis development in postterm embryos

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ABSTRACT

Growth and histological differentiation were studied in 8 litters of embryos and 4 litters of neonate grey short-tailed opossums, *Monodelphis domestica*. The embryonic litters included 2 that had passed their expected birth date, and whose weights exceeded the usual birthweights; we refer to these litters as 'postmature'. There was an abrupt increase in the growth rate of XY gonads after birth, but this was not seen in XX gonads. Although there was evidence of testicular differentiation in XY gonads on the day before the expected birth, testicular differentiation was found to be blocked in postmature litters. The growth of XX gonads in postmature embryos was not affected. In view of evidence that exogenous oestrogens feminise the gonads of genetic males in some species of marsupials including *Monodelphis domestica*, the question arises whether oestrogen is responsible for the failure of testes to continue their development in utero. We suggest that the ability of functional testes to develop in the presence of oestrogen may be a fundamental requirement distinguishing eutherian mammals from other vertebrates, including marsupials.

INTRODUCTION

Marsupials differ from eutherian mammals in having a shorter gestation period and the young being born at a less developed stage. This could be relevant to the process of gonadal sex differentiation, which in Eutheria occurs to a large extent in utero. It has often been stated that the gonad of marsupials is indifferent at birth and differentiation into testis or ovary occurs postnatally. However, McCrady (1938) found the first histological signs of testicular differentiation in the Virginia opossum, *Didelphis virginiana*, at developmental stage 35, i.e. the time of birth; and although O et al. (1988) originally described the gonads of the tammar wallaby, *Macropus eugenii*, as undifferentiated at birth, a more recent investigation by Renfree et al. (1992), including electron microscopy, found the male gonad at birth to have features characteristic of a testis. Baker et al. (1990) examined pups of the grey short-tailed opossum, *Monodelphis domestica*, on the day of birth, and found the gonads of XY individuals to be both histologically and quantitatively different from those of their XX litter mates. We now present our findings on the growth and differentiation of embryonic gonads of the grey opossum and compare gonadal growth rates before and after birth.

MATERIALS AND METHODS

Embryos and neonates were taken from a breeding colony maintained at the Institute of Zoology. Details of the breeding regime have been published previously (Baggot et al. 1987; Baker et al. 1990). In classifying all unborn opossums as 'embryos', rather than 'fetuses', we follow the nomenclature of Kaufman (1992), validated by McLaren (1992).

The exact time when mating occurred was established using closed-circuit video recordings (Baggot et al. 1987) and the age of embryos was determined from this point. Birth normally takes place after 14.4 ± 0.4 d, which includes an interval of about 18 h between mating and ovulation. The time of

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Table 1. *Bodyweights and gonadal volumes in litters of opossum embryos*

Litter no.	No. of days after mating	Sex chromosomes	No. of individuals	Mean body weight (mg)	Mean gonad volume (mm^3)	S.E.M.	Ratio gonad volume/body weight	Ratio XY/XX
951	13.3	XY	2	51.4	0.00721	0.00046	0.000144	1.07
		XX	5	50.2	0.00663	0.00025	0.000134	
1004	13.4	XY	6	55.6	0.00732	0.00016	0.000136	1.05
		XX	6	59.1	0.00759	0.00036	0.000129	
952	14.2	XY	4	69.1	0.00796	0.00075	0.000116	1.00
		XX	10	69.3	0.00790	0.00037	0.000116	
960	14.0	XY	3	98.2	0.00943	0.00028	0.000096	1.23
		XX	3	109.6	0.00855	0.00032	0.000078	
954	14.4	XY	5	105.1	0.00954	0.00046	0.000091	1.14
		XX	8	98.1	0.00776	0.00030	0.000081	
1073	14.1	XY	4	112.7	0.01000	0.00029	0.000089	1.10
		XX	4	122.6	0.00995	0.00041	0.000081	
1096	15.4	XY	3	111.9	0.00965	0.00037	0.000087	1.05
		XX	3	114.7	0.00954	0.00032	0.000083	
1052	15.5	XY	3	118.0	0.00988	0.00017	0.000084	1.02
		XX	4	122.1	0.01001	0.00039	0.000082	

birth was determined by continuous observation of females close to parturition.

Embryos and newborns were processed in an identical fashion. To obtain embryos, the mother was killed by an intraperitoneal injection of Euthatal (May & Baker) on d 13, 14 or 15 after mating. Each embryo was dissected out, weighed, and killed by decapitation. Chromosome preparations were made from tail tips by the method previously described (Moore & Thurstan, 1990). The bodies were trimmed, fixed in Bouin's solution and prepared for serial sectioning at 7 μm, followed by staining with haematoxylin and eosin. Volumes of left and right gonads were computed from section areas transposed onto a digitising tablet via a Leitz camera lucida (Baker et al. 1990). Measurements were carried out in the absence of knowledge of chromosomal sex, and the gonads were evaluated histologically at the same time.

Quantitative and histological data are available on 8 embryonic litters containing a total of 73 individuals. Two of the litters, having failed to be born the day after the expected day of birth, were judged to be postmature, and this was supported by their bodyweights. The mean birthweight ± S.E.M. of 5 litters totalling 25 young has previously been found to be 98.52 ± 1.37 mg (H. D. M. Moore, unpublished data), compared with 97.9 ± 2.3 mg by (VandeBerg, 1990). In an additional litter, 3 d before the expected day of birth, the gonads were too small to be measured.

For the purpose of illustrating the relationship of gonadal growth to bodyweight in Figure 1, embryos and neonates were classified according to bodyweight into groups of 20 mg. The data include 4 neonatal litters on the day of birth, published by Baker et al. (1990).

RESULTS

Quantitative data on the 8 prenatal litters in which gonads were measured are shown in Table 1 and those of 4 postnatal litters in Table 2. The ages of prenatal litters were calculated from the time of mating, and variation in time between mating and ovulation could contribute to the observed differences in weight of embryos at 14 d.

The mean gonadal volumes of XY individuals exceeded those of XX individuals in all 4 postnatal litters and in 6 out of 8 litters of embryos, while for mean gonadal volumes relative to bodyweights, XY individuals exceeded XX individuals in 7 embryonic litters, while in the 8th the ratios were equal.

In Table 3, a comparison is made between the gonadal volumes of postmature embryos and neonates having similar bodyweights. It will be seen that XY gonads increased by about 50% following birth, whereas there was no significant difference between the gonadal volumes of postmature XY embryos, postmature XX embryos, and XX neonates.

The relationship of gonadal growth to bodyweight is illustrated in Figure 1. Gonadal growth rate increases after the birth of XY neonates and this increase in gonadal growth does not occur in the postmature embryos, even though their bodyweights were similar to those of neonatal pups (Fig. 1*a*). By contrast, there is no acceleration in the growth of the

Table 2. *Bodyweights and gonadal volumes in litters of opossum neonates*

Litter no.	Age after birth (h)	Sex chromosomes	No. of individuals	Mean body weight (mg)	Mean gonad volume (mm^3)	S.E.M.	Ratio gonad volume/body weight	Ratio XY/XX
1033	41	XY	2	144.3	0.0139	0.00074	0.000097	1.23
		XX	4	123.0	0.0097	0.00112	0.000079	
950	51–55	XY	1	124.3	0.0144	—	0.000115	1.58
		XX	3	109.2	0.0080	0.00043	0.000073	
1042B	59	XY	4	165.8	0.0180	0.00117	0.000109	1.51
		XX	4	163.9	0.0118	0.00089	0.000072	
938	75	XY	2	181.8	0.0211	0.00204	0.000116	1.97
		XX	2	181.8	0.0107	0.00113	0.000059	

Table 3. *Comparison of gonadal volumes in XY and XX embryos and neonates of* M. domestica *weighing between* 110 *and* 132 *mg* (*means* ± S.E.M.)

	Chromosomes	n	Weight (mg)	Gonadal volumes (mm^3)
Embryos	XY	9	118.01 ± 1.43	0.009926 ± 0.000172
Neonates	XY	5	118.66 ± 2.37	0.014898 ± 0.000572**
Embryos	XX	13	119.63 ± 2.09	0.009492 ± 0.000297
Neonates	XX	12	119.99 ± 1.87	0.009810 ± 0.000323

** Value differs from that of XY embryos at probability level 0.005.

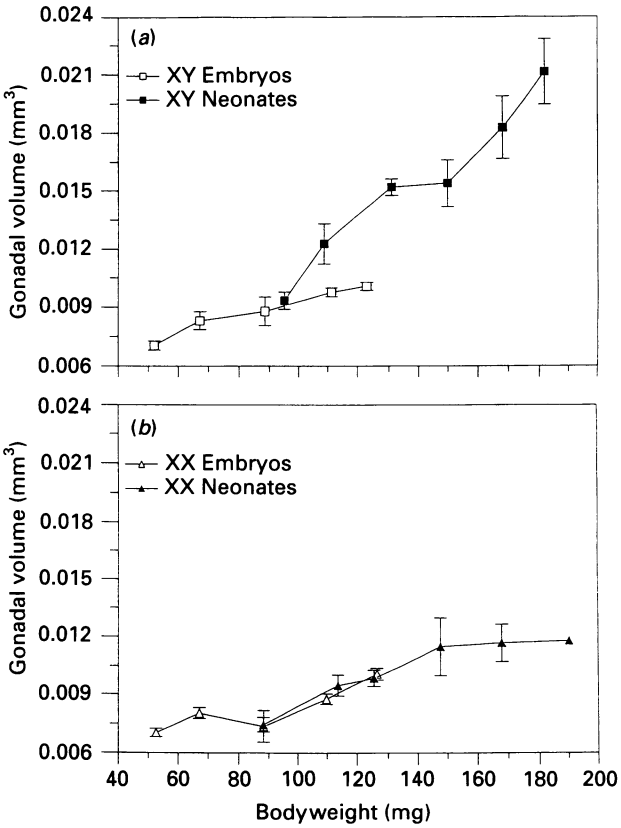

Fig. 1. Graphs illustrating the relationships of gonadal volumes to bodyweights in (*a*) 30 male opossum fetuses (open squares) and 23 neonates (solid squares), and (*b*) 43 female opossum embryos (open triangles) and 24 neonates (solid triangles).

gonads in XX neonates, which continues at the same rate as that in embryonic gonads (Fig. 1*b*).

The histology of the gonads is illustrated in Figure 2. There is evidence of the beginning of histological differentiation of the gonads in embryos on the day before birth. In Figure 2*a* we see the gonad of an XY embryo of body weight 97.2 mg. The gonad is surrounded by a tunica albuginea, while internally the cells are beginning to divide into right and left compartments, separated by a slightly oblique vertical boundary line that is free of cell nuclei. In addition, there is a tentative suggestion that each of the 2 compartments is about to break up into an upper and a lower portion. At the top of the gonad, a triangular wedge of preinterstitial cells (arrow) is separating the outer portions of 2 developing sex cords in much the same way as can be seen in the more advanced testis of a neonate illustrated in Figure 2*c*. The gonad illustrated in Figure 2*b* is from an XX embryo (bodyweight 103.7 mg), litter mate of 2*a*. This gonad, too, is surrounded by a tunica albuginea, and some of its inner cells are arranged in whorls; but, in contrast to Figure 2*a*, the spaces not containing cell nuclei do not effect a compartmentalisation of the gonad.

The gonad of an XY neonate (bodyweight 118 mg) is easily recognisable as a testis by the presence of distinct cords (Fig. 2*c*). As in Figure 2*a*, the cords are separated by triangular wedges of preinterstitial cells (arrows), but these have now penetrated to the centre of the gonad, thereby completing the separation of individual cords. The developing testis is surrounded by a tunica albuginea of 5 to 6 cells in thickness. The gonad of a postmature embryo (bodyweight 125.2 mg), that was dissected from its mother's uterus 24 h after its expected birth, is illustrated in Figure 2*d*. This shows only minimal signs of testicular differen-

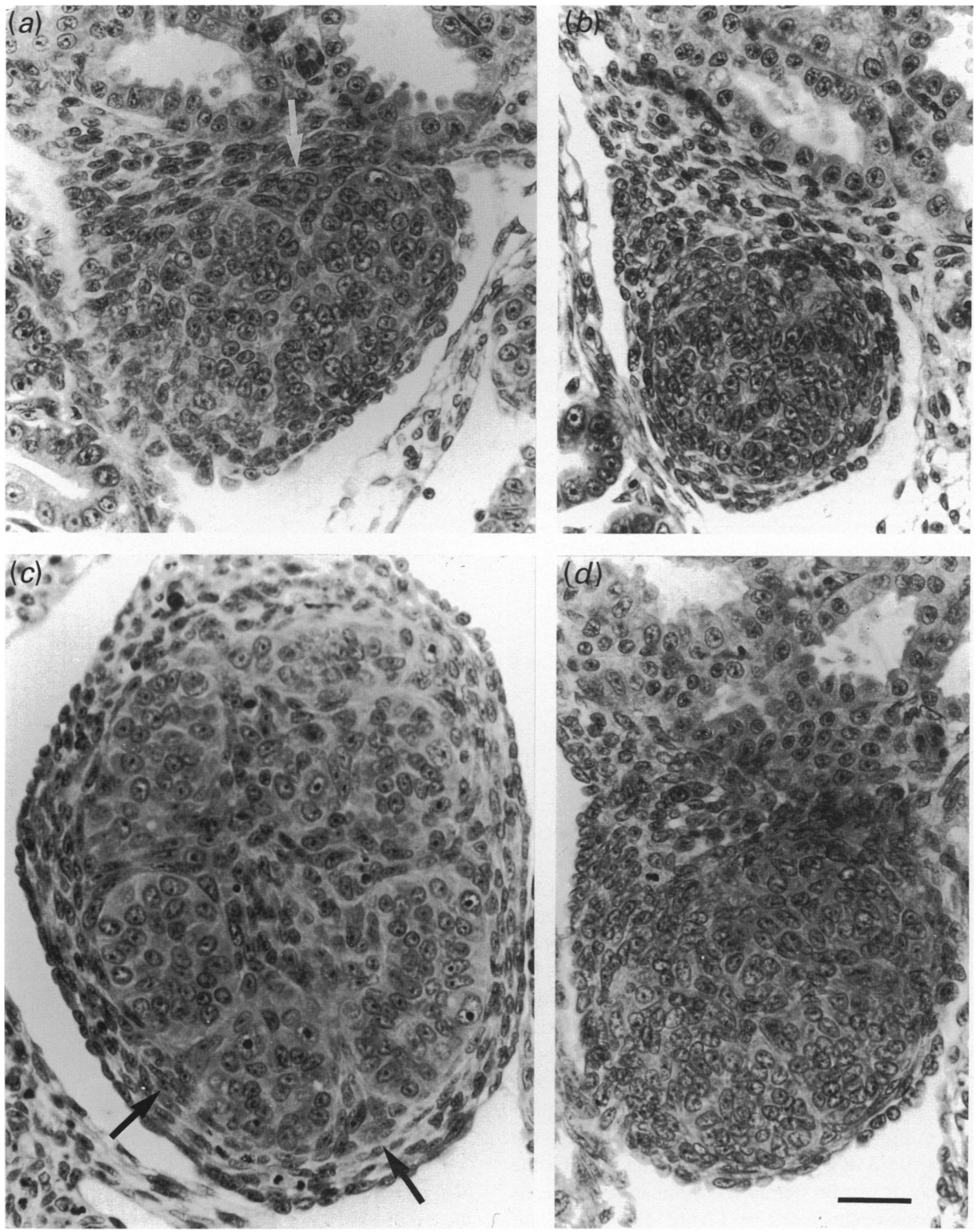

Fig. 2. Histological appearance of gonads. (*a*) Male embryo (bodyweight = 97.2 mg, gonad volume = 0.0095 mm^3). The gonad is surrounded by a tunica of 2–3 cells in thickness. A wedge-shaped mass of preinterstitial cells, resembling those in (*c*), is beginning to separate 2 masses of inner cells and, from the point of the wedge, an oblique vertical line lacking in nuclei effects the compartmentalisation of the gonad. (*b*) Female embryo from same litter (bodyweight = 103.7 mg, gonad volume = 0.0079 mm^3). This gonad is also surrounded by a tunica of 2–3 cells in thickness, and the inner cells appear to be arranged in whorls, but spaces between cell nuclei do not effect any compartmentalisation of the gonad. (*c*) Male neonate (bodyweight = 118 mg, gonad volume = 0.014 mm^3). The gonad is surrounded by a tunica 5–6 cells in thickness. Groups of wedge-shaped preinterstitial cells (arrows), resembling that in (*a*), have penetrated to the centre of the gonad, thus completing the separation of the sex cords. (*d*) Male postmature embryo (bodyweight = 125.2 mg, gonad volume = 0.0097 mm^3). The gonad is surrounded by a tunica of 2–3 cells, and the compartmentalisation of the inner cells shows little advance over that seen in (*a*). Bar, 30 μm.

tiation and seems hardly more advanced than the gonad in Figure 2*a*, belonging to an embryo before its expected birth, and weighing only 97.2 mg.

DISCUSSION

Our finding that the beginning of histological differentiation of the testis is detectable in embryos of *Monodelphis domestica* shortly before birth is in agreement with our previous conclusion that sexual differentiation in this species begins in intrauterine life (Baker et al. 1990). It is also in line with the finding by McCrady (1938) that evidence of testicular differentiation in the related *Didelphis virginiana* can be seen immediately after parturition. Although Fadem et al. (1992) described the gonads of neonate grey opossums as 'not differentiated' on the day of birth, the authors did not karyotype their specimens, so that the genetic sex of the neonates is unknown. In view of the recent report by Renfree et al. (1992) that in the tammar wallaby, *Macropus eugenii*, histological features characteristic of the testis are already present at birth, it seems likely that many species of marsupials resemble eutherian mammals in that gonadal differentiation is initiated in the embryo.

The data further suggest that quantitative differences between XX and XY gonads may likewise begin before birth. We previously found (Baker et al. 1990) that the gonadal volumes of XY neonates on the day of birth exceeded those of their XX litter mates in all 4 litters examined. Our present data on embryos, summarised in Table 1, includes 2 litters, 960 and 954, with bodyweights close to those expected at birth (H. D. M. Moore, unpublished; VandeBerg 1990). In litter 960, the mean gonadal volume of XY embryos was 10% greater than that of their XX litter mates, whereas the mean XY bodyweight was 10% less than in XX embryos, while in litter 954 the difference in mean gonadal volume was 23% compared with 7% for bodyweight. These 2 litters have the highest ratio of male, compared with female, gonadal volumes relative to bodyweight in embryos. As would be expected, this difference is smaller in embryos with small gonadal ridges, and the difference decreases in postmature embryos, in which the XY gonads stop growing, while the XX gonads continue to grow. The difference in relative volume between XY and XX gonads increases in postnatal litters (Table 2).

As can be seen in Figure 1, there is an abrupt increase in the gonadal growth rate of genetic males, but not in postmature XY embryos. In XX gonads, there is no postnatal growth spurt, and the gonads of embryos and neonates grow at the same rate.

There is at present no explanation for the prolonged gestation length in some females. It is unlikely to be due to an error in estimation of gestation time, since the bodyweights of these embryos exceeded the usual birthweights.

These findings suggest that, in order to continue its normal development, the opossum testis must find itself outside the maternal environment. This raises the intriguing question as to whether there might be a connection with the apparently lesser propensity of eutherian, compared with marsupial, testes to become feminised by exogenous oestrogen. Burns (1955, 1961) showed that by treating pouch young of *Didelphis virginiana* with oestradiol dipropionate, testes were modified into ovotestes or even ovaries, some of which contained germ cells. Treatment of genetically male neonates of *M. domestica* with oestradiol benzoate by Fadem & Tesoriero (1986) and by Moore & Thurstan (1990) resulted in streak gonads and feminisation of internal and external genitalia, apart from the presence of the scrotum, which is not testosterone-dependent in marsupials (McCrady 1938; O et al. 1988). However, treatment with oestradiol of pouch young of the tammar wallaby, *Macropus eugenii*, by Shaw et al. (1988) resulted only in undescended, histologically abnormal testes and stimulation of the müllerian duct, and this is similar to the effects obtained in eutherian mammals. Greene (1942) reported that high doses of oestrogen administered to pregnant rats resulted in male offspring with undescended testes, partially developed oviducts, uteri and a well developed upper vagina, while the phallus was small and hypospadiac. McLachlan et al. (1975) found similar abnormalities in many, but not all, male offspring of oestrogen-treated pregnant mice. There appears to be no report of the conversion of testes into ovotestes or streak gonads as a result of oestrogen treatment in a male eutherian mammal.

There are as yet no data on circulating oestrogen levels in *Monodelphis domestica* during pregnancy, but a number of reports suggest that measurable levels are likely to be present. Harder & Fleming (1981) measured oestradiol and progesterone profiles during the reproductive cycle of *Didelphis virginiana* and concluded that the ratios of these hormones during pregnancy did not differ from those during equivalent days of the oestrus cycle. Fadem (1989) found significantly higher levels of peripheral plasma oestradiol in *Monodelphis domestica* during oestrus than in dioestrus females, and Hinds et al. (1992) have recently demonstrated the presence of progesterone in the maternal circulation of this species. It seems likely, therefore, that oestrogen is present during the gesta-

tional period of *M. domestica*, and that the developing embryos are exposed to it. It will be important to ascertain whether this hormone could explain the blocking in the development of the XY gonads that we have observed, or whether other factors might be involved.

In Eutheria, maternal hormones cross the placenta (Diczfalusy & Mancuso, 1969), so that embryos of both sexes are exposed to them. While the bulk of these oestrogens are rapidly sulphurulated and thereby presumably inactivated, the levels of unconjugated oestradiol in fetal serum are nevertheless high (Winter et al. 1981). Yet the differentiation of the embryonic testis proceeds to the point of producing testosterone and antimüllerian hormone in sufficient quantities to masculinise the reproductive tract. It may indeed be that the early rapid growth and differentiation of the fetal eutherian testis, producing its own sex hormones, is a necessary adaptation for the eutherian male developing in a maternal hormonal environment (Mittwoch, 1971, 1986, 1992; Mittwoch & Burgess, 1991).

The feminisation of gonads of genetic males by oestrogen is widespread among vertebrates. Apart from marsupials, it is known to occur in teleost fishes (Yamamoto, 1969), amphibians (Chang & Witschi, 1956), and birds (Wolff & Ginlinger, 1935; Haffen, 1965; Scheib, 1983; Perrin et al. 1992), as well as in a turtle with temperature-dependent sex determination (Wibbles & Crews, 1992). The ability of testes to develop in such an environment of female sex steroids may thus be a fundamental requirement distinguishing eutherian mammals from other vertebrates, including metatherian mammals.

This difference gives added significance to certain differences in the sex chromosomes and their effects in marsupials and eutherians (Graves, 1990; Hayman, 1990; Sharman et al. 1990). Recently a homologue of the eutherian *SRY* gene has been identified on the Y chromosome of 2 species of marsupials, *Sminthopsis macroura*, and *Macropus eugenii*, but comparison between eutherian and metatherian Y-located *SRY* sequences suggest rapid evolution of these genes (Foster et al. 1992). Obviously the genetic mechanism responsible for testis formation must ensure that a functional testis develops in an environment appropriate to the biology of the species. It is noteworthy that in the marsupial, *Monodelphis domestica*, testicular differentiation begins in utero, as in eutherian mammals, but in contrast to eutherians, seems unable to proceed until after birth. It will be of the greatest interest to discover the similarities and differences in genotypes that are responsible for these effects, and their function in the environments in which they are acting.

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