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Mammary glands in male marsupials:

I. Primordia in neonatal opossums *Didelphis virginiana* and *Monodelphis domestica*

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Summary

Neonates of the American didelphid marsupials *Didelphis virginiana* and *Monodelphis domestica* were sexed by karyotype and histologically examined on the day of birth. Mammary anlagen were found in both sexes of both species, but the neonatal males had less than one-third of the full female complement of mammary glands. Male neonates of both species also had paired scrotal bulges anterior to the genital tubercle but these were never present in females, once again raising the question of whether the pouch and scrotum are homologous structures. Mammary anlagen are not found in male neonates of the Australian marsupial species so far studied, which suggests a dichotomy in the control of some aspects of sexual differentiation in the two marsupial lineages.

Key words: marsupial, mammary glands, opossums, sexual differentiation, scrotum, neonate, sexual morphogenesis.

Introduction

Many male eutherian mammals (e.g. Primates, Artiodactyla, Perissodactyla, Carnivora) have mammary glands, and retain the ability to lactate if provided with the appropriate hormonal stimulus. The phenomenon of the lactating billygoat (e.g. Bourdelle and Bressou, 1929) was first described by Aristotle (*c*350B.C.), and John Hunter provides details of a man who lactated to 'assist' his wife who had twins (see Owen, 1861). However, mammary glands and teats have never been described in any adult male marsupial (see Tyndale-Biscoe and Renfree, 1987 for review). In an extensive survey of Australian marsupials, Sharman *et al.* (1970) were unable to find any evidence of mammary or teat tissue in the male pouch young, juveniles or adults they examined. The significance of observations on two unrelated species, the tammar, *Macropus eugenii* (Alcorn, 1975), and the brown marsupial mouse, *Antechinus stuartii* (Bolton, 1983), describing scrotal bulges but no mammary anlagen in male fetuses and neonates was overlooked until recently. Detailed studies of sexual differentiation in the tammar (Renfree *et al.* 1987; Short *et al.* 1988; O *et al.* 1988; Renfree and Short, 1988; Shaw *et al.* 1988, 1990; Hutson *et al.* 1988) have shown that mammary anlagen are found only in karyotypically female embryos and are first seen on day 22 of gestation, which is 4–5 days before birth. Scrotal anlagen are only found in karyotypically male fetuses, and cannot be induced in female tammars, even after prolonged treatment with androgen (Shaw *et al.* 1988).

Despite these unambiguous findings for two Australian marsupials, there are several early reports on the North American Virginia opossum, *Didelphis virginiana*, indicating that mammary glands are present in newborn males. McCrady (1938) described the appearance of mammary anlagen in full-term fetuses of opossums, and clearly stated that these were present in both male and female fetuses. He further stated that although sex could be distinguished externally 12–14 days after birth (by the appearance of the pouch rudiments in the female and the scrotal rudiments in the male), the male gonad can be identified on the day of birth (McCrady Stage 35) by clearly defined testis cords containing primordial germ cells, at a time when the female gonad is still indifferent. Burns (1939*a*) concurred with McCrady on both observations, but found male type sex cords in the testes of some, but not all individuals on the day of birth. He also cited unpublished results of Carl Hartman who could recognise the sex externally in *Didelphis* after birth by the presence of a full complement of six or seven paired and one median mammary 'rudiments' in females, while in males only a limited number of the more caudal rudiments were found, irregularly arranged. Recently, male *Monodelphis domestica* pouch young were shown to have rudimentary indifferent gonadal anlagen on the day of birth (Moore and Thurstan, 1990). These authors first identified scrotal bulges on the abdomen and sex cords in the testes by late day 2 post-partum. However, no mention was made of the presence or absence of mammary anlagen at either birth or in the early neonatal period.

These results suggest that male tammars and Virginia opossums differ with respect to mammary development. Does this reflect a fundamental difference between Australian and American marsupials? We report here on the mammary anlagen and scrotal anlagen in karyotyped offspring on the day of birth in the Virginia opossum *Didelphis virginiana* and the grey short-tailed opossum *Monodelphis domestica*.

Materials and methods

Animals

Females of both species were derived from the breeding colonies held at the Department of Genetics, Southwest Foundation for Biomedical Research (Samollow *et al.* 1987; VandeBerg, 1990). Adult females were paired with males, checked for births twice daily, between 8 and 9 a.m. and 4 and 5 p.m., beginning two weeks after pairing. *Didelphis* neonates have been reported to have a mean weight of 133 mg at birth (Hartman, 1928) and we found that *Monodelphis* neonates had a mean weight (±S.D.) of 98.8±7.4 mg (n=25) when weighed between 0 and 8 h after birth. Adult *Didelphis* females have a well-formed deep pouch whilst *Monodelphis* is a pouchless species.

Six male and female *Monodelphis* neonates were selected at random from three litters of 8, 4 and 6. The *Didelphis* neonates were the complete litter of a single female who produced 3 males and 3 females.

Karyotyping

Neonates were removed from the teat and killed by decapitation. Brain tissue was chopped in Hams F10 medium containing colchicine ($1\,\mu g\,ml^{-1}$) for 1 h at 37°C followed by incubation in hypotonic sodium citrate for 10 min. The tissue was then fixed in methanol:acetic acid (3:1 v/v) for 1 h and air-dried chromosome spreads made after transfer to 60% acetic acid (Johnston and Robinson, 1986). In *Didelphis*, the chromosome number is 2n=22, and in *Monodelphis* 2n=18 and both have an XX/XY sex chromosome constitution, with a very small and hence easily recognized Y-chromosome.

Light microscopy

The bodies of 3 ♀ and 3 ♂ neonates of *Didelphis*, and 6 ♀ and 6 ♂ neonates of *Monodelphis* were fixed in neutral buffered formalin, and shipped to Australia. After processing, embedding in paraffin and transverse serial sectioning, sections at 8–10 μm were examined for the presence and location of mammary and scrotal anlagen.

Results

Mammary anlagen

In adult *Didelphis* females, there are between 13 and 15 mammary glands and teats, arranged as six or seven pairs and one median gland in a semi-circular pattern inside the well developed pouch. Rarely, the most anterior pair is missing (McCrady, 1938; Hartman in Burns, 1939*a*). In neonatal *Didelphis* females, a full complement of the adult pattern of mammary primordia was observed microscopically. In adult *Monodelphis* females, there are 11 to 13 (5 to 6 pairs and one median) mammary glands and teats, and rarely the most anterior pair is absent (E.S. Robinson, unpublished observations). There is no trace of a pouch. In neonatal *Monodelphis* females, we also found 11 to 13 mammary anlagen. Male neonates of both species had clearly defined mammary anlagen (Figs 1 and 2), and these were always located cranial (between 0.3 and 0.5 mm) to the scrotal bulges (Fig. 1). The number and pattern of the mammary anlagen was variable. In *Didelphis*, the three males had 1, 2 and 5 mammary anlagen, respectively. The six *Monodelphis* male neonates had 0, 1, 2, 2, 4 and 4 mammary anlagen, respectively. The positions of these mammary anlagen corresponded to the most cranial locations in the neonatal females.

Scrotal anlagen

In both species, paired bulges anterior to the genital tubercle could be observed macroscopically in some karyotypic male neonates on the day of birth prior to embedding. These were evident in all male neonates after serial sectioning (Fig. 1). These scrotal bulges, which later fuse in the midline to form the scrotal sac, consisted of connective tissue swellings covered by a thickened squamous epithelium. Scrotal bulges were never observed in female neonates.

Discussion

In the American didelphid marsupials, *Didelphis virginiana* and *Monodelphis domestica*, mammary anlagen are clearly discernible in males and females on the day of birth. However, males of both species had less than one third of the full female complement of mammary anlagen, and these were always located in the most cranial positions. In only one neonatal male were no mammary anlagen found. Scrotal anlagen were present in all male neonates of *Didelphis* and *Monodelphis*, and these were always caudal to the mammary anlagen. Scrotal anlagen were never present in female neonates.

The possession of mammary anlagen by these didelphid males suggests a dichotomy in the control of some aspects of sexual differentiation as between Australian and American marsupials. The differentiation of the mammary gland, pouch and scrotum is apparently under primary genetic control in marsupials (O *et al.* 1988; Renfree and Short, 1988; Shaw *et al.* 1990). The complete lack of expression of the gene(s) responsible for development of mammary anlagen in male Australian marsupials, in contrast to their variable expression in didelphid males, suggests a difference in the two marsupial lineages. Whether genes on the X or Y or the autosomes are involved in the control of mammary development has yet to be determined, but the X-dosage mechanisms already suggested (Cooper *et al.* 1977; Renfree and Short, 1988; Shaw *et al.* 1990; Sharman *et al.* 1990), where one X gives rise to a scrotum and two X's to mammary glands and a pouch, is thought to be responsible. This theory must now be amended to take into account the initial presence of mammary primordia in didelphid males. The time of appearance of the mammary anlagen in the fetal stages of didelphid males has been clearly shown to precede

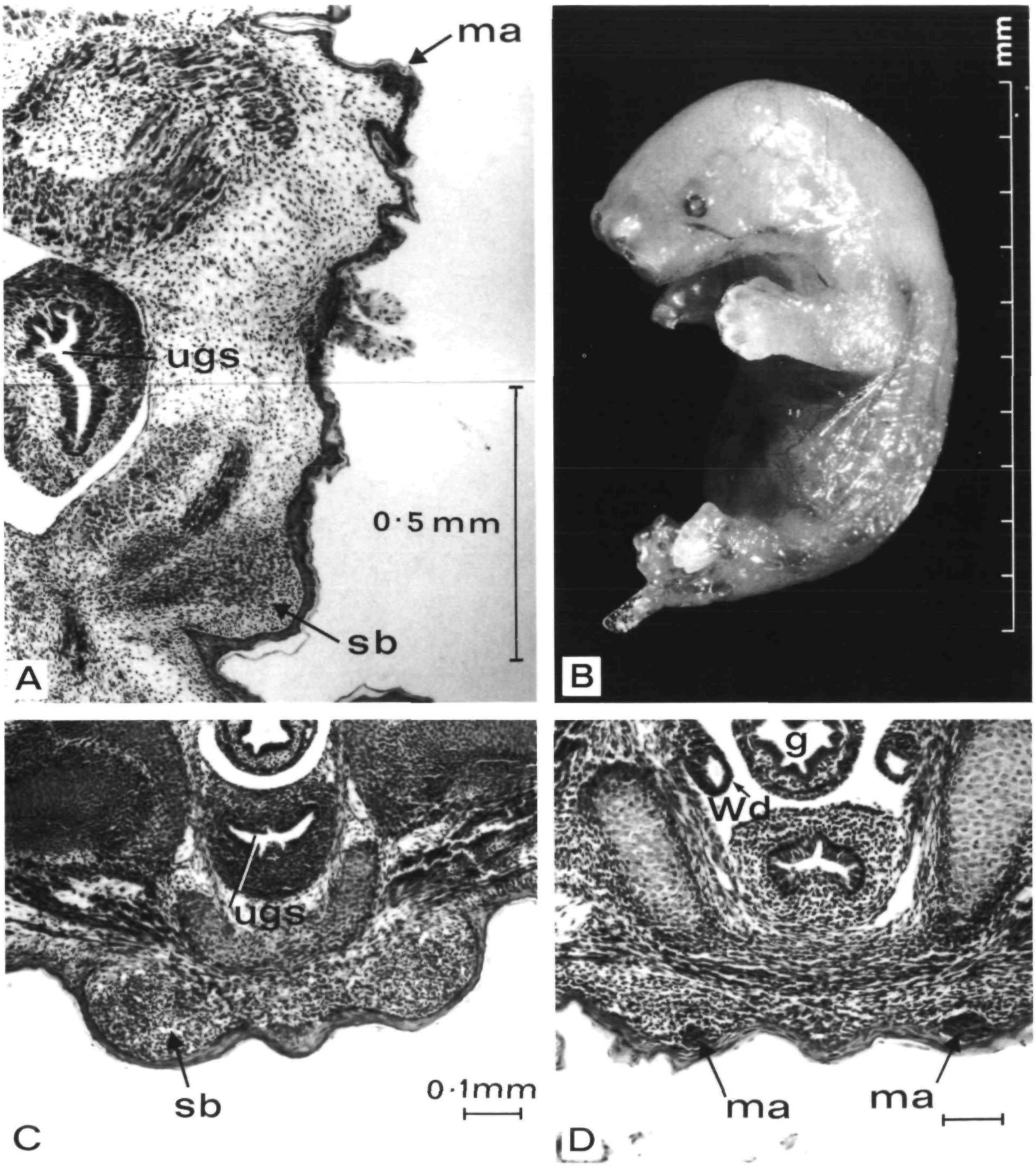

Fig. 1. (A,C,D) Representative transverse sections of *Monodelphis* and *Didelphis* male and female neonates. In A, a scrotal bulge in a male *Didelphis* neonate appears in the same section as a single mammary anlage. This section is slightly oblique and the mammary anlage is actually cranial to the scrotal bulge. (C) Caudal section of a neonatal male *Monodelphis*, showing the paired, but still separate scrotal bulges, compared to D showing a similar, but slightly more cranial, transverse section of a neonatal female *Monodelphis* with one pair of mammary anlagen. This was the most caudal pair of mammary primordia in this neonate. (B) A neonatal *Monodelphis domestica*, photographed within 5 h post-partum. As in all marsupial neonates, the anterior part of the body is better developed than the posterior, and the curvature of the body and hind limbs hides the scrotal bulges. Scale in mm. Abbreviations: g: gut; ma: mammary anlage; sb: scrotal bulge; ugs: urogenital sinus; Wd: Wolffian duct.

the time of gonadal differentiation (McCrady, 1938), so mammary development is unlikely to be under the control of gonadal steroids. This conclusion is supported by the findings of Renfree and Short (1988) who showed that mammary anlagen were present in female and scrotal anlagen in male tammar fetuses 5 days

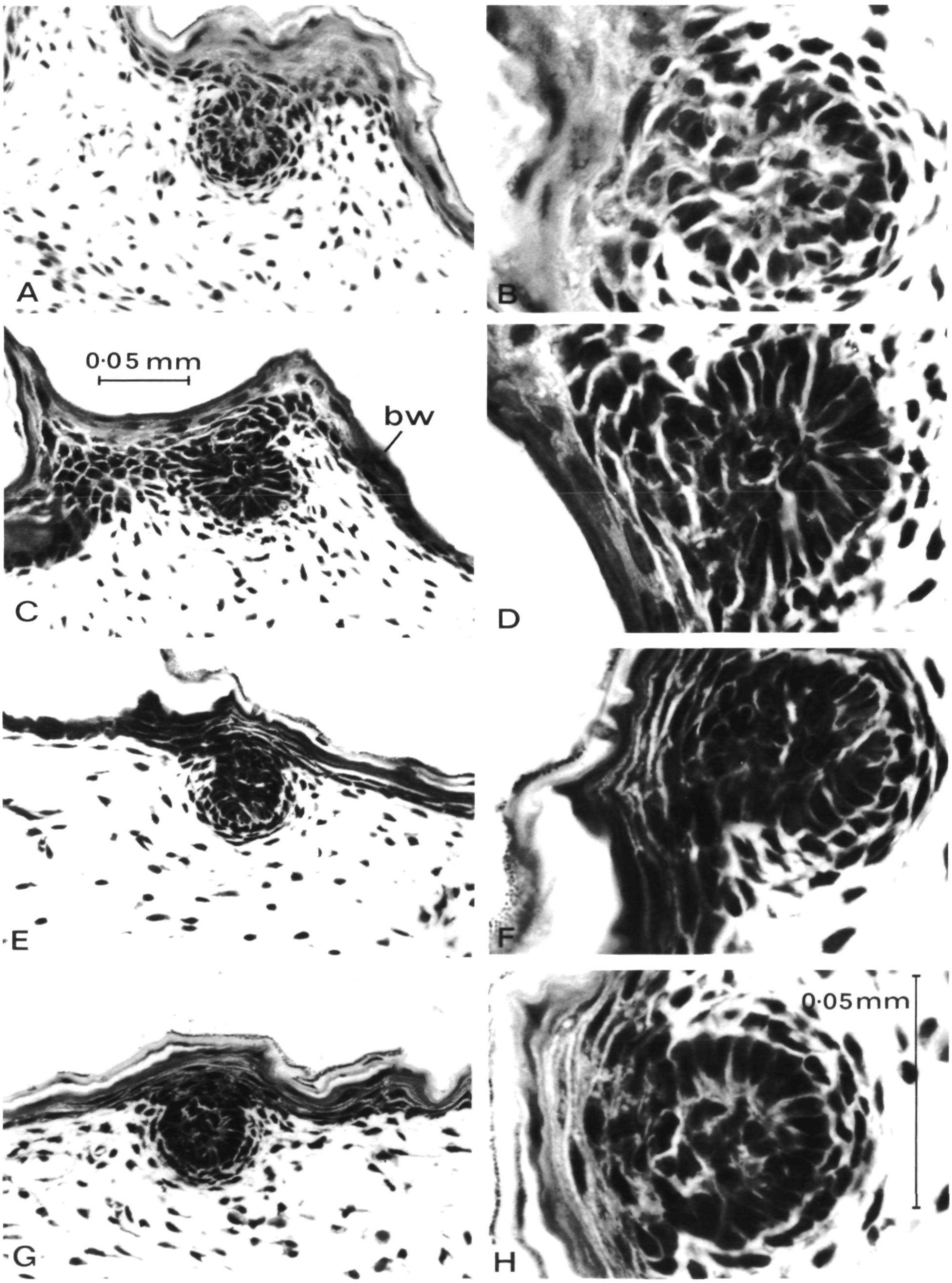

Fig. 2. Mammary anlagen in male *Didelphis* (A,B) and female *Didelphis* (C,D) and male *Monodelphis* (E,F) and female *Monodelphis* (G,H) neonates at low power and high power. There is no difference in size or morphology of the mammary anlagen between sexes or species. Abbreviation: bw, body wall.

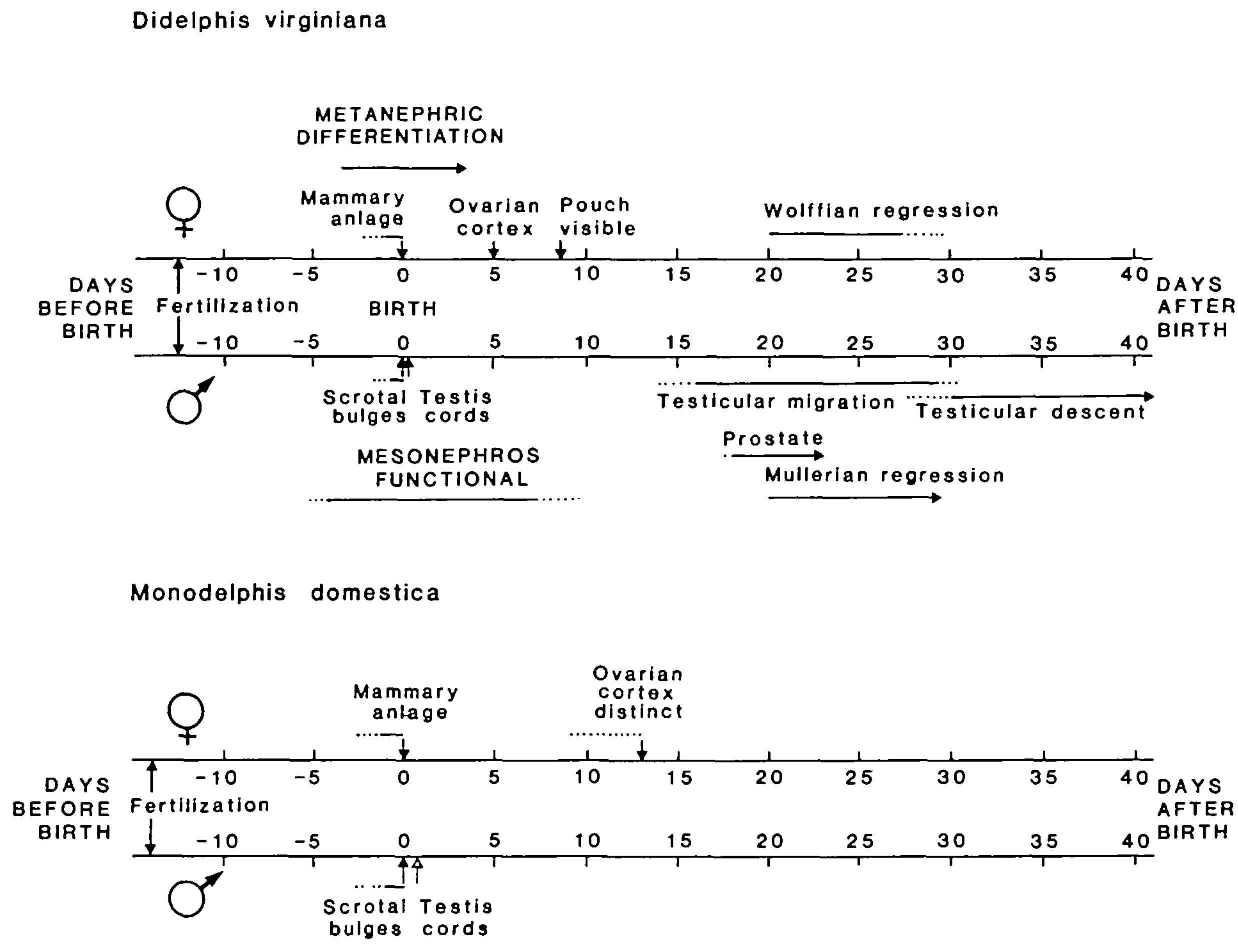

Fig. 3. Schematic summary of the known steps in sexual morphogenesis in the two didelphids, *Didelphis virginiana* (top) and *Monodelphis domestica* (bottom), taken from the descriptions of McCrady (1938), Moore (1939, 1945), Morgan (1943), Burns (1939*a*,*b*), Fadem and Tesoriero (1986), and Moore and Thurstan (1990), and from the results of this study. *Didelphis* has a gestation period of 12.5 days (McCrady, 1938), and *Monodelphis* 14 days (Fadem *et al.* 1982). Open arrow indicates putative time of appearance of sex cords; closed arrows indicate confirmed times.

before birth, when the gonadal ridge is only three cell layers thick. Shaw *et al.* (1988) in the tammar, and Fadem and Tesoriero (1986) and Moore and Thurstan (1990) in *Monodelphis*, found that neither androgen nor oestrogen treatment of neonates had any influence on mammary or scrotal development. Similarly, Burns (1939*a*, 1939*b*) failed to affect the pouch, mammary glands or scrotum after treatment of *Didelphis* neonates with oestrogen or testosterone.

The sexual morphogenesis of these two New World didelphid marsupials is similar (Fig. 3), but differs from that of Australian marsupials in the presence of some mammary anlagen in males on the day of birth. There also appears to be a slight difference between American and Australian marsupials in the time of differentiation of the gonads, since testicular development is apparent in *Didelphis* on the day of birth but not earlier (McCrady, 1938; Burns, 1939*a*), whereas in the tammar, sex cords form between birth and day 2 postpartum. It will be interesting to study the fate of the male mammary primordia in adult didelphids, and in other marsupial groups, and to explore further the claimed homology between pouch and scrotum (reviewed by Tyndale-Biscoe and Renfree, 1987) and the evolutionary significance of this apparent difference in mammary development between Australian and American marsupials.

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