

## Variation in phenotype due to random intrauterine positioning of male and female fetuses in rodents

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**Summary.** Rodents are polytocous mammals, and male and female fetuses can develop *in utero* contiguous to fetuses of the same or opposite sex. This paper describes experiments demonstrating that random intrauterine positioning of male and female fetuses results in within-sex variation in phenotype in mice and rats. This phenomenon provides a clear example of the degree to which the intrauterine environment can bias development in terms of effects on morphology, physiology and behaviour. I propose that individual differences in reproductively-related characteristics based on prior intrauterine position may play a role both in the regulation of population size in rodents and in the reproductive success of individuals as changes in population size occur.

### Introduction

The process by which testicular androgen alters the course of development has been elucidated by examining genetic errors (Ohno, 1976), errors of metabolism (Ehrhardt & Baker, 1974), and the results of experimental administration of androgen or androgen blockers (reviewed by Neumann & Elger, 1966; Wilson, 1978; Donovan, 1978; Gorski, 1979). In mammals, it is now established that differentiation into the male phenotype begins during prenatal life as a result of the secretion of testicular androgen (Block, Lew & Klein, 1971; Jost, 1972). In mice and rats, unlike mammals with long gestational periods (e.g. primates; Phoenix, 1974), this process continues at least through the first week of postnatal life as well. At birth, though, male and female rats and mice can be easily distinguished by external examination of the length between the anus and genital papilla or by internal examination of the accessory sex organs and gonads. Early experiments with rats (Pfeiffer, 1936; Harris, 1964; Young, Goy & Phoenix, 1964; Barraclough, 1966; Arai & Gorski, 1968) and mice (Barraclough & Leathem, 1954; Edwards & Burge, 1971) suggested that there was a critical developmental period that commenced shortly after birth, with both physiological and behavioural masculinization and defeminization occurring in response to androgen exposure during this time. This assumption was based on the finding that castrating males at birth resulted in their being capable of producing gonadotrophins in a cyclic pattern characteristic of females when implanted with an ovary in adulthood. In addition, the potential to exhibit female sex behaviour (e.g. lordosis) was increased in males castrated at birth and decreased in females injected with testosterone propionate shortly after birth. Subsequent to these initial observations, it became apparent that exposure to androgen during both the prenatal and neonatal periods influenced sex-related behaviour patterns during later life as well as morphology and physiology (De Moor, Verhoeven & Heyns, 1973). In rats, for instance, it is possible that masculinization and defeminization by androgen may to some degree be independent processes, with the maximum sensitivity to the masculinizing effects of

androgen occurring prenatally, while the defeminizing action of androgen occurs primarily during postnatal life (Ward & Renz, 1972; Davis, Chaptal & McEwen, 1979). However, some degree of defeminization in response to androgen occurs prenatally, as evidenced by the finding that exposure to androgen antagonists such as flutamide during prenatal life increases the potential to exhibit female sex behaviour in both male and female rats (Gladue & Clemens, 1978). Thus, prenatal androgen exposure via exogenous treatment (Ward & Renz, 1972) as well as from some endogenous source (Gladue & Clemens, 1978) can to some degree defeminize female rats. The timing of the masculinization and defeminization processes is an important issue. The results of my experiments concerning the intrauterine position phenomenon suggest that in mice, at least, exposure of certain female fetuses to elevated concentrations of testosterone *in utero* does not impair their capacity to reproduce.

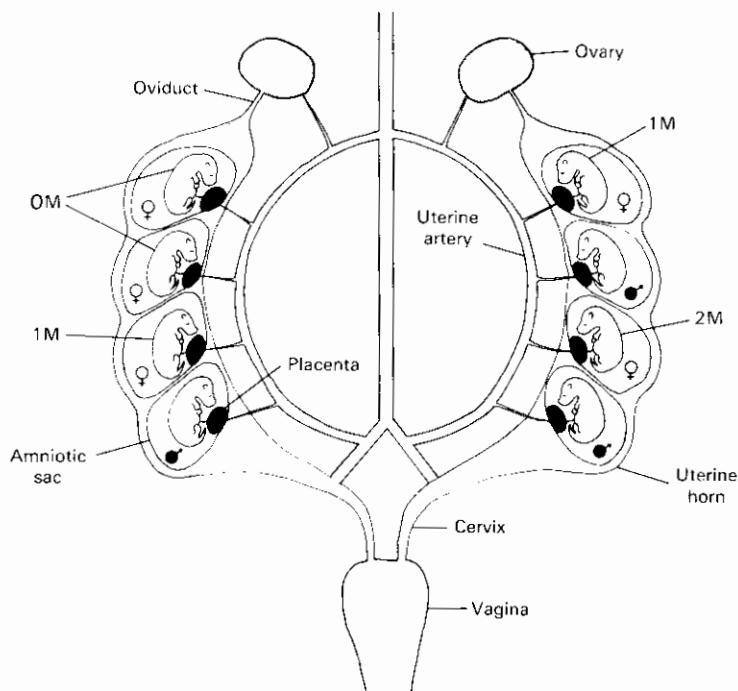
Numerous investigators have reported that male mice castrated at birth and treated with testosterone in adulthood would not exhibit aggression towards another male (see Edwards, 1969; Peters, Bronson & Whitsett, 1972). These data were interpreted as supporting the concept of a critical neonatal period in mice during which masculinization and defeminization occurred in response to androgen exposure. Again, subsequent experiments have revealed that androgen exposure during both prenatal and neonatal life influences the exhibition of aggression in adult mice (vom Saal, Svare & Gandelman, 1976; vom Saal, 1979).

In rats and mice, then, androgen exposure influences morphological, physiological and behavioural characteristics, with the period of sensitivity to androgen extending from the later part of gestation into the early post-natal period. Of particular interest is the finding that a part of the medial preoptic area, referred to as the sexually dimorphic nucleus, differs in volume in male and female rats at birth, with the magnitude of the difference increasing throughout the first 10 days after birth (Gorski, Harlan, Jacobson, Shryne & Southam, 1980; Jacobson, Shryne, Shapiro & Gorski, 1980). The medial preoptic area has been proposed to be the neural area that triggers the preovulatory surge of luteinizing hormone (LH) in female rats and also is involved in the mediation of female sexual behaviour (Gorski, 1979). The finding that perinatal exposure to elevated levels of androgen produces a change in morphology in this area of the brain suggests that both the capacity to secrete gonadotrophins in a cyclic (female-like) pattern and the capacity to exhibit either male or female sex behaviour may be influenced by androgen via a direct effect on brain morphology.

#### The intrauterine position phenomenon

*General methods.* Adult virgin female mice are time-mated with males for 2 h beginning at 08:00 h. Insemination is verified by the presence of a vaginal plug. Disturbing female mice during the first week of pregnancy results in a high rate of pregnancy loss (20–30%; see Chipman & Fox, 1966). To minimize pregnancy loss, the male rather than the female should be removed to another cage after mating. In rats, insemination must be verified by the presence of spermatozoa in the vagina. The pregnant females are not disturbed after Day 13 of pregnancy. It has been reported that stressing a pregnant rat during the last third of gestation alters the timing of testosterone secretion in male fetuses, with the result that masculinization is interfered with (Ward & Weisz, 1980). It is possible, therefore, that the effects on female fetuses of developing between male fetuses might be attenuated or even eliminated by stressing the mother, particularly during the last part of pregnancy. CF-1 mice in my laboratory reliably give birth 19 days after insemination, with surprisingly little regard to the light:dark cycle. Beginning at 08:00 h on Day 19 (Day 0 is the morning of insemination), females are killed by cervical dislocation, and the fetuses are rapidly removed from the uterine horns. Fetal sex is determined by the length of the anogenital space (for mice, mean  $\pm$  s.e.m.: males =  $1.63 \pm 0.02$  mm, females =  $0.85 \pm 0.02$  mm). Sprague-Dawley rats (ARS) exhibit considerable variation in the time of parturition,

the mean being 22 days. To obtain surviving young, they must be delivered by Caesarean section during the evening of Day 21. Further advancing the time of delivery results in an increase in mortality of the young. It is imperative to remove the amniotic fluid from the respiratory tract of the young immediately after each fetus is removed from its amniotic sac. This is accomplished by placing the mouth and nose against tissue paper and gently palpating the flanks. Fetuses have three intrauterine positions, determined at random, that they can occupy relative to fetuses of the opposite sex. For females, positioning can occur between two male fetuses (2M females), next to one male (1M females), or not next to a male (0M females; see Text-fig. 1). All experimental young are kept under heat lamps until they are fully active, at which time they are fostered to mothers that had delivered naturally within the preceding 24 h. Each mother is removed to a holding cage, its own young are removed and replaced with the young of known intrauterine position, and the mother is then returned to the home cage. During the next 5 days, an attempt is made not to disturb the foster mothers. For rats and mice, mortality with this procedure is approximately 7%. In experiments in which the young have been gonadectomized at birth, 17% mortality has been recorded. A toe-clipping pattern is utilized to identify individual young, and information concerning the positioning of individual fetuses in each litter is recorded. Foster litters consist of 8–10 young, usually with 5 1M females being placed with either 5 0M or 5 2M females. In previous studies, different housing conditions have been utilized to control for postnatal effects. No differences between 0M and 2M females based on post-natal litter composition have been found. Weaning occurs on Day 23 after birth. Comparisons are made using *t* tests or  $\chi^2$  analyses in most cases.



**Text-fig. 1.** Schematic diagram of a mouse uterus showing the 3 potential intrauterine positions that female fetuses can occupy relative to male fetuses: 2M female = between 2 males; 1M female = next to 1 male; 0M female = not next to a male. (After McLaren & Michie, 1960.)

**Background.** Within any adult population of males or females, variation in phenotype is observed. As indicated in the Introduction, gross differences between males and females in morphology, physiology and behaviour are mainly due to the fact that males are exposed to high

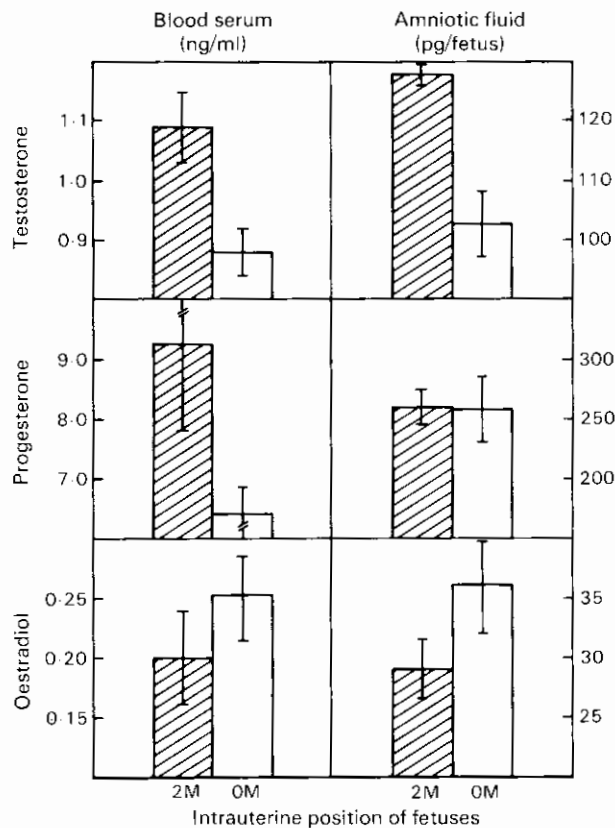
levels of androgen during early life. It is reasonable to presume that variation in adult phenotype amongst males and amongst females might, in part, be due to differences in androgen levels during perinatal development. Traditionally, however, females that have been exposed to elevated levels of androgen during development have been viewed as being abnormal. The assumption has been that androgenized females should be at a reproductive disadvantage relative to other 'normal' females.

Clemens (1974) observed that the length of the anogenital space of female rats at birth (which provides a sensitive bioassay for prenatal androgen exposure) was longer in females that resided in the uterus between 2 male fetuses than in females not next to a male. This finding raised the possibility that, as a normal part of development in polytocous mammals, females are exposed to different levels of androgen based on their proximity to male fetuses. This finding has since been replicated in mice (vom Saal & Bronson, 1978). But, in both males and females, no differences in body weight based on prior intrauterine position have been found either at birth or in adulthood (F. S. vom Saal, unpublished; Ward, Karp & Aceto, 1977; vom Saal & Bronson, 1978). In addition, Clemens, Gladue & Coniglio (1978) working with rats, and vom Saal (1978) working with mice found that administration of an anti-androgen to pregnant females during the last third of pregnancy eliminated differences in anogenital distance between females based on their proximity to male fetuses. The hypothesis that this morphological difference results from females that develop between male fetuses being exposed to elevated levels of androgen during prenatal life was recently examined by vom Saal & Bronson (1980a). We found that on Day 17 of gestation, when the male accessory sex organs are differentiating (Rugh, 1968), blood testosterone concentrations of male mouse fetuses are three times higher than in females. No sex differences in blood oestradiol-17 $\beta$  or progesterone concentrations were found. Blood and amniotic fluid levels of these steroids were then compared in 0M and 2M female fetuses. The 2M females had significantly higher concentrations of testosterone in their blood and amniotic fluid than did the 0M female fetuses. No differences in oestradiol-17 $\beta$  or progesterone concentrations were found (Text-fig. 2). Importantly, 0M and 2M females do not differ in their blood testosterone concentrations during postnatal life. We have proposed that testosterone passes via the amniotic fluid from males to contiguous females. An important related observation is that, at this stage of gestation, mouse and rat fetuses are packed tightly in each uterine horn, and the individual placentae are spaced fairly evenly. There is therefore a large area of contact of the placental membranes surrounding contiguous fetuses. It is possible that, in those mammals in which multiple uterine residence occurs, if the placental membranes of individual fetuses of opposite sex are in contact during the time of sexual differentiation (when the males' testes are secreting high levels of androgen), then female fetuses contiguous to a male will also be exposed to elevated levels of androgen.

The possibility that 2M female fetuses might have higher blood concentrations of testosterone because they tend to come from litters containing more male fetuses than do litters containing 0M females has been examined. Blood was collected from mothers at the time that the fetal blood was collected on Day 17 of pregnancy and testosterone, oestradiol-17 $\beta$  and progesterone concentrations were measured. Blood from mothers carrying 9 male and 3 female fetuses or 3 male and 9 female fetuses was examined, but no differences in the hormone concentrations were found (vom Saal & Bronson, 1980a). In some comparisons of 0M and 2M females, an analysis of covariance has also been performed, with the number of males found in the litter from which each female was obtained utilized as the covariate. In all such analyses, the number of male fetuses in the litter has not been found to be a significant factor. In mice, therefore, the available evidence is that 2M females are influenced by contiguous male fetuses independently of the maternal circulation.

The nature of blood flow to the uterus, and the possibility of vascular interconnections between fetuses have been examined in mice and rats. In pregnant mice, offshoots from a main loop artery lead to the individual placentae. This main artery branches from the iliac artery

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**Text-fig. 2.** Concentrations of testosterone, progesterone and oestradiol-17 $\beta$  in the serum and amniotic fluid of 17-day-old OM and 2M female mouse fetuses.

caudally and the abdominal aorta rostrally. The result is that maternal blood flow through the artery feeding each uterine horn is bidirectional (McLaren & Michie, 1960). In the rat and guinea-pig, however, uterine blood flow is not bidirectional (Del Campo & Ginther, 1972; Egund & Carter, 1974). McLaren & Michie (1959) also reported no evidence of vascular interconnections of placentae or fetal membranes in mice, even in rare cases (1% of all fetuses) in which the placentae of contiguous fetuses were in contact with each other. These findings suggest that the aetiology of the intrauterine position phenomenon in mice and the freemartin in cattle is quite different. The freemartin refers to the situation in cattle in which a female born co-twin with a male is usually sterile (Marcum, 1974). The male co-twin has also been found to be sterile or to exhibit a decrease in fertility (Dunn, McEntee, Hall, Johnson & Stone, 1979). Lillie (1916) assumed that the freemartin resulted from the transfer of androgen from the male to the female fetus via anastomoses of the chorionic vessels that he observed. Jost (1972) has reported that the freemartin (the female) does not result from exposure to abnormally high androgen levels, since androgen injections to pregnant cattle did not produce the freemartin syndrome in female offspring. Ohno, Christian, Wachtel & Koo (1976) have proposed that disruption of differentiation of the embryonic gonad of a freemartin results from the presence of tissue from the male co-twin that has the surface H-Y antigen coded for by the Y chromosome, since it has now been demonstrated that the male and female co-twin have both XX and XY tissue and are thus chimaeras. Presumably, it is the presence of this antigen that induces development of the female gonads into ovotestes, with the result that the gonads are non-functional. Again, the evidence is that this either does not occur or is very rare in polytocous

species such as mice (Marcum, 1974). Quite likely, protection against the possibility of female offspring being sterile as a result of developing next to a male fetus *in utero* has evolved in polytocous mammals.

The strategy for examining the consequences of exposure to elevated levels of testosterone in 2M females is straightforward. There is an extensive literature on the physiological processes and behaviours that are influenced by exposure to androgen during early life in mice. Of course, the capacity for androgen to influence specific traits depends ultimately on genotype (Simon, 1979). When considerable within-sex variation in such a trait is observed, the degree to which prior intrauterine position contributes to this variation is examined. Examples of such sex-related characteristics are within-sex aggression (Beatty, 1979), urine marking of a novel environment (Bronson, 1976), copulatory behaviour (Beach, 1979; Södersten, 1979), infanticide or maternal behaviour towards young (Svare, 1979), and maternal (lactation-induced) aggression (Svare, 1980). In addition, the timing of puberty and length of oestrous cycles are markedly influenced by exposure to testosterone during both prenatal (Turner, 1939) and neonatal life (Peters & Sorensen, 1971). The following discussion will concern comparisons of these types of characteristics in 0M and 2M females and males and the possible relevance of variation in these characteristics to the reproductive ecology of rodents.

In most of these experiments, only comparisons of 0M and 2M females have been made. But 1M females represent 50% of the female population. In all experiments in which 1M females have been examined for length of anogenital space at birth, adult aggressiveness and timing of puberty (vom Saal, 1976; and unpublished), they have been intermediate in their characteristics between 0M and 2M females.

### Female mice

#### *Physiological comparisons*

*Social cueing.* The regulation of puberty in female mice by cues produced by both sexes has been proposed to play an important role in controlling population size (Bronson, 1979; Massey & Vandenberg, 1980). Female mice that live in environments containing relatively few females can ovulate and mate when only 4 weeks old if they are housed with an adult male. However, as the number of females housed together is increased, the sexual maturation of female mice is delayed or even totally inhibited (Vandenberg, 1973; Bronson & Desjardins, 1974). While agonistic encounters and resulting stress (Christian & Davis, 1964) probably play a role in the total suppression of reproduction observed in some high-density populations (Christian, 1971), pheromonal cues that are produced by grouped female mice (Drickamer, 1977; McIntosh & Drickamer, 1977) are known both to delay the onset of puberty and lengthen subsequent oestrous cycles.

The nature of the effects of prior intrauterine position on the timing of puberty and length of oestrous cycles during adolescence reflects the fact that gonadotrophin secretion in peripubertal females is regulated by cues emitted by males and other females in house mice (Bronson & Coquelin, 1980). Three facts concerning the ovulatory cycle of the house mouse are now established: (1) in juvenile females, both pheromonal and tactile cues produced by adult males accelerate the time of puberty and subsequent oestrous cycles (Bronson & Maruniak, 1975; Vandenberg, 1969, 1973); (2) pheromonal cues are produced by females (juvenile and adult) that can delay puberty in juvenile females and decelerate (lengthen) subsequent cycles (Vandenberg, Drickamer & Colby, 1972; Drickamer, 1977; McIntosh & Drickamer, 1977); and (3) the dominance relationship of a female's sensitivity to cues produced by males and females shifts during adolescence. Thus, in juvenile females, group housing with other females completely overrides the accelerating action of a male, but the cues produced by males completely override the inhibitory effect of grouping in adult females (Vandenberg, 1973). The

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experiments summarized in Table 1 have led to a number of observations concerning these phenomena in 0M and 2M females. When housed before puberty with other females from the same intrauterine position, 0M females ovulate and mate (i.e. enter puberty) significantly later than do 2M females, and the length of the first post-pubertal oestrous cycle is significantly longer in 0M females. However, when housed individually with an adult male, juvenile 0M females tend to ovulate and mate at a younger age and have significantly shorter post-pubertal oestrous cycles than do the 2M females (vom Saal & Bronson, 1978; vom Saal, Pryor & Bronson, 1981). In addition, 2M females exhibit prolonged oestrous cycles in adulthood relative to 0M females under all social conditions in which normal cycles are observed (vom Saal & Bronson, 1980b). These potentially confusing findings are explicable by proposing that prior intrauterine position biases both the intrinsic timing of the oestrous cycle as well as a peripubertal female's capacity to transmit puberty-inhibiting and oestrous cycle-decelerating cues and/or her sensitivity to such cues.

**Table 1.** The age of puberty as indicated by insemination and successful pregnancy and the length of oestrous cycles during adolescence and adulthood in 0M and 2M female mice when examined under different housing conditions

Developmental stage	Housing			
	Individual		Grouped (5 ♀/Cage)	
	No male	Male present	No male	Male present
Puberty* (first ovulation)	—	2M > 0M	—	0M > 2M
Early adolescent oestrous cycle	Both prolonged†	2M > 0M‡	—	0M > 2M‡
Adult oestrous cycle§	2M > 0M	2M > 0M	Both prolonged	2M > 0M

\* From vom Saal & Bronson (1978).

† Unpublished observation.

‡ From vom Saal *et al.* (1981).

§ From vom Saal & Bronson (1980b).

The previous experiments suggest that the potency of the cues affecting sexual maturation that are produced by female mice varies as a function of prior intrauterine position. Presumably, male mice are made aware of the reproductive status of a female by olfactory, visual (proceptive behaviour that signals behavioural oestrus), tactile and possibly even gustatory (via the vomeronasal organ) cues. To compare the degree of attractiveness of adult 0M and 2M females to male mice, a 0M and a 2M female were placed in separate chambers of a test apparatus when in early dioestrus. An adult male was then placed on a platform and allowed to jump into the chamber containing the 0M or 2M female. Significantly more males choose the 0M female: of 49 males that have been tested, 40 have chosen the 0M female while only 9 males have chosen the 2M female ( $P < 0.01$ ; vom Saal & Bronson, 1978, 1980a). The cue(s) mediating this difference in attractiveness is as yet unknown, but, given the mouse's reliance on olfactory cueing systems, it is probably pheromonal.

In another experiment adult 0M and 2M females were ovariectomized and given subcutaneous implants of empty capsules or capsules containing increasing doses of oestradiol-17 $\beta$ . The females carrying capsules containing oestradiol also received an injection of progesterone 4 h before being observed with an adult male for 30 min. There were no differences in the dose of oestradiol required to induce sexual receptivity or in the lordosis quotient (no. of females showing lordosis/no. of mounts  $\times$  100) in the sexually receptive 0M and 2M females, indicating no difference in the sensitivity to oestradiol or in the behaviour of 0M and 2M females towards males in this study. However, regardless of the presence or absence of oestradiol and

progesterone, the males attempted to mount the OM females significantly more than they did the 2M females (mean  $\pm$  s.e.m.: OM females =  $14.0 \pm 2.3$ ; 2M females =  $8.3 \pm 1.2$ ;  $P < 0.01$ ). Additionally, when the mounting attempts of the males towards the non-receptive females were rejected, virtually all males exhibited aggression towards the female partner. The males paired with the non-receptive OM females exhibited significantly more attacks than the males paired with the non-receptive 2M females (OM females =  $11.1 \pm 2.3$ ; 2M females =  $6.7 \pm 1.0$ ;  $P < 0.05$ ). No aggression was observed towards any of the sexually receptive OM and 2M females. Thus, independent of the presence of the ovary or ovarian steroids, OM females are more sexually arousing to males than are 2M females (vom Saal & Bronson, 1978).

*Sensitivity to oestrogen.* As indicated above, OM and 2M females do not differ in the dose of oestradiol required to induce sexual receptivity. The capacity of oestradiol to suppress LH secretion and to induce uterine growth were also compared in adult, ovariectomized OM and 2M females, but again, no difference was detected in this experiment (vom Saal & Bronson, 1978).

#### *Behavioural comparisons*

Female mice have traditionally been considered to be non-aggressive (Scott & Fredericson, 1951; Anderson & Hill, 1965; Moyer, 1968), except when lactating (female mice with young exhibit intense aggression when strange mice enter their nest area). This concept stems, in part, from laboratory observation that non-lactating adult female mice tend not to attack adult males unless they are treated with testosterone at the time of testing (vom Saal, Gandelman & Svare, 1976). There is now considerable evidence that in many species of rodents, inter-female aggression occurs, and dominance hierarchies among males as well as females are observed. Thus, while lactating females may exhibit aggression that is more intense than that exhibited by non-lactating females, it is incorrect to consider non-lactating female rodents to be passive. There are obviously many factors involved in regulating aggression in mice. For example, Mugford & Nowell (1970, 1971) have reported that male mice produce a urinary pheromone that elicits aggression from other males, while female mice produce a pheromone that reduces aggression by males. Thus, inter-male aggression is an androgen-dependent behaviour, and the pheromone that elicits aggression is also androgen-dependent. Female mice are generally smaller than males and body weight is positively correlated with dominance (Barkley & Goldman, 1977). Adult female mice have been reported to attack juvenile males and other females but not larger adult males (White, Mayo & Edwards, 1969; Gray, Whitsett & Ziesenis, 1978). Also, in populations of wild mice, females that are quite large have been observed to attack smaller adult males (O. A. E. Rasa, personal communication). Aggression between females is influenced by hormonal changes that occur during the oestrous cycle (Hyde & Sawyer, 1977), but because of the general assumption that females do not fight, little else is known about the regulation of aggression in females.

Observations of mice living in stable territories have led to the conclusion that the dominant male in a deme (breeding unit) accounts for about 90–95% of the offspring produced (DeFries & McClearn, 1970). In studies of freely growing populations of mice, all young that survive as population density becomes high have been reported to be produced by a few dominant females. The subordinate females either fail to ovulate and mate, fail to reach term, or are unable to protect, feed and care for their young (DeLong, 1967; Lloyd & Christian, 1969; Lloyd, 1975). The dominant females have also been reported to exhibit aggression toward intruders into the home territory. Male and female mice appear to attack intruders once stable territories have been formed. The result is that virtually all male and some female intruders into an established deme are killed (Reimer & Petras, 1967; Lidicker, 1976).

In general, then, the available evidence indicates that there is considerable variation among female mice in terms of aggressiveness, and that such variation may play an important role in determining a female's reproductive success at high population density. The role of prior

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intrauterine position in the exhibition of aggression in adulthood was examined by comparing the aggressiveness of OM and 2M females in a number of situations.

Post-partum or lactation-induced aggression is extremely intense in mice and can lead to the death of an intruder into the nest area if escape is not allowed. This behaviour requires continuous suckling stimulation to be maintained in mice (Svare & Gandelman, 1976). The intensity of post-partum aggression exhibited towards male or female intruders was compared in two groups of adult, lactating OM and 2M female mice. The females were singly housed and tested on Day 7 *post partum*. No difference in the intensity of aggression (duration of time spent chasing and biting the intruder) between the OM and 2M females was observed towards a male intruder ( $32 \pm 6$  sec for 15 OM females tested and  $38 \pm 6$  sec for 15/15 2M females); aggression by all of the females was quite intense and resulted in severe wounding of the male. The OM and 2M females differed considerably in the intensity of aggression exhibited towards a female intruder, the 2M females (15/18) attacking for  $39 \pm 6$  sec and the OM females (14/19) for  $16 \pm 4$  sec ( $P < 0.01$ ). The 2M females do not appear to discriminate between male and female intruders in terms of the intensity of aggression that they exhibit, but OM females exhibited very mild attacks, with little wounding, towards the female intruder, and allowed the female intruder into the nest area without attack (vom Saal & Bronson, 1978).

The exhibition of aggression by OM and 2M females was also compared when the females were placed in direct competition. A OM and 2M female were housed on either side of a barrier until both females were in dioestrus as indicated by vaginal lavage. The barrier was then raised and the pair was observed for 30 min. No aggression was observed in 8 of the 28 pairs tested. Tail rattling, aggressive grooming, chasing, and biting were observed in the other 20 pairs and in 17 the 2M female exhibited aggression towards and established dominance over the OM female opponent ( $P < 0.01$ ; vom Saal & Bronson, 1978). Thus, while in dioestrus or while lactating, 2M females are more aggressive towards other female mice than are OM females. Since OM and 2M females that were ovariectomized and implanted with capsules (empty or containing increasing doses of oestradiol) behaved similarly towards males, although the behaviour of males toward OM and 2M females did differ, prior intrauterine position appears to influence a female mouse's behaviour towards other females but not towards males.

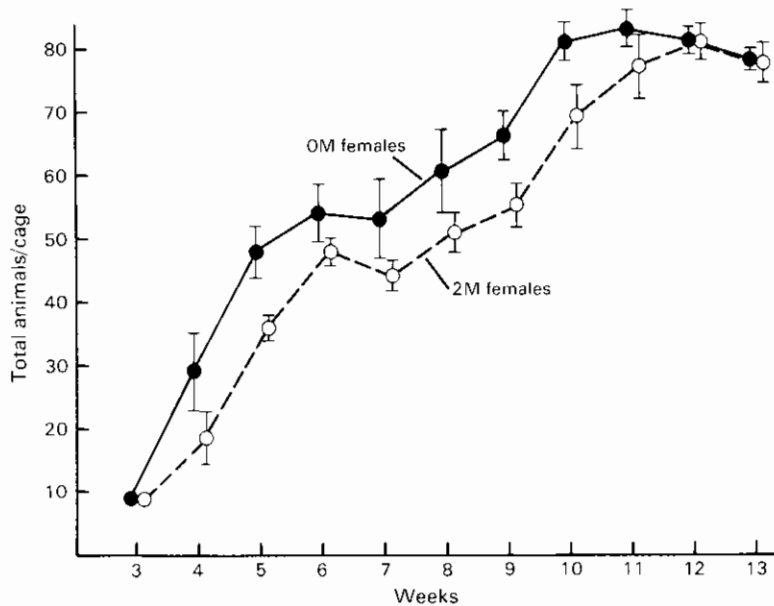
In male mice, the rate of urine marking of the environment is positively correlated with dominance status (Desjardins, Maruniak & Bronson, 1973; Maruniak, Desjardins & Bronson, 1977), and in some environments may serve to delineate territorial boundaries (Harrington, 1976). Adult 2M females mark a novel environment at significantly higher rates than do OM females ( $P < 0.05$ ; vom Saal & Bronson, 1978). This finding suggests that not only are 2M females more aggressive than OM females, but that in some environments, the 2M females might have a greater tendency to mark and defend a territory than would OM females.

### *Reproductive capacity*

The most fundamental question concerning the later effects of intrauterine position is whether 2M and OM females differ in their basic capacity to ovulate, mate, produce and raise healthy young. If rats and mice are exposed to testosterone during the neonatal period, they lose the capacity to produce gonadotrophins in a cyclic pattern (the androgen sterility syndrome; Barraclough & Leathem, 1954; Arai & Gorski, 1968). Since 2M females have longer, more irregular oestrous cycles than do OM females, there is obviously some effect of exposure to elevated levels of testosterone as a result of positioning between male fetuses on the feedback systems regulating cyclicity in females. However, when the oviducts of OM and 2M females were examined for the presence of ova on the morning after pro-oestrus, no difference in the proportion of animals ovulating or the number of ova shed was found (vom Saal & Bronson, 1980b).

The capacity to produce and raise healthy young was compared in adult OM and 2M females by housing them singly with a male. When the females were visibly pregnant, the male was removed. The number and weight of the young found at birth and at weaning were compared, and the entire process was repeated following weaning of the first litter. There were no differences, in relation to prior intrauterine position of the mother, in the number or weight of the young at birth or at weaning for the first or second litter (vom Saal & Bronson, 1978). Therefore, while adult 2M females have longer oestrous cycles than do OM females, their basic capacity to reproduce in an optimum laboratory environment is not impaired.

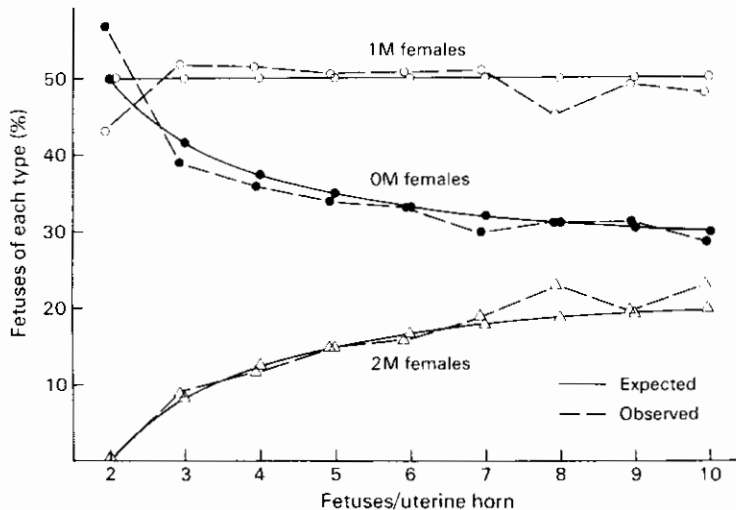
The obvious question that arises is, how might the reproductive performance of OM and 2M females differ in more complex social environments? At this time, only the first of a series of experiments addressing this question has been completed. Just after weaning, 8 OM or 8 2M juvenile females were placed with an adult male in  $60 \times 60$  cm chambers with 4 nest areas (5 replicates of each condition). The number of offspring of these females found alive at birth and the total number of animals alive within each box at the end of each week were compared. The OM and 2M females did not differ in the mean age at the time of delivery of the 1st, 2nd or 3rd litters, or in the length of the time between litters. Also, there was no difference in the number of young found alive on the morning of delivery. But at the end of each week there were significantly more young surviving in the boxes initially containing the 8 2M females ( $P < 0.05$ ; Text-fig. 3). There was no difference in asymptote between the boxes in which OM or 2M females served as the founder population: cessation of population growth occurred as a result of the death of all young produced, not as a result of an inhibition of reproductive capacity. By the end of the experiment, however, the founder females represented only 10% of the population. However, dispersal of the animals defeated in aggressive encounters was not possible in this study, although it is a critical density-regulating mechanism in many rodent species (Krebs, Gaines, Keller, Myers & Tamarin, 1973; Lidicker, 1975).



**Text-fig. 3.** The total number of mice found alive in boxes that initially contained 8 OM females and 1 male or 8 2M females and 1 male (5 replicates of each) at weekly intervals.

As a control procedure, juvenile 0M and 2M females were also housed singly with an adult male, and the number and weight of young produced and weaned over 2 litters were recorded. No difference between 0M and 2M females was found in this experiment (F. S. vom Saal & F. Bronson, unpublished). It seems likely, therefore, that the high mortality of young observed when 8 2M females were housed together probably occurred as a result of high levels of aggression between these females, since an increase in aggressiveness among mice in freely growing populations as density increases results in an increase in infant mortality (Vessey, 1967; Lloyd, 1975). Tentatively, then, these findings support the hypothesis that the behavioural and physiological differences that have been recorded between 0M and 2M females can influence their reproductive success in complex social environments.

If the intrauterine position phenomenon is to be considered as mediated by the intrauterine environment, then it is necessary to determine whether positioning of fetuses by sex *in utero* is random. The frequencies of 0M, 1M and 2M females that are expected based on random positioning of fetuses by sex in a uterine horn, as a function of the number of fetuses per horn, are presented in Text-fig. 4. The observed frequencies of these 3 types of females in an analysis of 1534 uterine horns (767 litters of mice delivered by Caesarean section) are also presented. The observed and expected frequencies do not differ significantly, thus supporting the hypothesis that intrauterine positioning by sex is random. The proportion of 0M and 2M females that are found as the number of fetuses in a uterine horn decreases changes markedly, such that no females positioned between males can be found with fewer than 3 fetuses in a uterine horn. It is apparent, then, that shifts in litter size, independent of shifts in sex ratio, will alter the proportion of 0M and 2M females that are produced (with  $>1$  fetus/uterine horn, 50% of the females produced are 1M females). This observation leads to the interesting possibility that this may represent a self-regulatory system in rodents whereby the characteristics of female (and male) offspring will be observed to change when shifts in litter size occur. A related observation from mouse population studies is that as population density increases, prenatal mortality also increases (Christian & Lemunyan, 1958). One consequence of this is that the frequency of females that develop in a uterine horn between male fetuses will decrease in proportion to the decrease in litter size, and the characteristics of females in the next generation should shift toward those of 0M females.



**Text-fig. 4.** The observed frequencies of 0M, 1M and 2M female mice from 1534 uterine horns and the expected frequencies of these females based on random positioning by sex as a function of the number of fetuses found alive at term in a uterine horn. Both the mean and the mode = 6 fetuses/uterine horn.

At high population density in many rodent species, an increase in aggressiveness is commonly observed. Chitty (1967) and Krebs *et al.* (1973) have proposed that this shift in phenotype in a population as density increases represents a greater probability of survival at high population density of animals with aggressive genotype. Aggressiveness between different strains of mice varies considerably, and this has been demonstrated to reflect differences in genotype. But, as the experiments on the intrauterine position phenomenon demonstrate, within a population of mice in which aggression is observed, aggressiveness of individual females varies as a function of their prior intrauterine proximity to male fetuses. Chitty (1967) has proposed that genotype shifts dramatically toward aggressiveness as population density rises and presumably then shifts again in favour of non-aggressiveness as density decreases. An obvious problem with this model is that such rapid shifts in gene frequencies, particularly as density decreases, cannot easily be explained. Also, the studies that have been conducted, particularly using electrophoretic analysis of proteins as an index of the degree of genetic polymorphism within a population, have attempted to test whether the frequencies of genes at any loci shift as cycles in population size occur, and have not examined genes that have been in any way linked to aggressiveness (Krebs *et al.*, 1973; Fairbairn, 1978). An alternative explanation for an increase in aggression within a population as density increases relates to the intrauterine position phenomenon. As competition between individuals increases, dispersal, particularly of juvenile and adolescent animals, occurs (Bronson, 1979). The results of the aggression experiments described above suggest that as population density and competition for space increase, a greater proportion of 0M females will be dispersed and the proportion of 2M females in the population would increase. When 8 0M or 8 2M female mice were confined together and allowed to breed freely with an adult male, the aggressiveness of the 2M females appeared to reduce their reproductive success. The above model predicts, however, that in a more naturalistic setting, i.e. one in which competition between 0M, 1M and 2M females as well as dispersal can occur, 2M females would disperse the other types of females and thus contribute more offspring than would the 0M and 1M females as population density increases.

Animals that are dispersed from high density populations are thought to have a low probability of survival (Lidicker, 1975). Thus, both the probability of surviving and of successfully reproducing should be greater for 2M females when population density is increasing. However, as density levels increase to the point where there is an increase in prenatal mortality, presumably as a result of high levels of aggression and resulting stress (Rowe, Taylor & Chudley, 1964; Christian, 1971), the proportion of 2M female offspring that are produced will decrease. As a result, there will be a decrease in the proportion of females in the next generation that are highly aggressive, and an increase in the proportion of females that are non-aggressive. Thus, if fetal death occurs before the onset of secretion of testicular androgen by male fetuses, and if litter size is reduced to only 1 or 2 fetuses per uterine horn, then the majority of female offspring produced will be 0M females (see Text-fig. 4).

The preceding discussion has focussed on the possibility that the characteristics of 2M females may provide them with a reproductive advantage as population density increases. It is considered just as probable that the characteristics of 0M females may place them at a reproductive advantage over 1M and 2M females when population density is low. For example, independently of their reproductive state, 0M females emit potent cues that attract and highly arouse male mice. Also, 0M females enter puberty sooner and subsequent oestrous cycles are shorter than those of 2M females when the density of females in the environment is low. The proportion of 0M females in litters that are produced by mothers that are stressed as a result of crowding should increase as a function of population density. The 0M females are the least suited to reproduce successfully when population density is high, and an increase in their numbers should serve to decelerate or even inhibit further population growth. The 0M females are non-aggressive and thus are the most likely to be driven out of the home environment when intense competition for space exists. Once they are dispersed, however, the 0M females should have the highest probability of reproducing successfully.

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Another interesting possibility is raised by the finding of Ward & Weisz (1980) that when pregnant rats are stressed the timing of the peak in testosterone secretion from the testes of male fetuses is disrupted. In adulthood, males produced by mothers that were stressed during pregnancy have been reported as showing evidence of being less masculine and more feminine in their characteristics, although the reliability of this finding has been questioned (Chapman & Stern, 1979). It is possible that if the secretion of testosterone by male fetuses carried by severely stressed mothers is disrupted, then females located next to these males *in utero* will not be influenced by the hormonal secretions of their male litter-mates. Thus, the female offspring of stressed mothers should all resemble OM females regardless of their intrauterine proximity to male fetuses. Since at high population density mice are found to be chronically stressed (Christian & Davis, 1964), this might provide an alternative self-regulatory mechanism whereby few (or no) male or female offspring that are highly aggressive would be produced by females that become pregnant while living in a very crowded environment.

At present, there is no information concerning whether offspring differ as a function of the prior intrauterine position of the mother. In this regard, an analysis of possible maternal effects, both prenatal and postnatal, needs to be conducted as does a comparison of OM and 2M females in terms of the effects of stress on reproductive performance.

In summary, considerable variation in phenotype exists in populations of female mice that, in part, can be traced to the circulating levels of testosterone that females are exposed to prenatally by their intrauterine proximity to male fetuses (see Table 2). The available evidence

**Table 2.** A summary of the experimental comparisons of OM and 2M female mice

Experimental comparison	Results*
<b>Physiology</b>	
Reproductive capacity	
Production of young	N.D.
Survival of young	
Individually housed	N.D.
Grouped	OM > 2M
Age of puberty	
Male + individually housed	2M > OM
Male + grouped	OM > 2M
Oestrous cycle length	
Postpubertal	
Male + individually housed	2M > OM
Male + grouped	OM > 2M
Adult	2M > OM
Sensitivity to group housing	
Postpubertal oestrous cycle	OM > 2M
Adult oestrous cycle	N.D.
Stimulus characteristics	
Inhibition of puberty	OM > 2M
Attractiveness to males	OM > 2M
Arousal of males	OM > 2M
Sensitivity to oestrogen	
Uterine weight	N.D.
LH negative feedback	N.D.
Lordosis	N.D.
<b>Behaviour</b>	
Aggression	
Interfemale	2M > OM
<i>Post partum</i>	2M > OM
Urine marking	2M > OM

\* N.D. = no difference. > refers to greater, longer or later than.

suggests that prior intrauterine position may interact with population density in influencing the reproductive success of individual female mice.

#### Female rats

Prior intrauterine position influences the morphology, physiology and behaviour in female rats. Clemens *et al.* (1978) reported that 2M females had longer anogenital spaces at birth than did 0M females and that 2M females exhibited more mounting (male sex behaviour) towards receptive females than did 0M females when treated with testosterone propionate in adulthood. When adult 0M and 2M female mice were treated with testosterone and paired with a sexually-receptive female, no differences in the number of mounts exhibited or the proportion of females that mounted were observed, but significantly more 2M than 0M females exhibited aggression toward the receptive female partner (vom Saal & Bronson, 1978). Species differences in the behaviours influenced by prior intrauterine position obviously exist.

The age at puberty and length of oestrous cycles in 0M and 2M female rats are affected by prior intrauterine position as in mice. There was no difference between 0M and 2M females in the mean  $\pm$  s.e.m. (21 rats/group) age of vaginal opening ( $36.0 \pm 0.4$  and  $36.2 \pm 0.04$  days respectively), but the first vaginal oestrus was significantly later in 2M females ( $38.9 \pm 0.8$  days) than in 0M females ( $36.9 \pm 0.4$  days) ( $P < 0.05$ ). Thus, 2M females enter puberty significantly later than do 0M females. 2M females tended to have longer oestrous cycles than did 0M females for the first 3 cycles after puberty (mean  $\pm$  s.e.m. 0M females =  $4.6 \pm 0.1$  days; 2M females =  $5.0 \pm 0.2$  days;  $P = 0.055$ ). However, few of the adolescent females exhibited consistent 4- or 5-day cycles. In adulthood, the 0M females had significantly shorter cycles than did the 2M females (0M females =  $4.7 \pm 0.1$  days; 2M females =  $5.7 \pm 0.3$  days;  $P < 0.01$ ; F. S. vom Saal, unpublished). In mice, singly housed 2M females enter puberty later and have longer post-pubertal and adult oestrous cycles than do 0M females (vom Saal & Bronson, 1978, 1980b; vom Saal *et al.*, 1981).

#### Male mice

Male mice that developed *in utero* between 2 male fetuses (2M males) or between 2 female fetuses (0M males) have also been compared. There are no differences between 0M and 2M males in amniotic fluid or blood concentrations of testosterone on Day 17 of gestation. Also, neither body weight at birth or in adulthood nor anogenital distance at birth varies as a function of prior intrauterine position in males (F. S. vom Saal, unpublished). Male fetuses secrete sufficient quantities of testosterone to be maximally masculinized, since exposure to very high concentrations of testosterone via injections to a pregnant female is without apparent effect on the male offspring, while the female offspring are completely virilized (vom Saal, 1979).

Based on the above information, there would appear to be little reason to expect that the intrauterine position of a male fetus should influence its development. However, in a comparison of oestradiol-17 $\beta$  concentrations in the amniotic fluid of 17-day-old mouse fetuses, female fetuses had significantly higher concentrations than male fetuses (mean  $\pm$  s.e.m.: females =  $33.6 \pm 1.5$  pg/fetus; males =  $20.9 \pm 2.8$  pg/fetus;  $P < 0.005$ ) and those of 0M males were higher than those of 2M males (0M males =  $23.8 \pm 9$  pg/fetus; 2M males =  $16.3 \pm 1.1$  pg/fetus;  $P < 0.01$ ; F. S. vom Saal, unpublished).

Oestrogen in the blood of fetuses is bound (inactivated) by  $\alpha$ -fetoprotein, a blood-binding protein (Raynaud, Mercier-Bodard & Baulieu, 1971). But  $\alpha$ -fetoprotein has been found in brain cells of fetal rats (Benno & Williams, 1978), although the significance of the presence of an intracellular pool of  $\alpha$ -fetoprotein is, as yet, unclear (McEwen, 1980). It has been proposed, however, that some oestrogen may enter target tissues (such as specific brain areas) and

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influence development even when  $\alpha$ -fetoprotein is present in the circulation (McEwen, Plapinger, Chaptal, Gerlach & Wallach, 1975; Gorski, 1979).

Experiments are currently being conducted concerning the consequences of developing *in utero* between male or female fetuses on morphology, physiology and behaviour in adult male mice. Results so far clearly indicate that prior intrauterine position does influence the behaviour of male mice.

Adult, naive 0M, 2M and 1M males were compared for the tendency to kill or behave parentally toward newborn mice. Approximately 45–50% of CF-1 male mice will kill 1-day-old young that are placed into a male's home cage for 30 min, while 30–40% exhibit parental behaviour (retrieve the young to a nest and hover over them). As shown in Table 3 prior intrauterine position significantly influences the probability that a male will exhibit killing or parental behaviour in adulthood ( $P < 0.01$ ; F. S. vom Saal, unpublished).

**Table 3.** The number (and proportion) of 0M, 1M, and 2M adult male mice that killed, were parental towards, or ignored 2 newborn mouse young that were placed in the male's cage for 30 min

Behaviour to young	Intrauterine position of male			Total
	2M	1M	0M	
Killed	7 (23)	12 (40)	19 (63)	38 (42)
Parental	20 (67)	11 (37)	8 (27)	39 (43)
Ignored	3 (10)	7 (23)	3 (10)	13 (15)

The differences between 0M and 2M females can be understood in terms of their exposure to androgen *in utero* (Jost, 1972), but there is considerably less information available concerning the role that oestrogens may play in development. The data presented above for male mice are difficult to interpret but the results do suggest that variation in oestrogen concentrations during fetal life may influence brain development and thus adult behaviour.

### Conclusion

It is proposed that the intrauterine position phenomenon in mice, i.e. the capacity of male and female fetuses to have their development modified via exposure to steroids secreted by contiguous litter-mates, may have co-evolved with multiple uterine residence in response to positive selective pressure, while other developmental possibilities provided negative selective pressure. An example of the latter is the safeguard against vascular connections between fetuses that appears to have evolved in mice to protect against the transfer of blood tissue between fetuses of the opposite sex and thus the occurrence of the freemartin syndrome (sterility of the offspring).

About 70% of female mice develop *in utero* next to at least 1 male fetus and hence experience some elevation in blood testosterone titres during the last third of gestation. The elevation in blood testosterone titres in these females is terminated at parturition, however. Testosterone can sterilize female mice if present in the circulation in sufficient concentrations within the first few days following birth. This set of conditions may have produced selective pressure such that parturition in mice occurs just before the developmental period of maximum neural sensitivity to the defeminizing and potentially sterilizing effect of testosterone in female mice.

An important aspect of the evolution in mice of multiple uterine residence is the capacity of mice to exhibit eruptive population growth. At high population density, the stress relating to crowding can disrupt the timing of testosterone secretion by male fetuses with the result that variation in the reproductive characteristics of offspring based on intrauterine position may be eliminated, and all females produced may resemble 0M females. Environmental stress also increases fetal death, thus reducing litter size and increasing the proportion of 0M females

produced (see Text-fig. 4). It is predicted that as a mouse population increases in density, the highly aggressive 2M females should have a reproductive advantage over the less aggressive 0M and 1M females. But, if 2M females have a reproductive advantage over 0M and 1M females when population density starts to increase, one might expect the evolution of a system that maximizes production of 2M rather than 0M female offspring. It is possible, however, that when population density reaches the point at which the stressful effects of crowding influence litter size or testosterone secretion by male fetuses, little further successful reproduction within the population can occur. There would therefore be a selective advantage to maximizing the production of 0M females that should have the highest probability of reproducing successfully when they leave the home environment. Animals leaving the home environment have a low probability of survival, but the overwhelming success of *Mus musculus* as a colonizing species demonstrates that survival of dispersed or emigrating animals does occur.

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