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Husbandry Of Monodelphis Domestica In The Study Of Mammalian Embryogenesis

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Monodelphis domestica: an alternative laboratory animal for the study of mammalian embryogenesis

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***Monodelphis domestica* is a useful laboratory animal for use in studying marsupial embryonic development. A breeding protocol which reliably produces timed-pregnant animals is described. Additionally, techniques for animal husbandry as well as embryo collection and handling are described.**

Mammalian embryogenesis has traditionally been studied in *Mus musculus*, the laboratory mouse, for a variety of reasons. The ability of this small rodent to thrive in tight quarters in indoor housing, as well as its fecundity and short (~21-day) gestation period made it an early favorite as a model system for studying genetics, physiology and development[1]. Immense strides have been made in acquiring and maintaining mouse genetic lines, both from spontaneous mutations as well as genetically engineered embryos. The sequencing of the mouse genome[2,3] plus its amenability to molecular manipulation[4] and well-documented applicability to human genetic studies[5] ensure that the laboratory mouse will continue to be the premier experimental system for investigating mammalian embryonic development.

The study of marsupial embryogenesis, on the other hand, has had a late start. The relatively recent practice of systematically obtaining embryos from the Australian dasyurid marsupial *Sminthopsis macroura* [6-9] is largely responsible for most of what is known about embryonic development in these mammals. The focus of current work in our laboratory is reproduction and embryogenesis in the didelphid, *Monodelphis domestica*, the only New World marsupial currently grown and bred routinely in laboratory colonies around the world. Derived from a small founding population originally collected in the 1970s in Brazil and imported into the United States[10], the gray short-tailed opossum (or laboratory opossum as it has come to be called[11]) has been useful in wide-ranging studies of animal biology: immunology[12-14], evolutionary biology[15, 16, 51], physiology[17, 18], anatomy[19, 20], genetics[21, 22], and nutrition[23]. The recent sequencing of the genome of this marsupial[24] has already generated important information[25, 26] and provides a useful tool for discerning the genetic details of embryonic development in marsupials and understanding the evolutionary relationships between marsupial and placental mammals.

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In this report we describe reliable protocols for obtaining timed-pregnant *M. domestica* for use as embryo donors and for explanting and growing embryos *in vitro*. While we specifically acknowledge references for those protocols not originated from our laboratory, we describe in detail a complete protocol for ensuring the availability of specifically staged embryos from timed pregnancies. Additionally, we include explantation and culture techniques we have found useful. To our knowledge, no superovulation protocol has been devised for this laboratory animal, We therefore deem it useful at this point to describe a successful regimen for obtaining embryos year-round from this polyovular species, the only such marsupial maintained today in indoor breeding laboratory colonies. All the protocols described which have been performed in our laboratory have been approved by the Institutional Animal Care and use Committee of Oberlin College.

General Husbandry Practices

The laboratory opossum has been successfully raised in laboratory colonies in North America, Europe and Australia since it was introduced in the 1970s into the United States as a laboratory animal[10]. Since then, various husbandry practices have been found useful[10, 11, 27, 28, 38,41,50] for maintaining laboratory colonies of different sizes and longevities. Here, we describe amendments to these practices appropriate for a sustainable breeding colony of between 25 and 75 animals at any one time and specifically maintained for producing time-mated females to obtain precisely staged embryos at different stages of pregnancy.

Most polycarbonate or polypropylene cages used for laboratory rats are suitable for use with the laboratory opossum. A standard rat cage has sufficient floor area (~0.12 m^2) to hold not only bedding but also a nest jar and food dish (Fig. 1). Both male and female laboratory opossums build nests by lining a cage corner or a suitably sized container with soft material such as shredded paper towels. Unlike pine bedding, non-aromatic bedding material such as shredded or chipped aspen wood[10] absorb animal urine without generating strong odors. Sterilizable liter-capacity glass jars, such as those used for preserving fruits and vegetables, make excellent nests because they withstand disinfection by heat. Additionally, their transparent walls permit observation of these nocturnal animals when they are at rest in the daytime or, in the case of nursing females, monitoring the health of a litter with minimum disturbance.

Adult laboratory opossums subsist on a staple of fox chow[27] but we supplement this diet twice a week with ~10 g thinly sliced fruit (apple, pear, banana, grapefruit, or orange) as a vitamin source and ~10 g previously frozen lean ground beef enriched with $CaCO_3$ and KI, as has been reported for the Australian marsupial, *Sminthopsis macroura*[9]. Because the animals consume meat within a few minutes of serving, and fruit within a day, deterioration of these foodstuffs within the animal cages is not a concern. Placing food materials in heat-sterilizable glass custard bowls prevents spillage onto the bedding-covered cage floor and possible contamination with excrement and urine, reducing the necessity for changing bedding and replenishing the staple food, fox chow, to once every

3 or 4 days. Drinking water is supplied *ad libitum* via heat-sterilizable sipper bottles held at a slant through the metal grid covering the top of the cage.

Unlike laboratory rodents and guinea pigs, laboratory opossums are solitary animals. We keep adults (unless they are being pair-mated) in individual cages (Fig. 2) in a windowless but ventilated room maintained on a 12:12 light:dark cycle and held at 27^{o}C and ~70% relative humidity. Pups are born during the dark portion of the cycle and navigate their way to the dam's teat field within minutes of birth. If more than 13 pups are born, only those able to attach individually to the 13 nipples on the teat field will have the opportunity to survive. Attachment to the teat is mandatory and permanent for each pup for the first 3 weeks of its life (Fig. 3). Should a pup be detached from its nipple during this period, re-attachment is rarely successful. Juveniles wean themselves within 10 weeks of birth although they continue to nurse at irregular intervals, remaining close to the dam (Fig. 4) and supplementing the food they eat with maternal milk. It may be possible to wean pups earlier if a fresh supply of moistened fox chow or suitable foodstuff can be provided reliably. At 12 to 13 weeks, same-sex pairs of pups are transferred two to a cage and fed regular 'adult' food. By 15 weeks of age, juveniles are best caged individually to maintain an acceptable level of cage hygiene and prevent aggressive displays between sub-adult males. With our husbandry scheme, juveniles attain adult weight (~120 g in males, ~70 g in females) within 20 weeks of birth (Fig. 5). For up to 36 and 18 months, respectively, males and females will mate reliably and produce viable young.

The average litter size (N=72) is 6.92 $\pm$ 3.87 at weaning, although it is 9.08 $\pm$ 2.88 at birth. This is in agreement with earlier observations reported for what must certainly be ancestors of the animals we currently use in our laboratory[28]. Given that the average size of a litter of embryos we obtain routinely from pregnancy days 1 through 13 is 11.40$\pm$ 2.19 (N= 68), a significant decrease occurs between conception and weaning. This decrease may result from the combination of embryonic failure *in utero* and death between the times of birth and weaning. While the loss of one or two neonates between birth and weaning may be attributed to their failure to thrive (and thus become subject to cannibalism), the loss of entire litters within four weeks of birth is unlikely to be due to poor fetal health, particularly when otherwise normal-appearing neonates disappear one or two at a time over a period of several days. It appears therefore that at least some mothers are cannibalistic.

Cannibalism is unlikely to be due solely to inadequate protein in the diet, although in rodents, this behavior has been correlated negatively with availability of food[29]. Indeed, this behavior may be an extension of aggressive or infanticidal behavior, as has been shown in several species of laboratory rodents[29-34]. Because rodents are not mainly carnivorous, however, aggression in these animals is unlikely to result automatically in cannibalism. By contrast, the laboratory opossum is omnivorous, with strong carnivorous preferences when given the choice. We think it possible that an aggressive dam's interest in her pups as dietary meat may be deflected by the availability of other meat sources. Supplementation of the laboratory opossum diet with ground beef is our attempt to discourage cannibalism. While we have no definitive, empirical evidence that this

practice eliminates cannibalism, we have noticed less frequent cannibalism since adding meat to our animals' diet. Anecdotal evidence from other laboratories raising laboratory opossums solely on fox chow suggests that cannibalism is rather more frequent in their circumstances.

Breeding Protocol and Mating Behavior

Obtaining timed-pregnant laboratory opossums has been successfully accomplished with the use of a video camera to record the actual time of mating[35, 36, 45, 52.] However, because mating in these nocturnal animals occurs during the dark portion of a 12:12 light:dark cycle, the frequency of matings recorded with a video camera in a lighted room is likely to be lower than what an infra-red camera can record. To maximize the number of timed matings possible given the modest size of our animal colony, we employ an infra-red camera to document the activities of a maximum of four couples, each in a cage, during the dark cycle in our animal room. A polypropylene cage with transparent walls and a floor area of approximately 2000 sq cm suffices for each couple; four such cages fit well within the view of most infra-red cameras without sacrificing image resolution. The female laboratory opossum is an induced ovulator[18, 37], requiring 6.46 ± 0.42 (N = 43) days of "overnight" (5 to 6 hr during the dark cycle) exposure to a male to come into estrus. Nightly exposure of a female to a male is not required, however; exposure every other night is just as effective in inducing estrus within a week. Our mating set-up (Fig. 6) allows us to alternate two groups of four females each caged individually with a male for the maximum recording capacity of VHS video tape (6 hr). This procedure gives a reliable source of time-mated females as well as a record in real time of when copulation occurred. As a result, we are able to confirm matings and obtain embryos at every stage of pregnancy.

Although female laboratory opossums are sexually mature at ~20 weeks (or ~60 grams body weight), they do not go into estrus spontaneously. A female may be induced into estrus, however, by confinement in a cage with a mature male[45]. Although holding male and female animals in the same room has been reported to induce estrus in females[44], our experience indicates that a male must be in close proximity (within the same cage, for instance) to a female in order for estrus to be induced in the latter. This is consistent with the report that a non-volatile pheromone secreted by the male suprasternal gland is mainly responsible for estrus induction[18, 38.] Indeed, if presence in the same room with males were sufficient for a female to go into estrus, our videotaping observations should have revealed that mating occurs with equal likelihood on each "overnight". The fact that approximately one week of "overnights" is required argues strongly against same-room confinement as sufficient to induce estrus in females. As in other marsupials[39], lactation suppresses estrus in the laboratory opossum. However, estrus occurs spontaneously within two weeks of weaning or removal of pouch young, as determined by histological analysis of cell smears from the urogenital sinus and serum estradiol radioimmunoassay[40].

Mating behavior in the laboratory opossum consists of an invariant succession of activities leading to copulation. Our use of an infra-red video camera to record male-female encounters during the dark cycle has afforded specific detailing of mating behavior in these animals first described in 1982[50]. Male opossums initiate contact by sniffing the hind quarters of a female, or excrement and urine she may expel upon being introduced into a mating cage containing a male. The female, if responsive, reciprocates by first slowly, then briskly, moving in a circular path. The two animals then walk head-to-tail in increasingly tighter circles. This proximity gradually positions the male for mounting, then immobilizing, the female with his legs and wrestling her to the cage floor. Intromission occurs within seconds[41]. Over 94% of all copulations (N = 43) observed on video were followed by 1 to 2 min of copulatory lock. However, copulatory lock does not ensure insemination, as evidenced by unfertilized eggs being occasionally (< 5%) recovered from mated females confirmed videographically to have undergone up to 30 seconds of copulatory lock. For any "overnight" during which a copulation is to occur, about 95% of all copulations occur within 2 hr of confining a male and a female in the same cage during the dark cycle.

Inbreeding of laboratory opossums poses a major concern for maintaining a sustainable breeding colony. Animals now in established laboratory colonies worldwide are descended mainly from the small number of founders introduced into the United States over 30 years ago[10] and supplemented by other introductions since then[11]. To slow down the rate of inbreeding, we exchange animals with operators of other breeding colonies. In addition, we ensure that in our colony, animals with common progenitors going back two generations are not paired for the purpose of producing animals to be used in propagating the colony.

Obtaining and Culturing Embryos

Animals to be dissected for embryos must first be anesthetized with a suitable inhalant, such as isoflurane, in a vented fume hood. A small wad of cotton wetted with approximately 500 μL of inhalant and introduced into a screw-top, liter-capacity glass jar in which the animal has been previously placed anesthetizes an average-sized female within 2 min. Following disinfection of the animal's venter with 70% ethanol, the ventral body wall is cut open, first with an incision to the skin, then with a congruent second incision to the abdominal muscle wall. Exsanguination using a 30-mL syringe fitted with a 21-gauge hypodermic needle applied to the inferior vena cava at the level of the kidneys minimizes the amount of blood released when major blood vessels are severed during subsequent uterine explantation. This, in turn, significantly minimizes contamination and decreases the amount of blood introduced into the liquid dissection medium into which explanted uteri are transferred. The choice of dissection media depends on whether embryos will be fixed immediately or grown *in vitro*. Sterile phosphate-buffered saline (PBS) is adequate for holding uteri to be dissected for embryos, but a serum-free culture medium such as DMEM[44] is better suited for embryos to be grown *in vitro*. A warming plate kept at 33°C is useful for holding culture media or PBS within reach during dissection (Fig. 7a).

How embryos are handled depends on their developmental age (Fig. 8). Embryos younger than day 10 are approximately 300 μm in diameter and are easily aspirated individually into a pre-sterilized glass Pasteur pipette operated with a hand-operated micrometer syringe (Fig. 7b). Fire-polishing the aperture of the pipette to melt any sharp glass edges prior to use prevents damage to embryos. If a larger aperture is desired, the pipette can be truncated along its taper with a diamond pencil prior to fire-polishing. It is important to keep embryos fully immersed in a small volume of liquid medium during all aspects of handling, as when aspirating them from one container to another. Particularly with older embryos, handling should be done with extreme care to prevent the superficial cells of the embryo from losing their adhesion to the interior surface of the shell. Our experience has been that collapsed embryos day 7 or older are unable to re-inflate *in vitro*.

Marsupial embryos typically implant late in the gestation period and then only in a relatively superficial to moderately invasive manner. With the exception of bandicoots[42, 43], marsupial embryos adhere to the uterine endometrium via the so-called yolk-sac or chorio-vitelline placenta. In the laboratory opossum, this period of adhesion is confined to the last 48 hr of the 14-day pregnancy. Thus, obtaining embryos $\leq$ 12 days old is mechanically straightforward, requiring only that the uteri be dissected and carefully emptied of their contents in a suitable dissecting or culture medium[44]. Day-13 laboratory opossum embryos adhere to the uterine endometrium via numerous fine villus-like projections of the bilaminar yolk sac[46, 47, 51] (Fig. 8c). Explantation of embryos during this stage requires gentle coaxing with special tools to prevent damage to the highly distended and fragile bilaminar yolk sac. A useful tool for this purpose is a pre-sterilized glass Pasteur pipette pulled over a small flame and heated so as to bend the narrow end and melt its tip to form a small bead of glass.

Embryos survive *in vitro* culture for up to 96 hours in various culture media[35, 36, 44, 45] if kept at 33°C to 37°C in a humid atmosphere containing 5% CO_2. These conditions are adequate for growing cleavage-stage embryos (days 1 – 5) and early blastocysts (days 6 – 9). Commencing on day 9, blastocysts expand dramatically (Fig. 8b) as gastrulation approaches. Additionally, the rapid proliferation of pluriblast cells at this time renders one hemisphere significantly heavier than the other, causing the embryo to be oriented pluriblast-side down in the culture vessel. A device which can gently shake or rock culture vessels during incubation is useful in ensuring that the embryo is not unduly exposed to high concentrations of nitrogenous waste excreted into the culture medium[48]. Although cleavage-stage embryos readily survive without a daily change of culture medium, older embryos do not.

Conclusion

Monodelphis domestica is an excellent laboratory animal which can be bred sustainably in indoor laboratory colonies. Females breed year-round and produce litters after a gestation period of 14 days, for up to 18 months of age. Males remain fertile for up to 4

years of age. Timed pregnancies are easy to obtain, thus making it possible to obtain embryos of any desired age.

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