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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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44 In this report we describe reliable protocols for obtaining timed-pregnant *M. domestica*
45 for use as embryo donors and for explanting and growing embryos *in vitro*. While we
46 specifically acknowledge references for those protocols not originated from our
47 laboratory, we describe in detail a complete protocol for ensuring the availability of
48 specifically staged embryos from timed pregnancies. Additionally, we include
49 explantation and culture techniques we have found useful. To our knowledge, no
50 superovulation protocol has been devised for this laboratory animal, We therefore deem
51 it useful at this point to describe a successful regimen for obtaining embryos year-round
52 from this polyovular species, the only such marsupial maintained today in indoor
53 breeding laboratory colonies. All the protocols described which have been performed in
54 our laboratory have been approved by the Institutional Animal Care and use Committee
55 of Oberlin College.
56
57
58 **General Husbandry Practices**
59
60 The laboratory opossum has been successfully raised in laboratory colonies in North
61 America, Europe and Australia since it was introduced in the 1970s into the United States
62 as a laboratory animal[10]. Since then, various husbandry practices have been found
63 useful[10, 11, 27, 28, 38,41,50] for maintaining laboratory colonies of different sizes and
64 longevities. Here, we describe amendments to these practices appropriate for a
65 sustainable breeding colony of between 25 and 75 animals at any one time and
66 specifically maintained for producing time-mated females to obtain precisely staged
67 embryos at different stages of pregnancy.
68
69 Most polycarbonate or polypropylene cages used for laboratory rats are suitable for use
70 with the laboratory opossum. A standard rat cage has sufficient floor area (~0.12 m^2) to
71 hold not only bedding but also a nest jar and food dish (Fig. 1). Both male and female
72 laboratory opossums build nests by lining a cage corner or a suitably sized container with
73 soft material such as shredded paper towels. Unlike pine bedding, non-aromatic bedding
74 material such as shredded or chipped aspen wood[10] absorb animal urine without
75 generating strong odors. Sterilizable liter-capacity glass jars, such as those used for
76 preserving fruits and vegetables, make excellent nests because they withstand
77 disinfection by heat. Additionally, their transparent walls permit observation of these
78 nocturnal animals when they are at rest in the daytime or, in the case of nursing females,
79 monitoring the health of a litter with minimum disturbance.
80
81 Adult laboratory opossums subsist on a staple of fox chow[27] but we supplement this diet
82 twice a week with ~10 g thinly sliced fruit (apple, pear, banana, grapefruit, or orange) as
83 a vitamin source and ~10 g previously frozen lean ground beef enriched with $CaCO_3$ and
84 KI, as has been reported for the Australian marsupial, *Sminthopsis macroura*[9]. Because
85 the animals consume meat within a few minutes of serving, and fruit within a day,
86 deterioration of these foodstuffs within the animal cages is not a concern. Placing food
87 materials in heat-sterilizable glass custard bowls prevents spillage onto the bedding-
88 covered cage floor and possible contamination with excrement and urine, reducing the
89 necessity for changing bedding and replenishing the staple food, fox chow, to once every

90   3 or 4 days. Drinking water is supplied *ad libitum* via heat-sterilizable sipper bottles held
91   at a slant through the metal grid covering the top of the cage.
92
93   Unlike laboratory rodents and guinea pigs, laboratory opossums are solitary animals. We
94   keep adults (unless they are being pair-mated) in individual cages (Fig. 2) in a
95   windowless but ventilated room maintained on a 12:12 light:dark cycle and held at 27°C
96   and ~70% relative humidity. Pups are born during the dark portion of the cycle and
97   navigate their way to the dam's teat field within minutes of birth. If more than 13 pups
98   are born, only those able to attach individually to the 13 nipples on the teat field will have
99   the opportunity to survive. Attachment to the teat is mandatory and permanent for each
100   pup for the first 3 weeks of its life (Fig. 3). Should a pup be detached from its nipple
101   during this period, re-attachment is rarely successful. Juveniles wean themselves within
102   10 weeks of birth although they continue to nurse at irregular intervals, remaining close
103   to the dam (Fig. 4) and supplementing the food they eat with maternal milk. It may be
104   possible to wean pups earlier if a fresh supply of moistened fox chow or suitable
105   foodstuff can be provided reliably. At 12 to 13 weeks, same-sex pairs of pups are
106   transferred two to a cage and fed regular 'adult' food. By 15 weeks of age, juveniles are
107   best caged individually to maintain an acceptable level of cage hygiene and prevent
108   aggressive displays between sub-adult males. With our husbandry scheme, juveniles
109   attain adult weight (~120 g in males, ~70 g in females) within 20 weeks of birth (Fig. 5).
110   For up to 36 and 18 months, respectively, males and females will mate reliably and
111   produce viable young.
112
113   The average litter size (N=72) is 6.92 $\pm$ 3.87 at weaning, although it is 9.08 $\pm$ 2.88 at
114   birth. This is in agreement with earlier observations reported for what must certainly be
115   ancestors of the animals we currently use in our laboratory[28]. Given that the average size
116   of a litter of embryos we obtain routinely from pregnancy days 1 through 13 is 11.40$\pm$
117   2.19 (N= 68), a significant decrease occurs between conception and weaning. This
118   decrease may result from the combination of embryonic failure *in utero* and death
119   between the times of birth and weaning. While the loss of one or two neonates between
120   birth and weaning may be attributed to their failure to thrive (and thus become subject to
121   cannibalism), the loss of entire litters within four weeks of birth is unlikely to be due to
122   poor fetal health, particularly when otherwise normal-appearing neonates disappear one
123   or two at a time over a period of several days. It appears therefore that at least some
124   mothers are cannibalistic.
125
126   Cannibalism is unlikely to be due solely to inadequate protein in the diet, although in
127   rodents, this behavior has been correlated negatively with availability of food[29]. Indeed,
128   this behavior may be an extension of aggressive or infanticidal behavior, as has been
129   shown in several species of laboratory rodents[29-34]. Because rodents are not mainly
130   carnivorous, however, aggression in these animals is unlikely to result automatically in
131   cannibalism. By contrast, the laboratory opossum is omnivorous, with strong carnivorous
132   preferences when given the choice. We think it possible that an aggressive dam's interest
133   in her pups as dietary meat may be deflected by the availability of other meat sources.
134   Supplementation of the laboratory opossum diet with ground beef is our attempt to
135   discourage cannibalism. While we have no definitive, empirical evidence that this

136 practice eliminates cannibalism, we have noticed less frequent cannibalism since adding
137 meat to our animals' diet. Anecdotal evidence from other laboratories raising laboratory
138 opossums solely on fox chow suggests that cannibalism is rather more frequent in their
139 circumstances.
140
141

Breeding Protocol and Mating Behavior

143

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

163

164 Although female laboratory opossums are sexually mature at ~20 weeks (or ~60 grams
165 body weight), they do not go into estrus spontaneously. A female may be induced into
166 estrus, however, by confinement in a cage with a mature male[45]. Although holding male
167 and female animals in the same room has been reported to induce estrus in females[44], our
168 experience indicates that a male must be in close proximity (within the same cage, for
169 instance) to a female in order for estrus to be induced in the latter. This is consistent with
170 the report that a non-volatile pheromone secreted by the male suprasternal gland is
171 mainly responsible for estrus induction[18, 38]. Indeed, if presence in the same room with
172 males were sufficient for a female to go into estrus, our videotaping observations should
173 have revealed that mating occurs with equal likelihood on each "overnight". The fact
174 that approximately one week of "overnights" is required argues strongly against same-
175 room confinement as sufficient to induce estrus in females. As in other marsupials[39],
176 lactation suppresses estrus in the laboratory opossum. However, estrus occurs
177 spontaneously within two weeks of weaning or removal of pouch young, as determined
178 by histological analysis of cell smears from the urogenital sinus and serum estradiol
179 radioimmunoassay[40].
180

181 Mating behavior in the laboratory opossum consists of an invariant succession of
182 activities leading to copulation. Our use of an infra-red video camera to record male-
183 female encounters during the dark cycle has afforded specific detailing of mating
184 behavior in these animals first described in 1982[50]. Male opossums initiate contact by
185 sniffing the hind quarters of a female, or excrement and urine she may expel upon being
186 introduced into a mating cage containing a male. The female, if responsive, reciprocates
187 by first slowly, then briskly, moving in a circular path. The two animals then walk head-
188 to-tail in increasingly tighter circles. This proximity gradually positions the male for
189 mounting, then immobilizing, the female with his legs and wrestling her to the cage floor.
190 Intromission occurs within seconds[41]. Over 94% of all copulations (N = 43) observed on
191 video were followed by 1 to 2 min of copulatory lock. However, copulatory lock does
192 not ensure insemination, as evidenced by unfertilized eggs being occasionally (< 5%)
193 recovered from mated females confirmed videographically to have undergone up to 30
194 seconds of copulatory lock. For any "overnight" during which a copulation is to occur,
195 about 95% of all copulations occur within 2 hr of confining a male and a female in the
196 same cage during the dark cycle.
197
198 Inbreeding of laboratory opossums poses a major concern for maintaining a sustainable
199 breeding colony. Animals now in established laboratory colonies worldwide are
200 descended mainly from the small number of founders introduced into the United States
201 over 30 years ago[10] and supplemented by other introductions since then[11]. To slow down
202 the rate of inbreeding, we exchange animals with operators of other breeding colonies. In
203 addition, we ensure that in our colony, animals with common progenitors going back two
204 generations are not paired for the purpose of producing animals to be used in propagating
205 the colony.
206
207
208 **Obtaining and Culturing Embryos**
209
210 Animals to be dissected for embryos must first be anesthetized with a suitable inhalant,
211 such as isoflurane, in a vented fume hood. A small wad of cotton wetted with
212 approximately 500 μL of inhalant and introduced into a screw-top, liter-capacity glass jar
213 in which the animal has been previously placed anesthetizes an average-sized female
214 within 2 min. Following disinfection of the animal's venter with 70% ethanol, the ventral
215 body wall is cut open, first with an incision to the skin, then with a congruent second
216 incision to the abdominal muscle wall. Exsanguination using a 30-mL syringe fitted with
217 a 21-gauge hypodermic needle applied to the inferior vena cava at the level of the
218 kidneys minimizes the amount of blood released when major blood vessels are severed
219 during subsequent uterine explantation. This, in turn, significantly minimizes
220 contamination and decreases the amount of blood introduced into the liquid dissection
221 medium into which explanted uteri are transferred. The choice of dissection media
222 depends on whether embryos will be fixed immediately or grown *in vitro*. Sterile
223 phosphate-buffered saline (PBS) is adequate for holding uteri to be dissected for
224 embryos, but a serum-free culture medium such as DMEM[44] is better suited for embryos
225 to be grown *in vitro*. A warming plate kept at 33°C is useful for holding culture media or
226 PBS within reach during dissection (Fig. 7a).

227
228 How embryos are handled depends on their developmental age (Fig. 8). Embryos
229 younger than day 10 are approximately 300 μm in diameter and are easily aspirated
230 individually into a pre-sterilized glass Pasteur pipette operated with a hand-operated
231 micrometer syringe (Fig. 7b). Fire-polishing the aperture of the pipette to melt any sharp
232 glass edges prior to use prevents damage to embryos. If a larger aperture is desired, the
233 pipette can be truncated along its taper with a diamond pencil prior to fire-polishing. It is
234 important to keep embryos fully immersed in a small volume of liquid medium during all
235 aspects of handling, as when aspirating them from one container to another. Particularly
236 with older embryos, handling should be done with extreme care to prevent the superficial
237 cells of the embryo from losing their adhesion to the interior surface of the shell. Our
238 experience has been that collapsed embryos day 7 or older are unable to re-inflate *in*
239 *vitro*.
240
241 Marsupial embryos typically implant late in the gestation period and then only in a
242 relatively superficial to moderately invasive manner. With the exception of bandicoots[42,
243 43], marsupial embryos adhere to the uterine endometrium via the so-called yolk-sac or
244 chorio-vitelline placenta. In the laboratory opossum, this period of adhesion is confined
245 to the last 48 hr of the 14-day pregnancy.   Thus, obtaining embryos $\leq$ 12 days old is
246 mechanically straightforward, requiring only that the uteri be dissected and carefully
247 emptied of their contents in a suitable dissecting or culture medium[44].   Day-13 laboratory
248 opossum embryos adhere to the uterine endometrium via numerous fine villus-like
249 projections of the bilaminar yolk sac[46, 47, 51] (Fig. 8c) . Explantation of embryos during
250 this stage requires gentle coaxing with special tools to prevent damage to the highly
251 distended and fragile bilaminar yolk sac. A useful tool for this purpose is a pre-sterilized
252 glass Pasteur pipette pulled over a small flame and heated so as to bend the narrow end
253 and melt its tip to form a small bead of glass.
254
255 Embryos survive *in vitro* culture for up to 96 hours in various culture media[35, 36, 44, 45] if
256 kept at $33^{o}C$ to $37^{o}C$ in a humid atmosphere containing 5% CO_2. These conditions are
257 adequate for growing cleavage-stage embryos (days 1 – 5) and early blastocysts (days 6 –
258 9). Commencing on day 9, blastocysts expand dramatically (Fig. 8b) as gastrulation
259 approaches. Additionally, the rapid proliferation of pluriblast cells at this time renders
260 one hemisphere significantly heavier than the other, causing the embryo to be oriented
261 pluriblast-side down in the culture vessel. A device which can gently shake or rock
262 culture vessels during incubation is useful in ensuring that the embryo is not unduly
263 exposed to high concentrations of nitrogenous waste excreted into the culture medium[48].
264 Although cleavage-stage embryos readily survive without a daily change of culture
265 medium, older embryos do not.
266
267
268 **Conclusion**
269
270 *Monodelphis domestica* is an excellent laboratory animal which can be bred sustainably
271 in indoor laboratory colonies. Females breed year-round and produce litters after a
272 gestation period of 14 days, for up to 18 months of age. Males remain fertile for up to 4

273 years of age. Timed pregnancies are easy to obtain, thus making it possible to obtain
274 embryos of any desired age.
275
276
277
278
279
280 **Acknowledgment**
281
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