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Translating the Timing of Developmental Benchmarks in Short-Tailed Opossums (*Monodelphis domestica*) to Facilitate Comparisons with Commonly Used Rodent Models

Chris Bresee[a] Jules Litman-Cleper[a] Cindy J. Clayton[b] Leah Krubitzer[a, b]

[a]Center for Neuroscience, University of California at Davis, Davis, CA, USA; [b]Department of Psychology, University of California at Davis, Davis, CA, USA

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Abstract

Introduction: The gray short-tailed opossum, *Monodelhis domestica* (*M. domestica*), is a widely used marsupial model species that presents unique advantages for neurodevelopmental studies. Notably their extremely altricial birth allows manipulation of postnatal pups at timepoints equivalent to embryonic stages of placental mammals. A robust literature exists on the development of short-tailed opossums, but many researchers working in the more conventional model species of mice and rats may find it daunting to identify the appropriate age at which to conduct experiments. ***Methods:*** Here, we present detailed staging diagrams taken from photographic observations of 40 individual pups, in 6 litters, over 25 timepoints across postnatal development. We also present a comparative neurodevelopmental timeline of short-tailed opossums (*M. domestica*), the house mouse (*Mus musculus*), and the laboratory rat (*Rattus norvegicus*) during embryonic as well as postnatal development, using timepoints taken from this study and a review of existing literature, and use this dataset to present statistical models comparing the opossum to the rat and mouse. ***Results:*** One aim of this research was to aid in testing the generalizability of results found in rodents to other mammalian brains, such as the more distantly related metatherians. However, this broad dataset also allows the identification of potential heterochronies in opossum development compared to rats and mice. In contrast to previous work, we found broad similarity between the pace of opossum neural development with that of rats and mice. We also found that development of some systems was accelerated in the opossum, such as the forelimb motor plant, oral motor control, and some aspects of the olfactory system, while the development of the cortex, some aspects of the retina, and other aspects of the olfactory system are delayed compared to the rat and mouse. ***Discussion:*** The pace of opossum development is broadly similar to that of mice and rats, which underscores the usefulness of this species as a compliment to the more commonly used rodents. Many features that differ the most between opossums and rats and mice were either clustered around the day of birth and were features that have functional importance for the pup immediately after or during birth, or were features that have reduced functional importance for the pup until later in postnatal development, given that it is initially attached to the mother.

karger@karger.com
www.karger.com/bbe

Correspondence to:
Chris Bresee, csbresee@ucdavis.edu

Karger

Introduction

The gray short-tailed opossum, *Monodelphis domestica*, is a small pouchless marsupial from Brazil and northern Argentina [1]. In the wild, they occupy a range of habitats, from tropical jungle to more temperate wooded grassland [2]. They have adapted to living in close quarters with humans, and feeding on household pests, a fact that is the reason for the species name "domestica" [3]. These and a handful of other American opossums are the few remaining species of South American marsupials to survive the biotic interchange that occurred when the isthmus of Panama formed between North and South America [4, 5]. Therefore, the short-tailed opossum is one of a few extant New World species representing an ancient mammalian lineage that shares a common ancestor with eutherians ~170-160 million years ago (mya), before the K-PG extinction (see Fig. 1a), [4, 6–8].

The short-tailed opossum is one of the most used marsupial model species [1] but is still vastly underutilized in comparison to rodents [9]. Although the common ancestor of rodents and humans existed ~80 mya (Fig. 1b, c) mice and other rodents are in many ways exceptionally good models, with their small size, short generations, docility, ubiquity, ease of genetic manipulation, and a plethora of technologies that can be used to study their brains. While much of neuroscience is ultimately aimed at understanding human brains, one could describe modern neuroscience as the study of the mouse brain since the vast majority of studies in mammals are done in mice [9]. However, in order to understand human brains, we must: (1) understand the mammalian brain in general, (2) have outgroups with which to contrast results from euarchontoglires, and (3) not become so specialized in mouse neuroscience that findings do not apply to other species, particularly primates.

As a model species, short-tailed opossums present many of the same advantages of mice and rats (small, short generations, docile, and accessible [1–3, 10]), and to date it is one of only three marsupials to have had its genome sequenced and annotated [11, 12]. Additionally, short-tailed opossums possess many characteristics that make this species a particularly important complement to rodent research. On the one hand, they have some of the same traits as mice and rats, whether they are conserved (such as the presence of whiskers [13]), or convergently evolved (such as moving whiskers rhythmically to sample their environment, a.k.a. "whisking" [14]). Such shared traits allow researchers to test the generalizability of findings from rats and mice while controlling for or intentionally varying phylogenetic relatedness. On the other hand, short-tailed opossums possess some traits that rodents simply do not have (such as the marsupial reproductive strategy), or that are highly derived in rodents compared to the more basal condition in opossums (such as a full heterodont dentition [15]), both of which allow researchers to conduct experiments that would not necessarily be feasible or informative in rodents. These advantages all help fill in important gaps with regard to research with mice and rats: allowing comparative evolutionary studies that are difficult or impossible if restricted to these rodents.

Here, we present data to facilitate the use of short-tailed opossums in comparative developmental neuroscience research. First, we present diagrams describing externally observable morphological characteristics typifying important postnatal developmental benchmarks, from observations of 22 pups from 6 litters at 25 timepoints. Second, we present timepoints at which various neurological structures develop, comparing data between mice, rats, and opossums, taken from primary literature and the Translating Time dataset (translatingtime.org [16]).

These data are designed to facilitate wider adoption of short-tailed opossums as a model species. It is our hope that the staging guide and tables will allow researchers to generalize results from rodents, by identifying the age at which manipulations should be done in opossums, while at the same time highlighting morphological features that allow confirmation of pup staging.

Methods

Subjects

Six liters of short-tailed opossums, with a total of 22 pups, were photographed over 25 timepoints, with 207 photographs, for a total of 563 observations of individual pups (see Table 1).

Colony Maintenance, Animal Handling

As shown in Figure 2a, adult opossums are housed in clear acrylic 19" × 10.5" × 8" caging with wire tops and sipper-tube water bottles, with 12 oz. clear acrylic tumblers for hides, soft paper granule bedding (Care-Fresh) for substrate, and pleated paper strip bedding (EnviroDri) for nest building. Animals were fed ad lib either Complete Reproduction Fox Food Pellets or Purina Cat Chow. Opossums cannot gnaw food pellets through the wire grate as rodents do, so food is left directly in the substrate (Fig. 2a). Animals were acclimated to human interaction with weekly handling during cage cleaning and intermittent handling for health checks and breeding.

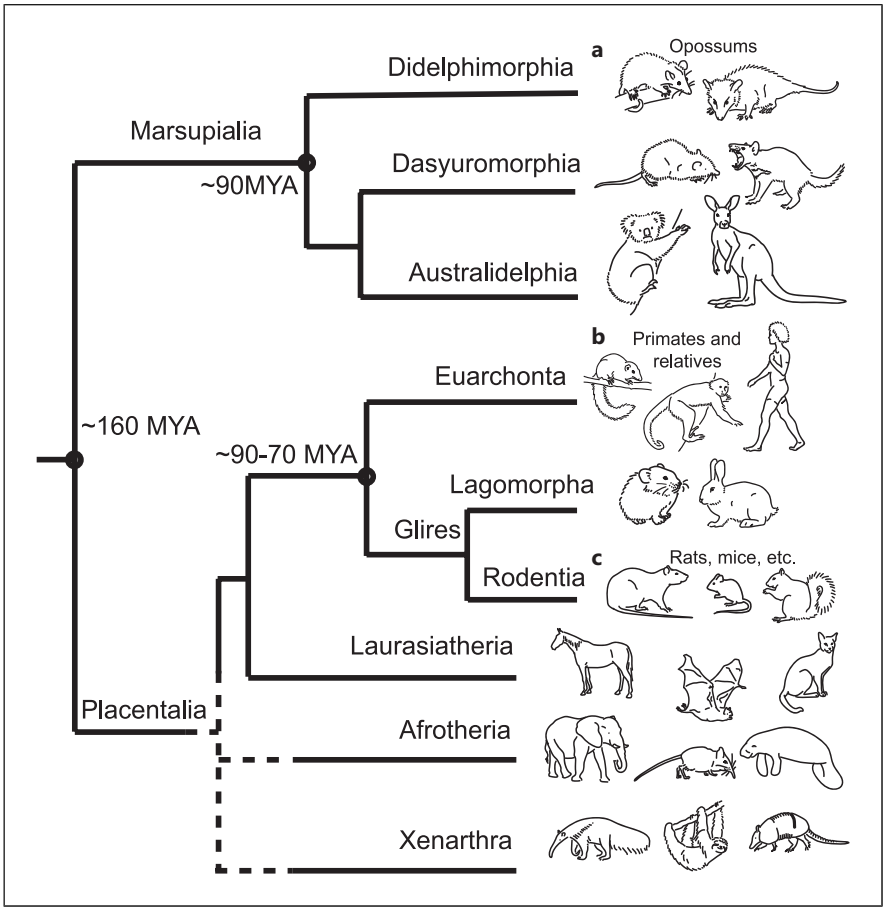


Fig. 1. Phylogenetic tree, illustrating that opossums and rodents are distantly related mammals, with rodents being relatively closer to the human clade, Euarchonta, although still quite distantly related. Branch order reflects relative last common ancestors, lengths are not to scale. Order and clade names adapted from [6, 7]. Dashed lines represent branches with ambiguous relationships.

Table 1. Number of observations of individual pups at each age

	Postnatal day																								
	0	3	6	8	9	12	15	16	18	20	21	24	27	30	31	32	33	34	45	40	45	50	55	60	90	Total
Number of observations	55	60	39	10	42	48	68	4	30	7	27	31	9	18	3	10	10	7	26	10	9	10	9	8	13	563

Data are from 178 photographs of 40 individual pups, in 6 litters. Number of pups ≠ number of observations because some photographs contained multiple individuals, some individuals/litters were photographed multiple times on the same day (for multiple angles), and some individuals/litters were not photographed on a given day.

Females were bred at 6–7 months of age, and males at 4–5 months. One female and one male are first moved to the same room, in adjacent cages, to acclimate to each other's smell. The animals are then housed together for 2 weeks, with the male moving to the female's cage. After 2 weeks, the male is separated, and the female is checked for pups each day for an additional 2 weeks. When pups are present a microisolator lid is used to prevent escapes, until pups are weaned.

Imaging Techniques

For ages P0–P24 short-tailed opossum dams were anesthetized via isoflurane and individual pups were manually positioned for better viewing. For P25+ individual pups were placed outside of the home cage and if necessary movement was constrained by gently holding the tail. Animals were placed in front of a ruler and photographs were taken.

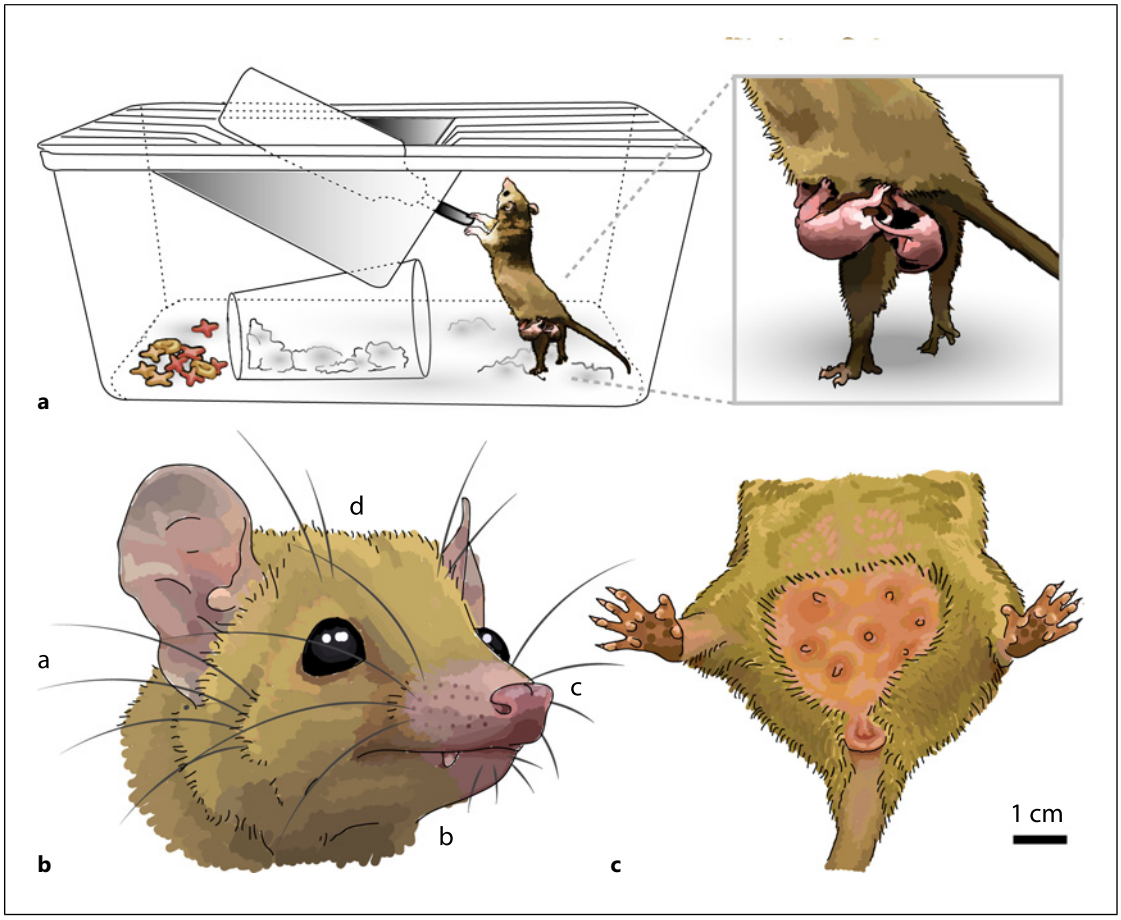


Fig. 2. Short-tailed opossum husbandry and adult morphology. **a** Opossums are housed singly, or with pups, except during breeding, when one male and one female are co-housed. Inset shows a female opossum with young clinging to her abdomen and hind legs. **b** Schematic of an adult opossum face highlighting some distinctive characteristics, including: large genal whiskers sprouting from a highly developed muscular genal pad (a), long canines (b) that are slightly visible even when the mouth is fully closed, a large furless rhinarium (c), large jaw muscles attaching to a midsagittal ridge on the dorsal surface of the skull, forming a slight trough at the midline of the head (d), and a prominent mandible, forming a more distinct chin than in rodents. **c** Ventral view of female opossum while nursing a litter. Unlike rodents the arrangement of nipples is not highly stereotyped; they are frequently arranged in a circle, with one or two in the center, but can also be arranged asymmetrically. Females that have never been pregnant do not have visible mammary glands or nipples. Newly pregnant females begin to show developing mammary glands, as well as thinning of the abdominal fur.

Qualitative Observation of Physical Features

After visually inspecting all 207 photographs for qualitatively varying features, we chose 10 physical characteristics that varied during development: skin/fur, whiskers, eyes, ears, mouth, nose, tail, body posture at rest, limbs, and head size/shape (see Table 2). We then systematically inspected each photograph, again in series, while focusing on one feature at a time. This systematic inspection was repeated for each physical feature. Some physical features tended to change simultaneously for all 22 pups (skin thickness, presence of whiskers, ear morphology in the first 2 weeks, lip morphology, body posture, and digit morphology), while other traits (eye opening, dorsal skin pigmentation, paw pad pigmentation, and nose pigmentation) were more variable. For these variable traits, we chose the time point that the majority of animals had a feature at a particular level of maturation.

Illustrations

Illustrations were produced using Adobe Illustrator on an MS Surface Book 3 tablet. We first referenced one primary photograph for each animal, and referenced up to 20 additional photographs of other animals at that developmental timepoint, if particular features were obscured or otherwise difficult to visualize in the primary photograph. Illustrations were chosen over photographs as these allowed us to highlight physical features of interest while reducing extraneous visual stimuli. Reference photographs are available upon request.

Literature Search

We compiled dates for neurodevelopmental feature maturation in rats (*Rattus norvegicus*), mice (*Mus musculus*), and opossums (*Monodelphis domestica*), based on primary literature sources

Table 2. Opossum feature maturation from birth (P0) to independent subadulthood (P60)

Day	Skin/fur	Whiskers, eyes, ears	Mouth, nose	Tail, body posture	Limbs	Head
0	Skin red, shiny, and translucent. Vasculature, ribs, and skull sutures visible. Milk spot visible. No fur	No whiskers, eyes fully covered with skin, no external ears	Mouth open anterior only	Body curled, tail curled tightly under body	Forelimbs have distinct digits, with claws, mostly fused except tips. Hindlimbs paddle-like, fused digits. no visible knee joint	Snout blunt, very short. Head-to-body ratio (HBR) 1:1.5
3	Skin pales to pink					
9	Skin thickens to semi-translucent	Small ridges where pinnae will be		Body may partly uncurl	Hindlimbs have defined digits, but fused. visible knee joint	Snout visibly longer, still wider than long
12	Sparse white fur on face and head. Milk spot not easily visible	Small pinnae point rostrally		May be held away from body		HBR 1:2
15	Skin thicker, matte. May have very slight gray tint to skin dorsally. fur on head may be pigmented. white fur on body	Whisker follicles barely visible. Very short mystacial whiskers present. pinnae point caudally, but fused to head	Visible division between upper and lower lip, but still partially fused	May detach from mother. May lie fully uncurled	Forelimb claws may be pigmented. Hindlimb digit tips separated	
18	Dorsal side acquires gray tint. Skull sutures barely visible	Mystacial and genal whiskers present. Division between upper and lower eyelid visible. Pinnae more free from head		Dorsal side may begin to pigment	Hindlimb digits more distinct, but only partially separated	
21	Dorsal skin more gray than pink	Supra-ocular vibrissae present. Pinnae mostly free from head; tips darken		Tail still lighter than body		
24	Dorsal skin dark gray	Pinnae fully free, but point more caudally than in adults	Mouth fully open	Dorsal side of tail as dark as the body		Snout visibly longer, still wider than long
27	Short, sparse agouti fur dorsally. Very thin white fur on the belly and limbs. No distinct guard hairs	Fur appears on pinnae	The nose may begin to show darker pigment than rest of face		Hindlimb pads may have pigment	HBR 1:2.5
30	Agouti fur covering skin dorsally, darker than in adults. Some distinct guard hair and undercoat. Sparse fur on limbs	Pinnae angled outward				
31	Fur on forelimbs thicker and darker					Snout visibly longer, still wider than long. Slightly pointed

Table 2 (continued)

Day	Skin/fur	Whiskers, eyes, ears	Mouth, nose	Tail, body posture	Limbs	Head
32	Fur slightly paler dorsally. Dorsal forepaw fur begins darkening	Eyes may begin opening				
34	More distinct guard hair. Thin undercoat. Dorsal hindpaw fur begins darkening					Snout about as wide as long
35		Eyes open, but not fully. Pinnae adult-like				HBR 1:3
40	Thicker undercoat, but not adult-like. Dorsal coat similar to adult tint. Belly fur begins to darken	Eyes mostly open	The nose has adult-like pigment		Hindlimb paw pads likely pigmented	
45	Belly fur darker					Snout longer than wide
50	Coat longer, but not adult length	Eyes appear adult-like				
55	Undercoat noticeably thicker, not quite adult thickness					Snout adult-like. HBR 1:4
60	Coat very similar to adult length/thickness/tint					

found from searching PubMed and Google Scholar databases, with the search terms "neurodevelopmental feature" + "species name." In addition, we used empirical data points that were included in the Translating Time database http://translatingtime.org/). The overall dataset consists of regression models that were built from hundreds of data points for the timing of neurodevelopmental events across 10 mammalian species. These data allow the inference of relative timing of some unobserved features, based on temporal bracketing of features with established timelines (see [16–19] for details of the methods used). All the data points used here were based on empirical observations, not model inferred points.

Comparative Developmental Timeline

We compiled the data obtained from the literature into a spreadsheet (a csv file) and subsequently visualized the timeline using RStudio. The order of the features is sorted by chronology for the features in short-tailed opossums.

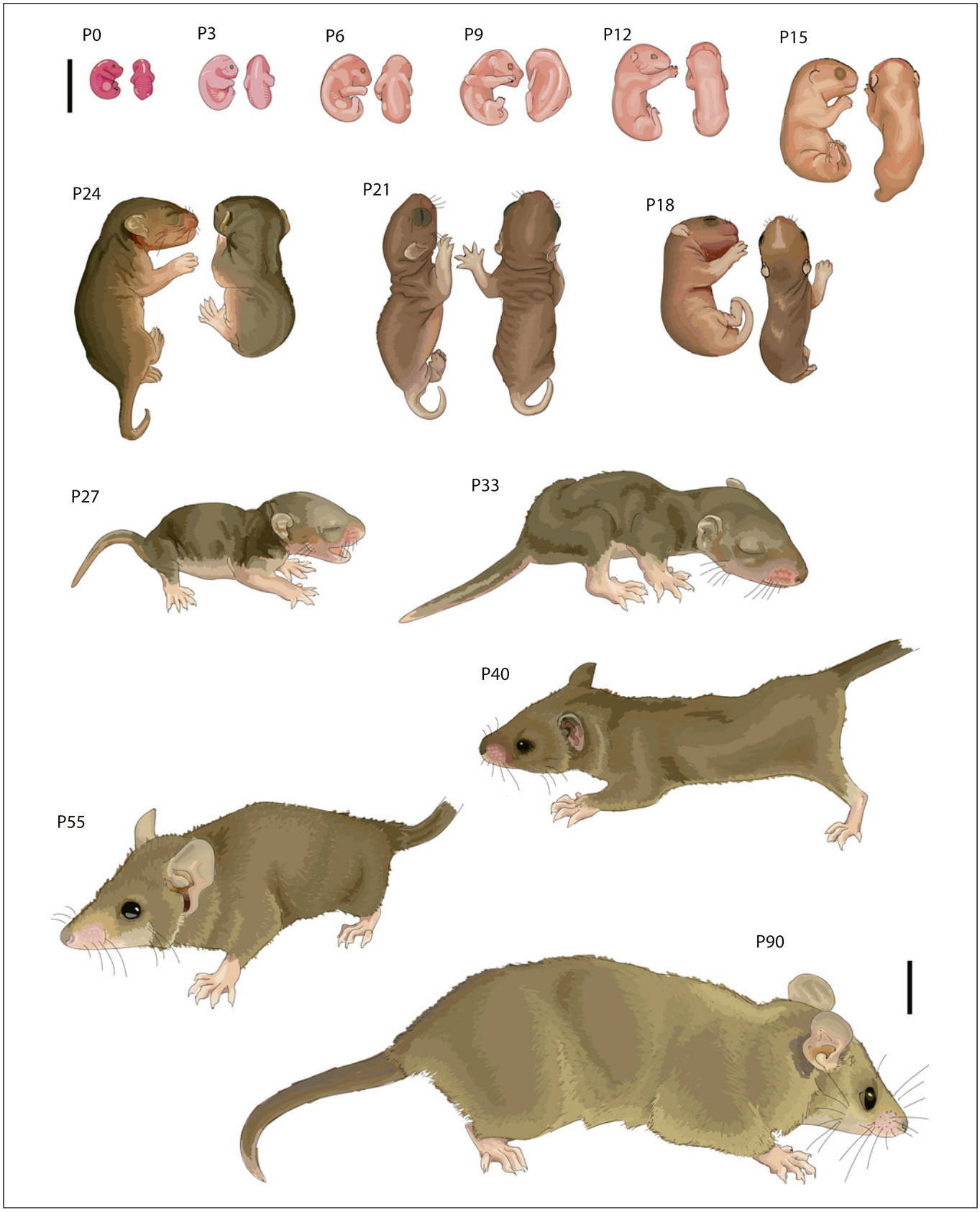
Statistical Models

All statistics were carried out in MATLAB. Only data points with values for all three species were included in analyses. Data for all species were corrected for nonlinearity via log transformation, and improved fit was assessed via significantly lower Akaike Information Criterion. Potential developmental heterochronies were identified by calculating Cook's Distance (CD) for each point for rat and mouse relative to opossum.

Results

Short-tailed opossum postnatal development can be divided into four stages: obligately attached (P0–P14), detach-relatch (P15–P54), independent subadult (P55–P90), and adult (>P90). Here, we first describe the entire developmental trajectory of these opossums, followed by a description of the two early developmental stages in detail, as these stages include the most drastic and numerous developmental changes.

We observed developing short-tailed opossum pups housed in our breeding colony. The rearing conditions are described in Figure 2. As can be seen in Figure 2c, female short-tailed opossums do not have a pouch but instead have a field of nipples on the abdomen. Nulliparous females do not have visually identifiable nipples on their ventral surface, but nipples develop during the first pregnancy (Fig. 1c). Rousmaniere et al. [20] report that short-tailed opossums have an average of 11.4 ± 2.19 (N = 68) embryos. Unlike many other marsupials, which produce far more embryos than there are nipples, short-tailed opossums have the potential to nurse up to 13



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young. However, the average litter size at weaning is 6.92 ± 3.87 (N = 72) [20], and these numbers are in line with observations from our own colony. As the embryos reach full term the fur between the dam's teats thins, allowing a clearer field for the newborn pups to navigate and find a nipple, which is likely achieved via tactile and/or thermal cues [21–23]. Because short-tailed opossums lack a pouch, pups are much more visible and accessible than in pouched marsupials. However, young pups tend to be cupped close to the female's body in a stance similar to the posture of a nursing rodent standing over a nest, so they can be difficult to visually identify. As pups grow in size, and begin to detach from the teat for short periods of time, they may cling to the dam's belly and flanks, as illustrated in Figure 2a. Females retain relatively prominent nipples after pregnancy, so one can distinguish nulliparous versus primi/multiparous females by this trait [24].

Many distinct morphological changes are observed throughout development, and these are illustrated in Figure 3. In this figure, pups are illustrated to scale, at each timepoint at which distinct morphological changes are observable. Table 2 lists 10 features that can be observed to change at particular timepoints. The most obvious changes include overall size (from ~8 mm at birth to ~12 cm nose-to-rump at adulthood), and skin and coat color (from a deep reddish-pink at birth, through pink, dark gray, and finally yellowish-agouti at adulthood). Significant changes are also observable in the major sensory accessory structures: eyes, pinnae, whiskers, rhinarium, mouth, and skin (all highlighted in subsequent figures).

In the early, obligately attached phase of development, pups are completely dependent on the mother and if a pup is removed from the mother it cannot relatch and will not survive. This phase of development roughly resembles the final week of mouse development, with the significant exception of the forelimbs and oral area. The general body shape is smoothly curved along the rostrocaudal axis, with a slight distinction between the head and neck, almost no distinction between the shoulders and trunk, and no distinction between the trunk and hips. Hindlimbs are barely more than undifferentiated paddles [25] and are qualitatively comparable to an E12–14 mouse embryo [26, 27]. However, because the limbs are small and held under the curled body, they are unlikely to be visibly observable without significant manipulation of the pup. The forelimbs, however, are roughly twice as large as the hindlimbs, and the digits of the forelimbs are unfused and tipped with claws (Fig. 4 inset), similarly to those of an E16–17 mouse [27, 28].

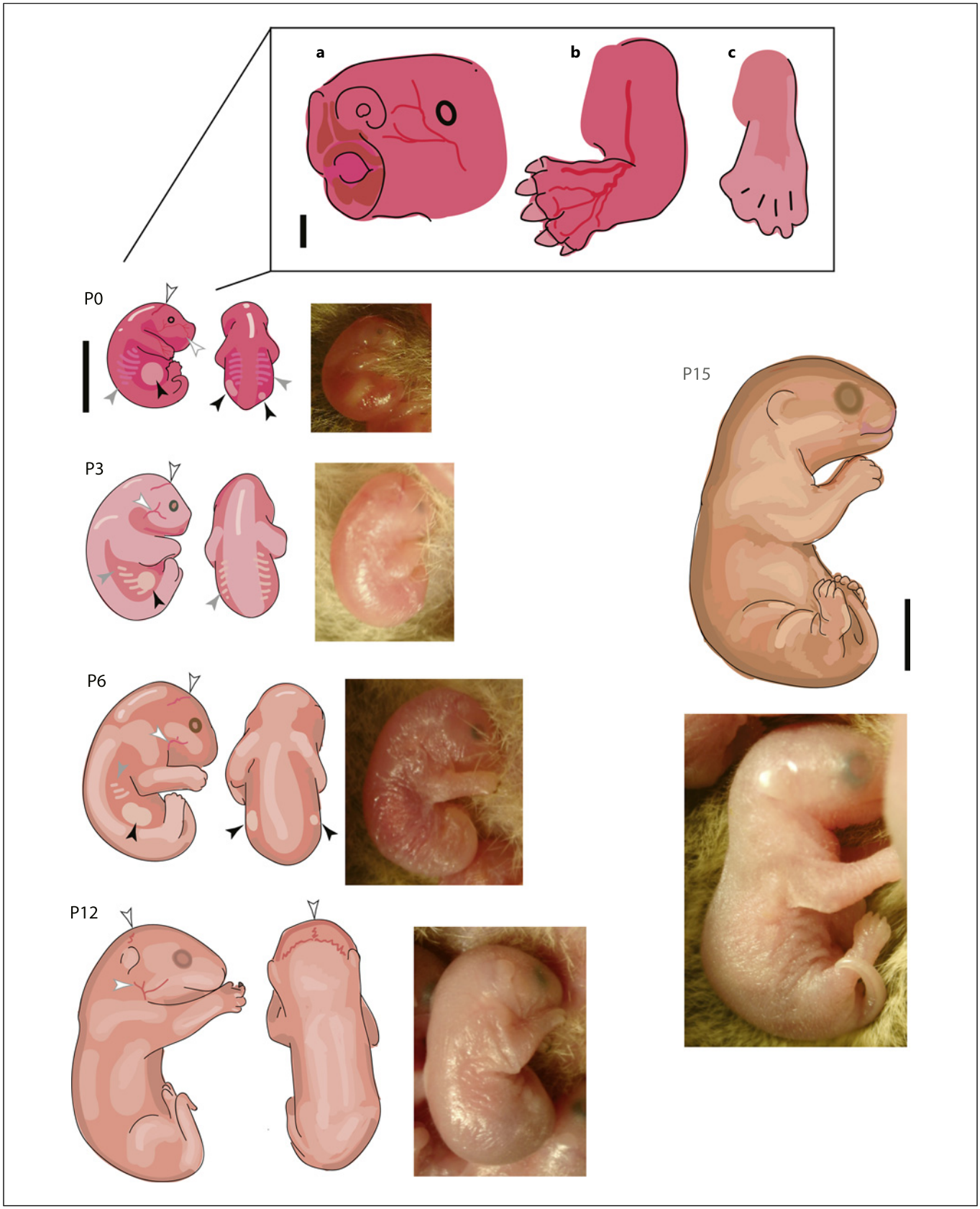
Much of the head is also similar to a mouse embryo in the last week of development, with the exception of the flat and ossified "oral shield," a structure adapted to facilitate latching to the teat [29, 30]. Like E18 mice [28] or E19 rats [31], short-tailed opossum pups are born without pinnae, and with no visible distinctions between upper and lower lips laterally.

The minute size of the neonates makes visible observation of behavior difficult, but not impossible. The posture of all pups is distinctly curled, again similar to many embryonic tetrapods well before birth. Pups physically cannot uncurl at this stage, as the musculature of the trunk and hindlimbs is not mature enough to produce movement. In general, the sensorimotor capabilities of newborns are limited, but particular modalities and motor abilities are precocial compared to placentals of an equivalent age. Specifically, the forelimb musculature and cervical spinal cord are much more developed compared to the hindlimbs and lumbar cord [32]. This accelerated cervical cord and forelimb development, combined with (potentially) functioning olfaction [33], the comparatively mature receptive fields of Merkel cells, and thermoreceptors in the snout, allow the senses of mechanical touch, temperature, and smell to influence rhythmic alternating forelimb movement [22, 23].

The skin of neonatal pups is visually distinctive compared to later ages. Pups are dark pink and red, with shiny, and almost transparent, very thin skin. This delicate skin means that bones, vasculature, and some internal organs are plainly visible. As can be seen in Figure 4, p0, a "milk spot" (milk in the stomach) is prominently visible laterally, and individual ribs are visible dorsally. These features are also visible at later stages as well but are particularly clear in neonates. The retinas are visible as a highly contrasting dark gray torus on each side of the head that, on P0 only, have a crisp border.

As postnatal development progresses, after P0, the skin pales and takes on a slight yellow tint, perhaps due to

Fig. 3. Illustrations highlighting visibly observable morphological changes in short-tailed opossums from birth to adulthood. All images are to scale, with scale bars representing 1 cm. The major features highlighted here include: overall size, whisker development, eye opening, pinnae growth, head-to-body ratio, skin texture (shiny at <p12, or matte at >p12), integument (translucent skin at <P15, increasingly pigmented skin at >P15, and significant fur at >P24), and color (from red at P0, to pink at <P15, to dark gray dorsal skin at >P24, and finally to increasingly yellow and lighter agouti as fur becomes progressively longer and thicker from P27 through P90).



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subcutaneous fat. As the skin starts to thicken, it becomes lighter pink, as can be seen in Figure 4, P3–P12. Due to this thickening, the ribs are increasingly less visible dorsally, the milk spot becomes less distinct, and the border of the retina becomes more blurred. By P6 pinnae begin to bud as very slight bumps on either side of the head. The hindlimbs are still nonfunctional and paddle-like, but the individual digits become more distinct. A clear distinction between the shoulder girdle and trunk begins to emerge, but the hips and hind legs are still not easily distinguished from the trunk. Starting at P9 and continuing through P12 the toes on the hindlimbs separate, and at P12 a knee becomes visible. Pinnae now appear as flaps, but fold rostrally, rather than their eventual caudal direction. Also, at P12 the first visible division between upper and lower lips can be seen laterally. Between P12 and P15 the pinnae reverse direction and point caudally. Finally, by P15, a full distinct hindlimb is visible, with well-defined toes, though they are still proportionately much shorter than in adults. At this stage, pups make well-coordinated rhythmic movements with the forelimbs, but although the hindlimbs move, the movements are not coordinated in a locomotor-like pattern. If detached, the pup may use its forelimbs, but not hindlimbs, to move in a goal-directed manner, though pups are still uncoordinated enough to be very poor at self-righting if placed on their backs.

After P18, pups can detach from the mother and relatch successfully. At this age, pups are very poor at locomoting independently, so are particularly vulnerable to temperature fluctuations if they detach out of the nest, similar to a P3 mouse pup. At first locomotion is almost exclusively accomplished by the forelimbs, with the hindlimbs being dragged behind. The hindlimbs begin to be used to support the weight of the body around the 4th week (P27), at the same time that fur begins to noticeably thicken, as shown in Figure 5. The four limbs are not well coordinated at this stage, potentially reflecting an immature propriospinal network [32]. Between P21 and P30, sensorimotor reflexes reflecting maturation of parts of the locomotor network, such as withdrawal and crossed extension, can be observed, and by P40 locomotion is adult-like.

During the entire detach-relatch phase the pups' eyes gradually open, as illustrated in Figure 3 and photographically documented in Figure 5. The exact timing of eye opening can vary but generally begins with a horizontal groove demarcating the division between the upper and lower eyelids appearing at P18. The lids may begin to separate around P31, but even in the same litter some individuals' eyes may not begin opening until P34. The distance between upper and lower lid then gradually widens until the eye appears fully round and adult-like circa P55. The external ears and nose also change significantly between P18 and 40. Between P18 and 21 the pinnae separate from the sides of the head. At first, they are pink, and proportionately very thick compared to those of the adult, but gradually become thinner and acquire pigmentation, until they appear adult-like at P40. The front of the nose begins as a very short and flat structure, and gradually elongates and becomes pointed until it also appears more adult-like at P40. Opossums are weaned at P50, but after P40 the most prominent morphological change is growth in size and reduction in head-to-body ratio, until the pup reaches adult proportions at P90 (see Fig. 3).

Compared to mice and rats, short-tailed opossums are born much earlier, with many neurological structures and connections developing after birth (Fig. 6, online suppl. Table S1; for all online suppl. material, see https://doi.org/10.1159/000538524). Although opossums are born at post-conception day (PCD) 14, their nervous systems are in some ways similar to that of an PCD10–PCD14 mouse and rat. For example, at half a day after birth opossum retinal ganglion cell neurogenesis is just beginning, while in mice these cells are born around PCD10, and in rats RGC neurogenesis occurs between PCD 11 and 12. Some features of a ~PCD10–14 mouse/rat brain do not mature until even later in opossums, such as axons from retinal ganglion cells reaching the optic chiasm on PCD 18 (postnatal day 4), while this occurs on PCD13 and 15 in mice and rats respectively. Taken together, the data in Figure 6 show variation in maturation timing between short-tailed opossums and mice and rats such that the later the developmental event is, the more it is delayed compared to these rodents.

Given this variation, we analyzed these data for possible statistical differences in overall timing, and possible heterochronies between opossums compared to rats and mice. Figure 6b–d shows the results of two regression

Fig. 4. Short-tailed opossum development while obligately latched to the teat. Filled black arrows: milk visible in the stomach. Filled gray arrows: ribs visible through skin. Open black arrows: skull sutures visible through skin. Open gray arrows: vasculature visible through skin. Eyes are covered by a thin layer of skin, with no obvious division between upper and lower lids. Pinnae begin as small bumps obvious at P6 and become flaps of skin at P12. Ears initially point rostrally at P12, but soon uncurl and point caudally by P15. All photographs and illustrations are to scale, with scale bars representing 5 mm.

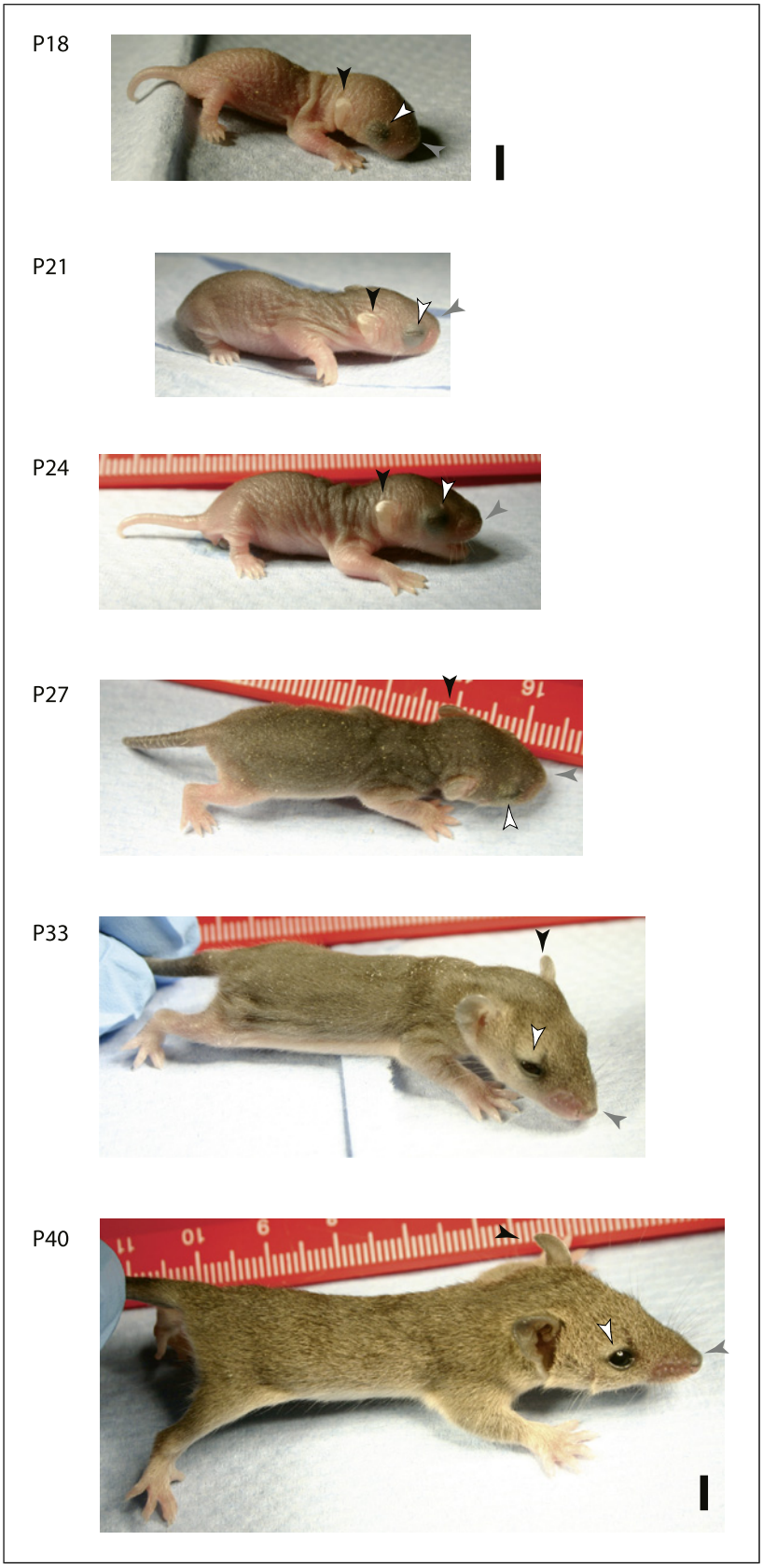
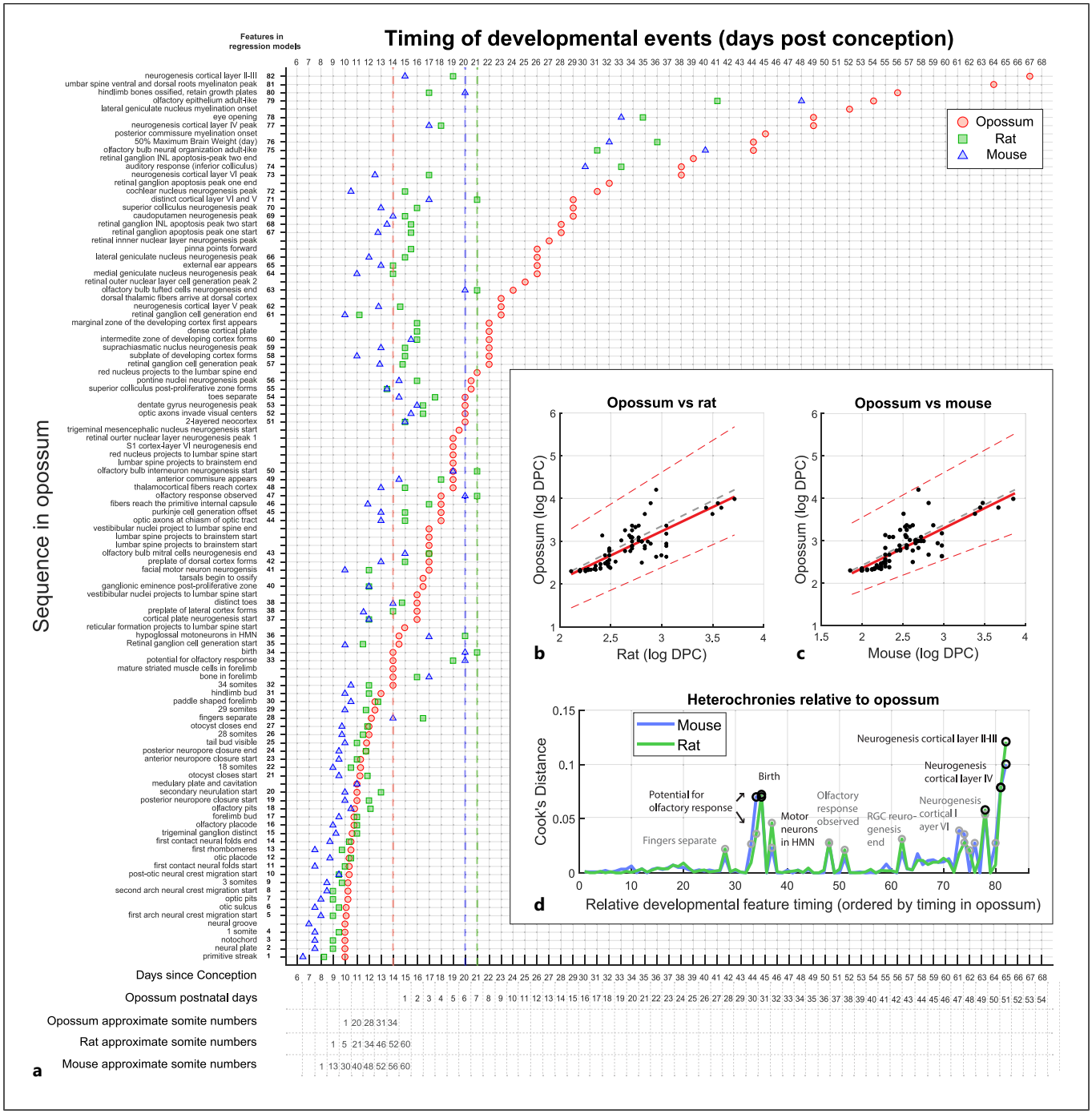


Fig. 5. Short-tailed opossum development in the detach-relatch phase. Black arrows: pinnae are initially thick immobile flaps barely separated from the skin of the head, and gradually elongate, become proportionately thinner, and acquire pigmentation. At P40 pinnae appear adult-like. White arrows: eyes are initially closed, with the first obvious division between upper and lower lids appearing at P18, and the eyelids beginning to separate at P32, but may not open fully until P55. Gray arrows: The nose is initially short and wide, and gradually elongates, with a noticeably more adult-like appearance at P33. All photographs are to scale, with scale bars representing 5 mm.



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models contrasting the days post conceptions (DPCs) for the timing of events in the opossum with the same events in rat (B) and mouse (C). In general, development proceeds very similarly between the opossums and the two rodent species, as indicated by the closeness of the fit and identity lines, and the fact that all points lie within the 95% confidence bounds. However, the different reproductive strategies of marsupials and placentals may impose distinct selective pressures on certain structures, and result in some differences in timing (Fig. 6d). While none of the points in the current dataset are influential in the statistical sense (all CD values are well below 0.5), we considered a CD of 0.02 or greater to be a potential heterochrony, based on qualitative assessment of the distribution of CD values.

Discussion

The data presented here are important for several reasons. First, on a practical note, while there have been other guides for the laboratory care of short-tailed opossum colonies [1, 20, 82], we believe that the detailed diagrams included here will complement these previous publications, as staging of pups helps maintain breeding colonies by aiding in planning and maintenance of the colony.

Second, these data are important for understanding similarities and differences in developmental programming in mammals. These results indicate that many neurodevelopmental events happen later in opossums, even though they are born earlier than most eutherians. Importantly, "earlier" here refers to both fewer gestational days, as well as being relatively more physically immature at birth. While this is well-established, to our knowledge no other study has compiled as extensive and detailed a comparative dataset for opossums, rats, and mice, showing the exact timing of developmental events. The finding that developmental events are qualitatively progressively delayed in opossums compared to rats and mice (as seen in Fig. 6) replicates studies of Darlington et al. [19]. Essentially, the authors found that a curve describing the timing of metatherian development appeared to be a somewhat horizontally stretched version of that same curve describing eutherian development: early events happen earlier (accelerated early development of metatherians), while later events happen later (delayed later development of metatherians). However, the statistical results of the regression models in this study indicate that neural development between opossums and rats, and opossums and mice, is overall quite similar. This lack of statistically significant differences

Fig. 6. Opossum developmental timing compared to that of rat and mouse highlights developmental heterochronies in forelimb, olfactory, retinal, and cortical development. **a** The order of features on the vertical axis is based on their relative timing of maturation in short-tailed opossums (red circles), which is shown alongside the rat (green squares), and mouse (blue triangles). Numbers on the **a** *y*-axis ("Features in regression models") represent the order of developmental events for which data for all 3 species was found (also reflect the order and names of the features referenced in the *x*-axis of **d**). The horizontal axis shows the number of days since conception, opossum postnatal days, and approximate number of somites. Dashed vertical lines represent the average PCD at which each species is born. Days since conception apply to all 3 species. This figure is intended to be used as a rough reference for making comparisons between timing of developmental features, and to help guide future experiments using short-tailed opossums within the large existing literature on the neurodevelopment of mice and rats. It should be noted that for all three species, feature maturation timing can vary considerably even between embryos in the same litter, so the times listed above should be taken as a reflection of the average developmental trajectory, not a strict prediction for any individual. Inset: regression models for opossum developmental feature maturation versus rat (**a**) and mouse (**b**), in log transformed days post conception (DPC). The red solid line represents the regression model, while the red dashed lines show 95% confidence intervals. The gray dashed line shows y = x, representing a theoretical identical relationship. The equation for rat is $\ln[y] = (1.1296*\ln[x])-0.1528$, $R^2 = 0.66$, $F(1, 75) = 143$, $p < 0.001$, 95% confidence interval: (0.7671, 1.1182). The equation for mouse is $\ln[y] = (0.9427*\ln[x])+0.4716$, $R^2 = 0.60$, $F(1, 75) = 114$, $p < 0.001$, 95% confidence interval: (0.9415, 1.3177). Bottom row (**c**) shows the degree to which data points for the rat (green) and mouse (blue) deviate from those predicted by the regression models (calculated as Cook's Distance [CD]), plotted in order of feature maturation in opossum (the numbers on the *y*-axis of **a** are the *x*-axis of **d**). Black circles highlight values greater than 0.05, while gray circles highlight values greater than 0.02. In the interest of visual clarity, some points are not labeled. These points, in order, are olfactory bulb interneuron neurogenesis start (50); auditory response (74); olfactory bulb neural organization adult-like (75); olfactory epithelium cellular and glandular composition adult-like (79); hindlimb bones ossified, retain growth plates (80). High values that appear before point 50 are generally accelerated in opossum, while high value points after point 50 largely represent delays in opossum maturation (note that this sign change does not apply to all points, but only to the points highlighted. Refer to panel A or table S1 for specific timing differences). Data sourced from the translating time database, as well as others: [16–19, 26, 28, 30–81]. A detailed list of the references that pertain to each feature, timepoint, and species can be found in online supplementary Table S1, as well as more detailed somite numbers, and developmental staging (Theiler staging for mouse, Witchi staging for rat, and McCrady staging for opossum). DPC, days post conception; HMN, hypoglossal motor nucleus; RGC, retinal ganglion cells.

is unexpected given the results of previous studies. Our current study did not find statistically significant differences between the species, but we did find a qualitatively similar curvilinear relationship in opossums compared to mice and rats (Fig. 6). We speculate that potential larger differences between other metatherian species and placentals may be enough to produce the statistically significant differences between metatherians and eutherians in general seen in Darlington et al. [19]. Ultimately, however, the similarity in developmental progression between the species found in the current study represents yet another advantage of the opossum as a model species that this paper aims to highlight.

However, not all events follow a smooth progression of timing. Many of the features that we found to differ the most between opossums and rats and mice were either clustered around the day of birth and were features that have functional importance for the pup immediately after or during birth, or were features that have reduced functional importance for the pup until later in postnatal development compared to rats and mice (given the extended period of relative physical inactivity of the pup while attached to the teat). For example, precocial development of digits on the forelimbs, and motor control of the tongue, facilitates locomotion from the cloaca to the nipple and latching onto the nipple, respectively. At the same time, delaying the maturation of metabolically costly structures such as the neocortex and retina until they are necessary (closer to the time when pups detach from the mother) may free up energy resources for other needs.

One unexpected and seemingly paradoxical result is that, while some functioning of the olfactory system may be accelerated in opossums (the potential for olfactory response occurs at birth, 5–6 days earlier than in mice and rats [33]), other experiments attempting to elicit changes in behavior of neonatal opossum by electrically stimulating the olfactory bulb essentially resulted in no effect [21]. Some features of the opossum olfactory system are very delayed compared to mice and rats, such as olfactory bulb neural organization and olfactory epithelium maturation; both become fully mature and adult-like 6–13 days in opossum [34–37], well after birth). One potential solution to this paradox could be that the opossum olfactory system may simply be partially functional at birth. Ultimately, this seeming contradiction highlights the opossum olfactory system as potential fertile ground for further research into developmental heterochrony.

Because neurodevelopmental differences are key to generating much of the diversity evident in the brains and bodies of mammals [83], opossums can be a powerful animal model for studying the diversity of extant mammals. Also, because short-tailed opossums are considered to be a relatively evolutionarily conserved mammal that may qualitatively resemble a basal eutherian in both brain and body morphology (for review and caveats, see [84, 85]), studying this species can give unique insights into mammalian evolution (e.g., [13, 14, 86–88]).

Statement of Ethics

All experimental protocols and animal husbandry were reviewed and approved by the UC Davis IACUC, under protocol number 22167.

Conflict of Interest Statement

The authors have no conflicts of interest to declare.

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Author Contributions

C.B. analyzed the data, conducted literature reviews, generated illustrations, generated tables, collaborated on database organization, collaborated on data visualization, and wrote and edited the manuscript. J.L.C. conducted literature reviews, collaborated on database organization, collaborated on data visualization, and edited the manuscript. C.J.C. oversaw animal husbandry and all data collection and edited the manuscript. L.A.K. conceived of the research, consulted on figures, and edited the manuscript.

Data Availability Statement

Data for Figure 6 are available as a supplemental table. Photographs from which observations were made can be made available upon request to L.A. Krubitzer and/or C. Bresee, as the large number of high-resolution image files is unwieldy to continually host on a file server. Further inquiries can be directed to L.A. Krubitzer and/or C. Bresee.

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