Experimental Biology 203, 1733–1740 (2000)
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JEB3669

PHYSIOLOGICAL VARIABILITY IN NEONATAL ARMADILLO QUADRUPLETS: WITHIN- AND BETWEEN-LITTER DIFFERENCES

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Accepted 13 March; published on WWW 10 May 2000

Summary

The role of genetics on neonatal physiological variability was examined in the nine-banded armadillo (*Dasypus novemcinctus*). Since armadillos give birth to only monozygous quadruplets, the genetic variation within litters is essentially zero. Quadruplets born in captivity were isolated and weighed within 8 h of birth. Oxygen consumption ($\dot{V}_{O_2}$) was measured in resting neonates by flow-through respirometry, heart rate obtained from an electrocardiogram and ventilation was measured by impedance techniques. Following the measurements, neonates were returned to the mother. Measurements were repeated at 4 and 8 days after birth. Mean heart rate significantly increased from 132 beats min^{-1} on the day of birth to 169 beats min^{-1} on day 8. Mean ventilation rate significantly decreased from 81 breaths min^{-1} on the day of birth to 54 breaths min^{-1} on day 8. During this same developmental period, mean mass significantly increased from 100 g to 129 g, and mean mass-specific oxygen consumption significantly decreased from 32.2 ml O_2 kg^{-1} min^{-1} to 28.6 ml O_2 kg^{-1} min^{-1}. For all variables measured, within-litter variability was always significantly less than between-litter variability, confirming a 'sibling effect' that we attribute to the genetic components determining physiological characters.

Key words: armadillo, quadruplet, *Dasypus novemcinctus*, metabolic rate, physiological variability, heart rate

Introduction

Most of our knowledge of animal development emerges either from the long-established field of embryology, which emphasizes morphological changes, or from more modern cellular and molecular approaches focusing on the mechanistic underpinnings of development. Far less is known about the development of physiological processes during the formative stages of an animal's life cycle. New technologies and experimental approaches for studying physiological processes in very small animals (see Burggren and Fritsche, 1995; Paul et al., 1997; Pirow et al., 1998; Burggren, 1998), have facilitated interest in developmental physiology in the past decade, particularly of the cardiovascular and respiratory systems (see Burggren and Pinder, 1991; Rombough, 1988; numerous chapters in Burggren and Keller, 1997).

Developmental studies at the biochemical, morphological or physiological level commonly attempt to answer the question 'What processes control and direct the outcome of development, manifested as the adult phenotype?'. While genetic instructions provide the basic template for development, we know that the ultimate phenotype also depends on environmental influences (see Burggren, 1999, 2000). Recent explorations of developmental physiology in birds (Burggren et al., 1994), amphibians (Burggren et al., 1997) and reptiles (Crossley et al., 1997, 1998) have begun to separate the influences of genetics and environment using a variety of established and emerging animal models (see Burggren, 2000). Variation in heart rate and other physiological processes is usually significantly less within sibling groups compared to non-sibling groups, a so-called 'sibling' or 'litter' effect. These observations strongly suggest that genetic templates for a population sub-group (i.e. a specific clutch or litter) may include very explicit instructions for physiological change. While the influence of 'maternal effects' on development is acknowledged (see Bernardo, 1996), there appear to be clear predetermined physiological trajectories that embryos must follow. As an example, multiple clutches of cliff swallow eggs reared under identical environmental conditions exhibit sharp increases and decreases in heart rate at specific points during development (Tazawa et al., 1994). Although similar patterns of changes were demonstrated in other clutches, the specific timing of these changes was unique to each clutch. These findings are significant for the field of evolutionary biology, which has traditionally not looked towards physiological patterns for insight because of a prevailing view that physiology is too labile, and too responsive to environmental conditions, to provide useful

indicators for cladistic or other analyses (Burggren and Bemis, 1990).

The role of genetics in dictating subtle yet heritable physiological characteristics during development has been explored primarily by minimizing (but not eliminating) genetic variation through the use of closely related sibling groups. The use of clonal animals, in which genetic variation is eliminated, has yet to be exploited in determining the role of genes and environment in dictating physiological development. In this study, we used the nine-banded armadillo, *Dasypus novemcinctus*, as an animal model that allows us to eliminate genetic variation within litters. *D. novemcinctus* invariably produces litters of four genetically identical quadruplets (see Loughry et al., 1998, for a description of the interesting reproductive biology underlying this phenomenon). This trait provides an opportunity to evaluate the potential contribution of genetics to the precise regulation of physiological processes. In this study, we measured body mass, heart rate, ventilation rate and oxygen consumption during the first eight days of development in eight different litters of *D. novemcinctus*. Physiological variation was compared within and between litters as development proceeded. Our results show that the cardio-respiratory physiology of an individual more closely resembles that of its genetically identical siblings than of non-siblings, suggesting that specific patterns of heart rate and metabolism are heritable characters.

Materials and methods

Animals

Pregnant nine-banded armadillos *Dasypus novemcinctus* (Simpson, 1945) (N=8; postpartum mean mass=4.45±0.19 kg) were captured near Clarksville, Arkansas, USA, and adapted to a laboratory diet of moistened cat food. The armadillos were shipped to our laboratory and housed in intermediate-sized dog kennels. A temperature-controlled room was used to maintain all animals at 25 °C and 60 % relative humidity. Routine animal maintenance was performed according to Storrs (1987). Kennels were checked 4-5 times daily for neonates, and if present, they were carefully removed from the mother and taken temporarily to our laboratory for data collection.

Metabolic measurements

Neonates were weighed, identified via a toenail clip code, then placed individually in metabolic chambers constructed from PVC tubes (diameter 8 cm, length 24 cm, approximate volume 1200 ml). The chambers were sealed, and immediately ventilated with room air (30 °C, 60 % relative humidity) at a rate of 565 ml min^{-1} (measured by a flow meter connected to the chamber outflow). Each animal was given a 15 min acclimation period (minimum), during which visual observation confirmed that the neonates had frequently fallen asleep. Oxygen consumption ($\dot{V}_{O_2}$, expressed as ml O_2 kg^{-1} min^{-1}) was calculated from the gas flow through the respirometers, and the difference in the percentage of oxygen in inflowing and outflowing gas was measured with a Beckman OM11 oxygen meter. Three separate oxygen consumption measurements were taken on each neonate to provide a mean value for the individual. Measurements were only taken when a given neonate was observed to be sleeping.

Heart rate and ventilation measurements

Following metabolic measurements, each neonate was briefly removed from its respirometer and fitted subcutaneously with a single pair of electrodes constructed from 40-gauge insulated stainless steel wire, designed to measure heart rate and ventilation rate. Electrodes were inserted subcutaneously on the lateral flanks using a 26-gauge needle. Neonates were returned to the respirometers and again allowed to acclimate, typically until they were again observed to be sleeping. Heart rate was determined from an electrocardiagram (ECG) recorded from the electrodes by using a high-gain coupler connected to a Narco Physiograph. Following a recording of at least 5 min of a clear ECG, ventilation was recorded for a 5 min period by passing the electrode signal into a BioCom 2991 impedance converter (Morrow Bay, California, USA). These measurements were repeated three times on each neonate to obtain an individual mean value. Once all variables were recorded satisfactorily from all four neonates, the electrodes were removed and the neonates were carefully returned to the mother, who invariably accepted back her litter.

The same series of metabolic, heart rate and ventilatory measurements was performed on the neonates again 4 and 8 days after birth.

Maternal DNA characterization

Observations on armadillos in the wild suggest that armadillo siblings do not venture far from each other to establish a territory, therefore it was possible that any of the pregnant females we obtained were themselves siblings, and that some litters might be more closely related than others. To investigate this we performed DNA analyses on heart, liver and kidney tissues. Samples were extracted from each of the mothers killed by direct cardiac injection of 10 ml of Euthanol into the deeply anesthetised animal (anesthesia obtained by intraperitoneal injection of 50 mg ml^{-1} sodium pentobarbitol at a dosage of 75–100 mg kg^{-1}). We then applied known primer sets to PCR-amplify microsatellite loci on these tissues using a modified technique of Prodohl et al. (1996). The microsatellite loci were resolved on polyacrylamide gels and stained with ethidium bromide to visualize the bands. Bands were labeled from largest allele (slowest running, designated A) to smallest allele (fastest running).

Statistical analyses

Age effects, litter effects and age-by-litter interactions were determined with a two-way repeated measures analysis of variance (ANOVA). To characterize the dynamics of variation within litters during development, a one-way repeated measures ANOVA was performed on the within-litter

variance. *Post-hoc* tests were performed using the Student–Newman–Keuls method. The fiduciary level of significance for all tests was $P<0.05$. All values presented are means ± 1 S.E.M. All statistical tests were performed with SigmaStat (Jandel Scientific).

Results

Body mass

The mean body mass of newborn *D. novemcinctus* was 99.8±2.7 g (N=28), increasing to 105.0±3.3 g at day 4 and 128.6±3.7 g at day 8 (Fig. 1A). These body mass increases during the 8 day development period were highly significant (Table 1). In addition, individual body mass was significantly different between litters ($P<0.0001$), which highlighted the similarity of body mass within each litter (Fig. 1B,C).

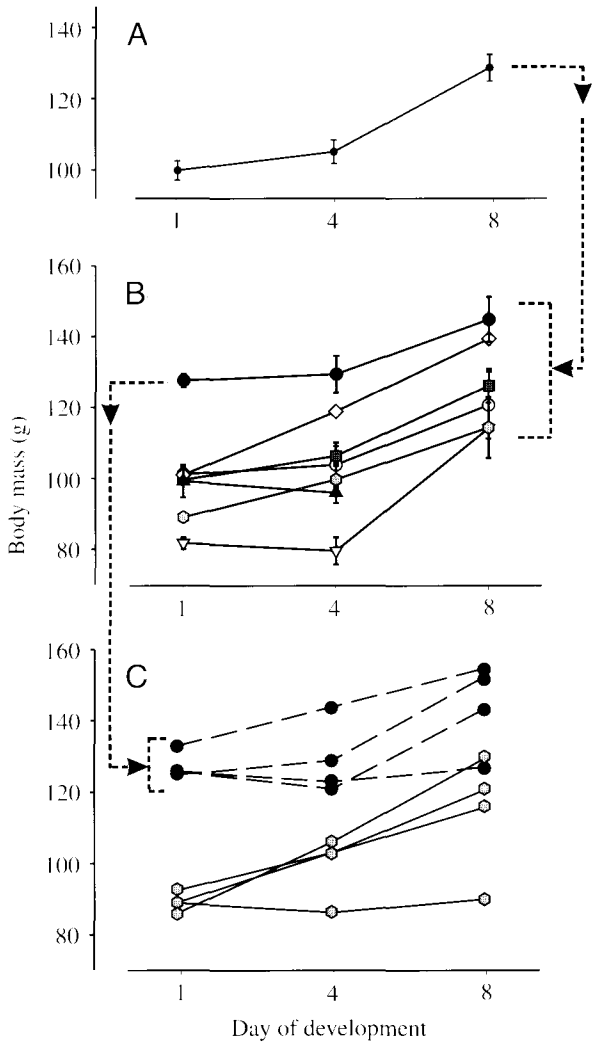

Fig. 1. Neonatal mass during development. (A) Each point represents the mean mass of all the neonates at a specific day of development (day 1, N=28; day 4, N=27; day 8, N=20). (B) Each symbol represents the mean mass of a specific litter of neonates followed through development. (C) Each line represents an individual neonate from the litter with the same symbol in B. Although the litters represented were chosen such that overlapping of symbols was reduced, they are typical results.

Metabolism

Oxygen consumption $\dot{V}_{O_2}$ at birth ranged from 22.7 to 50.0 ml O_2 kg^{-1} min^{-1}, with a mean of 32.3±1.1 ml O_2 kg^{-1} min^{-1} (N=28) (Fig. 2A). This did not change significantly at day 4, but by day 8, $\dot{V}_{O_2}$ was significantly lower (28.6±0.6 ml O_2 kg^{-1} min^{-1}) ($P<0.05$). Neonatal metabolic rate was significantly different between litters ($P<0.0001$), but remained very similar within each litter (Fig. 2B,C, Table 1). In addition, there was a significant interaction between litter and age when different litters were compared (Table 1).

Heart rate and ventilation rate

Fig. 3 shows a typical heart rate and ventilation rate tracing obtained at rest, while Fig. 4 shows heart rate changes during arousal. Heart rate is quite stable during rest, becoming very erratic when the animal wakes up or is manually aroused. Heart rate during sleep at birth ranged from 100 to 149 beats per minute (mean=132±2 beats min^{-1}, N=28) (Fig. 5A). Mean heart rate increased significantly to 150±8 beats min^{-1} at day 4 and 169±5 beats min^{-1} at day 8 ($P<0.05$). Heart rate was significantly different between litters ($P<0.01$) but was very similar within litters (Table 1, Fig. 5B,C).

Ventilation rate at birth ranged from 24 to 121 breaths per minute (mean=81±5, N=28) (Fig. 6A). This did not change significantly at day 4, but by day 8 ventilation rate was significantly lower (54±5 breaths min^{-1}) ($P<0.05$). Neonatal ventilation rate also showed a significant litter effect ($P<0.01$) (Fig. 6B,C). In addition, there was an interaction between litter and age in neonatal ventilation rate (Table 1).

Within- versus between-litter variability

Although the variation between litters remained fairly constant throughout the 8 days of development, the variation within litters tended to increase throughout development. This

Table 1. *ANOVA results showing* P *values of heart rate, ventilation rate, mass and metabolic rate for age affects, litter effects and litter-by-age effects*

	P values for effects tested			
Variable	Age	Between litter	Between litter-age interaction	Within litter-age interaction
Mass	<0.0001	<0.0001	<0.01	<0.01
Metabolic rate	<0.0001	<0.0001	<0.0001	NS
Heart rate	<0.01	<0.01	NS	NS
Ventilation rate	<0.01	<0.01	<0.05	NS

Values are taken from Figs 1, 2, 4–6.
Metabolic rate = oxygen consumption.
NS, not significant.
N = 20–28 neonates.

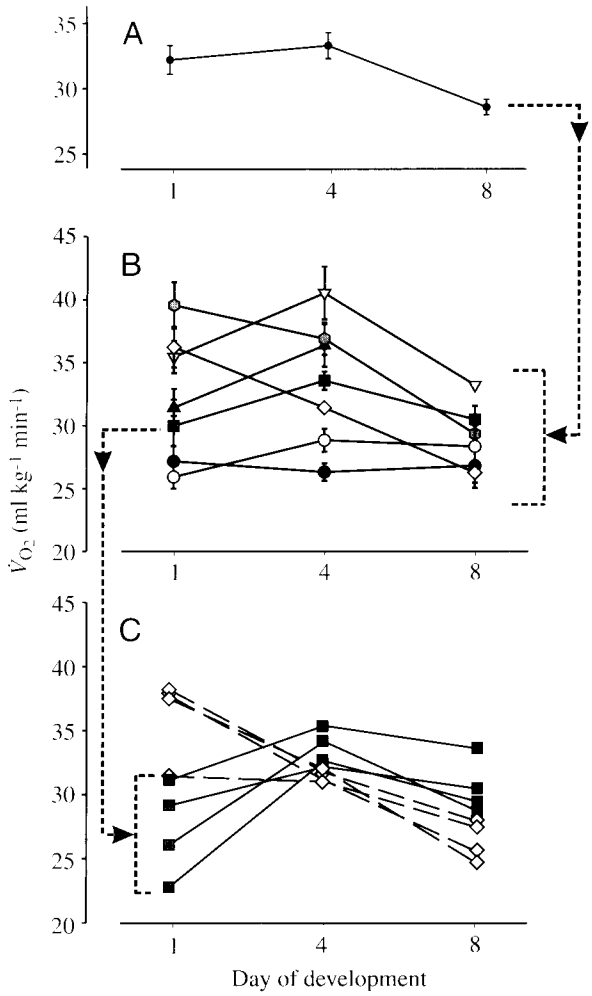

Fig. 2. Neonatal oxygen consumption ($\dot{V}_{O_2}$) measured in ml O_2 kg^{-1} min^{-1}. (A) Each symbol represents the mean of all neonates during a specific day of development (day 1, N=28; day 4, N=27; day 8, N=20). (B) Each symbol represents the mean of a specific litter of neonates followed through development. (C) Each line represents an individual neonate from the litter with the same symbol in B. Although the litters represented were chosen such that overlapping of symbols was reduced, they are typical results.

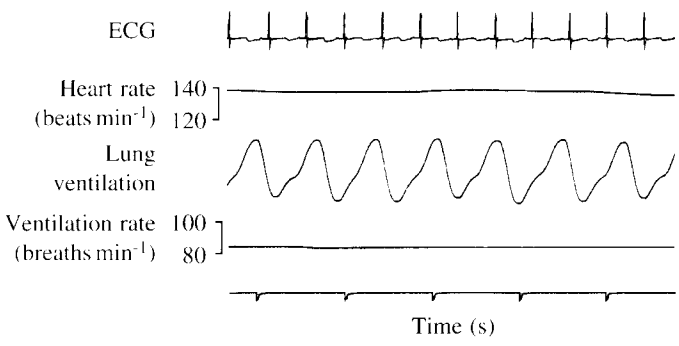

Fig. 3. Typical physiograph tracing of ECG, heart rate (beats min^{-1}), lung ventilation and ventilation rate (breaths min^{-1}) in a neonatal armadillo at rest.

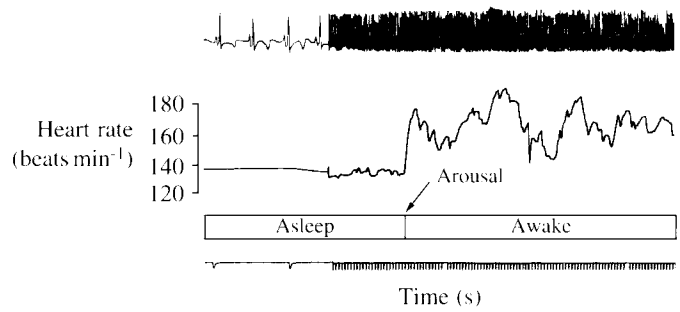

Fig. 4. Physiograph tracing showing ECG and heart rate (beats min^{-1}) at rest and during a period of arousal in a neonatal armadillo.

Table 2. *Microsatellite analysis of DNA maternal armadillo heart tissue*

Primer number	Female armadillo number 1	2	3	4	5	6	7	8
1	AD	BC	AC	AB	CC	AD	AC	AC
6	AF	DD	AF	BD	DD	DE	FF	DD
24	AB	AB	CC	AB	AB	BB	BB	AC

Known primer sets were applied to PCR-amplify microsatellite loci on maternal armadillo heart tissue using a modified technique of Prodohl et al. (1996). The microsatellite loci were resolved on polyacrylamide gels and stained with ethidium bromide to visualize the bands. Bands were labeled from largest allele (slowest running, A) to smallest allele (fastest running, F). The most informative three primer sets showing the different alleles of each armadillo are reported. A close relationship between females would be established if the profiles for all three primer sets matched.

increase, however, was only significant for neonatal mass (P=0.0118). To establish the extent to which the adult females were genetically related or identical siblings, DNA analysis was performed. DNA microsatellite analysis of the adult female armadillos showed in fact that none of these animals were identical siblings (Table 2).

Discussion

The nine-banded armadillo *D. novemcinctus* is the only species in the order Xenarthra that has invaded North America. Therefore, in addition to systematics, there are many other characteristics that make the armadillo a unique mammal in its north American habitat. Its body morph, feeding habits and the phenomenon of polyembryony, are all very atypical of other North American mammals. Since this is the first study to document physiological measurements of neonatal armadillos, it is of interest to compare our findings to those from other well-characterized mammalian species.

Neonatal physiology of armadillos

The length of gestation in mammals is, in general, positively

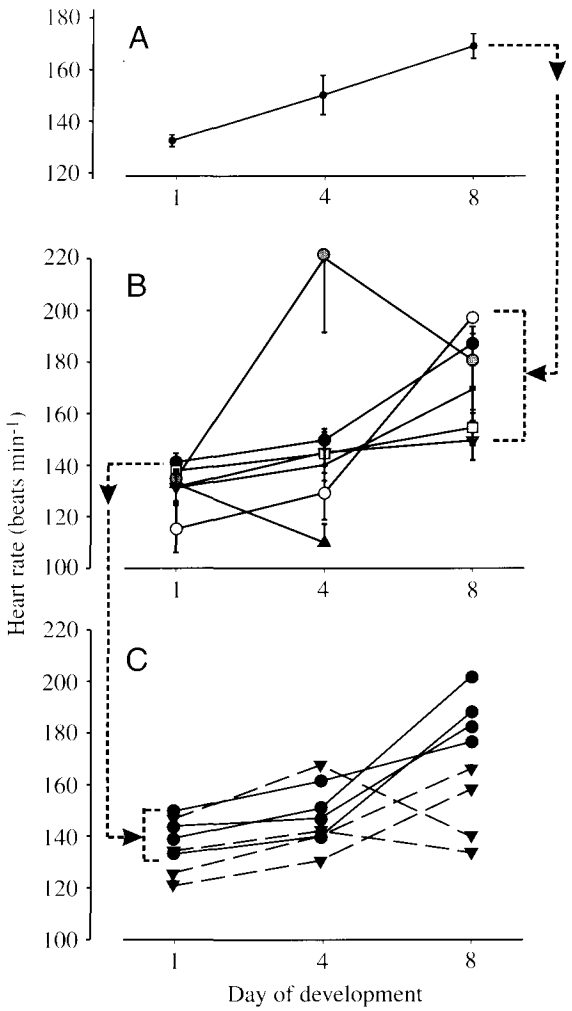

Fig. 5. Neonatal heart rate (beats min^{-1}). (A) Each point represents the mean of all neonates during a specific day of development. (day 1, N=28; day 4, N=27; day 8, N=20). (B) Each symbol represents the mean of a specific litter of neonates followed through development. (C) Each line represents an individual neonate from the litter with the same symbol in B. Although the litters represented were chosen such that overlapping of symbols was reduced, they are typical results.

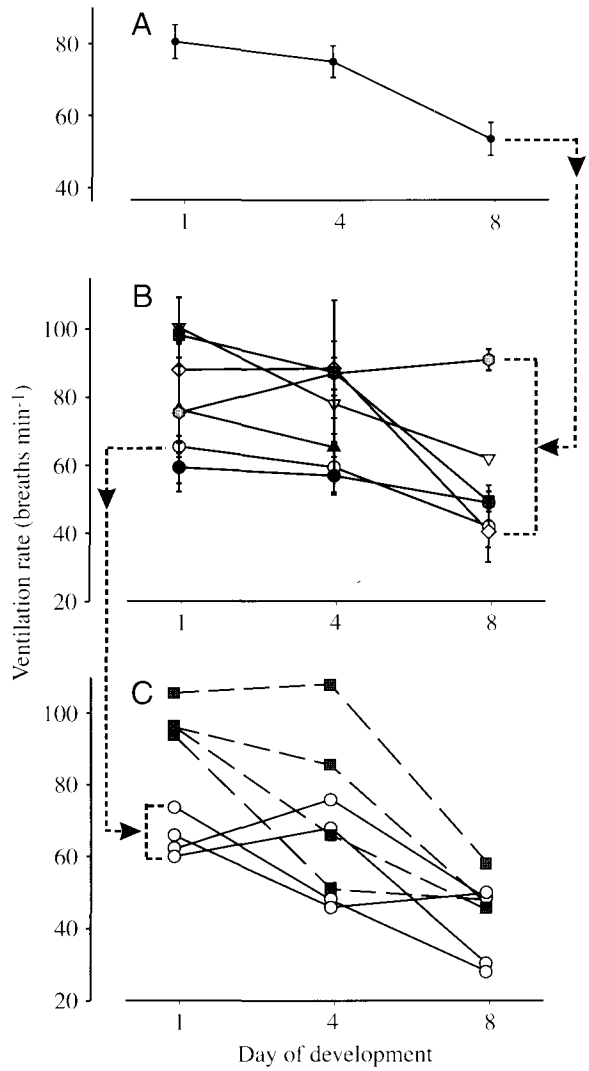

Fig. 6. Neonatal ventilation rate (breaths min^{-1}). (A) Each symbol represents the mean of all neonates during a specific day of development. (day 1, N=28; day 4, N=27; day 8, N=20). (B) Each point represents the mean of a specific litter of neonates followed through development. (C) Each line represents an individual neonate from the litter with the same symbol in B. Although the litters represented were chosen such that overlapping of symbols was reduced, they are typical results.

correlated with body mass as well as the degree of maturation of the newborn (Hugget and Widdas, 1951). However, the Xenarthra are an exception to this rule. Species in this order exhibit a long gestation period without a uniform progression toward longer gestation with increasing body size (Eisenburg, 1981). This is illustrated in the nine-banded armadillo, which has a gestation period of 120 days, considerably longer than other mammals in the 4 kg range.

As a broad rule, small mammals have higher growth rates than do large mammals, attaining adult mass at an earlier age (Hugget and Widdas, 1951). However, postnatal growth rates reflect many other factors including the life history of the animal. The nearly 30% increase in body mass exhibited by armadillos in this study during an 8-day neonatal period is slower than the growth rate of similar-sized rabbits, which can almost double their mass in 7 days (Spencer et al., 1985). However, since this growth rate is similar to that of other members of Xenarthra, the animals in this study were considered to be demonstrating normal, healthy development (Eisenberg, 1981). It is important to emphasize that in other mammals, low growth rates are associated with low metabolic rates, which may be the case for armadillos (Eisenberg, 1981).

At birth, the mean metabolic rate for the nine-banded armadillo was 32.2±1.1 ml O_2 kg^{-1} min^{-1}. This was higher than for mammals of similar neonatal mass such as the rabbit (20 ml O_2 kg^{-1} min^{-1}; Spencer et al., 1985), guinea pig (18 ml O_2 kg^{-1} min^{-1}; Towell et al., 1970) and domestic pig (13.4 ml O_2 kg^{-1} min^{-1}; Le Dividich and Noblet, 1984). This may be explained by the need for neonatal armadillos to use

shivering thermogenesis, which was observed quite often, to thermoregulate at 30 °C. Certainly as the armadillo grows, its mass-specific metabolic rate decreases. It is interesting to note that by adulthood, the metabolic rate of an armadillo is about half that of a similar sized rabbit (Johansen, 1961). The decline in oxygen consumption from postnatal day 4 to day 8 was paralleled by a similar decline in ventilation rate, which again is a common trait in developing mammals (Eisenberg, 1981).

Heart rate in neonatal armadillos increased progressively from birth to day 8, a physiological trait that differs from that in ovine as well as other newborn mammals (Unno et al., 1999; Daniel et al., 1996; Eisenburg, 1981). The degree of newborn development at birth may contribute to this finding, given the very immature nature of armadillo neonates. A similar pattern of increasing heart rate has also been reported in neonates of the domestic mouse, *Mus musculus*, which has a similar immature developmental status at birth (Hou and Burggren, 1989), and upon weaning, heart rate in the mouse begins to follow the standard allometric relationship with mass for mammals. Whether this change in pattern occurs in armadillos must await further study.

The initial resting heart rate of armadillos at birth (132 beats min^{-1}) is low in comparison to that of other similarly sized mammals, as represented by the classic 'mouse to elephant' curve of heart rate for adult mammals (Fig. 7). However, heart rate in adult armadillos is similar to that of other similarly sized adult mammals (Fig. 7, solid triangle) (Johansen, 1961). The extent to which the low neonatal heart rate in armadillos is an intrinsically lower overall heart rate than in other mammals remains difficult to assess. In this study, measurements were made on resting, apparently sleeping, animals. Such resting conditions may not have been used in other newborn studies with the exception of neonatal mice, which were in deep rest during measurement (Hou and Burggren, 1989). A second confounding factor might be the inability of newborn armadillos to thermoregulate effectively. All newborns were measured in humidified air at 30 °C, which is below adult body temperature at rest (35 °C; Johansen, 1961). If neonates are limited in their ability to maintain body temperature, this could account for lower heart rates, and body temperatures were not determined in this study. However, shivering thermogenesis was observed almost immediately after birth, indicating some thermoregulatory ability. Future analysis of the onset of thermoregulatory ability in these altricial mammals is needed to resolve this issue.

The 'sibling effect' in cardiac and metabolic physiology

The data presented, which reveal a 'sibling effect' for body mass, heart rate, metabolic rate and ventilation, clearly demonstrate a genetic component to these physiological characteristics and their pattern of development. Even subtle differences between litters in the pattern of physiological development (e.g. heart rate at day 4, but not at birth or day 8) can most simply be attributed to the different genetic makeup of each litter. Our previous studies used litters of genetically

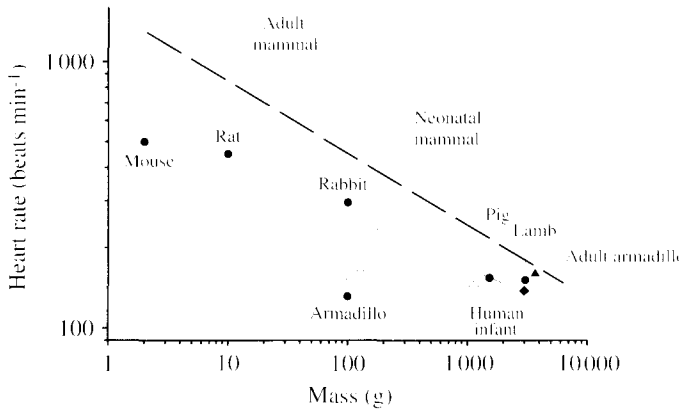

Fig. 7. Comparison of neonatal armadillo heart rate as a function of mass with other common mammals, as neonates and as adults. The allometric relationship of heart rate and mass for adult mammals is shown (broken line) (Stahl, 1967). Heart rates for neonatal armadillos are shown at day 1 (solid circle), day 4 (open circle) and day 8 (open square). These values are compared to other neonatal mammals including the mouse (Hou and Burggren, 1989), rat (Adolf, 1967), rabbit (Sebastiani et al., 1994), pig (Haaland et al., 1996), lamb (Tabsh et al., 1982), premature human (open diamond) (Low et al., 1991), and normal human (solid diamond) (Altman and Dittmer, 1971). A simple linear regression (solid line, $y=-0.0967x+375$; $r^2=0.966$) shows the difference between neonatal mammals and adult mammals. The mean heart rate for adult armadillos solid (triangle) is also shown for comparison (Johansen, 1961).

similar but not identical littermates (Burggren et al., 1999; Crossley et al., 1997), but this study of clonal littermates powerfully suggests that physiological patterns during development are heritable characters, and may therefore be useful indicators of evolutionary relationships.

'Maternal effects' on heart rate, that is, epigenetic influences of the mother on her offspring (see Bernardo, 1996), could be invoked as a possible explanation for these findings. Epigenetic effects commonly pertain to the external environment, but may also result from stochastic variation in cell signaling or developmental timing. Either internal or external epigenetic effects might cumulatively result in increasing within-litter variance. However, the likelihood that the environmental experiences of the mother were being passed epigenetically to her offspring, in equal amounts and across four measured traits, is remote, and is a highly unlikely explanation for our findings. Complete elimination of maternal effects (or rather appropriately, defining these effects within and between rearing females) in animals with lengthy generation times is difficult to achieve experimentally. However, the seven-banded armadillo (*Dasypus septemcinctus*) from South America, which produces 8 or 12 offspring comprising 2-3 groups of four clones each, might be a useful model for probing maternal effects on physiology in mammals.

Physiological patterns were similar but not identical in genetically identical litter mates. The prenatal environment in mammals, i.e. the placenta within the uterus, is heterogeneous

in important characteristics, notably in terms of placental blood flow. Thus, O_2 and nutrient delivery, as well as waste removal, is likely to vary between fetuses. This was reflected in the small but significant variation in within-litter body mass at birth. As neonatal armadillos developed, variability in both body mass and physiology between genetically identical litter mates became more pronounced. An 'environmental variable' as simple as gaining repeated access to a less or more productive mammary gland of the mother can have cumulative effects. If a neonate is not able to grow as rapidly as its siblings, its subsequent ability to compete for suckling may progressively decline in a downward spiral, ultimately leading to the cannibalization of that neonate by the mother. Thus, while environmental influences can be minimized (and controlled) in studies of the heritability of physiological characters during development, they are unlikely to be completely eliminated in complex animals with complex life cycles. Future studies on armadillos will be directed at examining at how the pre- and postnatal environment will influence the developmental trajectories followed by physiological processes, to establish further the relationship between genes and environment in directing physiological development.

We gratefully thank Dr Frank Knight, Amanda Withnell and the University of the Ozarks at Clarksville, AR, USA, for collecting armadillos and adapting them to the laboratory diet. Special thanks go to Greg Sawyer and Jef Jaeger for performing DNA extractions and PCR analysis.

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