Protein Malnutrition Affects the Growth Trajectories of the Craniofacial Skeleton in Rats

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ABSTRACT To investigate the effects of protein malnutrition on a normal growth trajectory, we radiographed *Rattus norvegicus* from 22 d (weaning) and continuing past adult size. We took measurements from longitudinal radiographs of rats fed a control diet and littermates fed an isocaloric low protein experimental diet. A Gompertz model was fit to each individual rat for body weight and 22 measurements of the craniofacial skeleton, producing parameters that described the rate and timing of growth. We tested for differences in these parameters due to diet, sex and litter with a mixed-model three-way ANOVA. Allometric analysis examined the scaling relationships between and within various regions of the skull. For most measurements, final sizes predicted by the model were not significantly different between rats fed the two diets, although the differences in final measurements showed small, but significant differences in growth between rats in the two diet groups. The instantaneous initial rate of growth, maximum rate of growth and deceleration of growth were significantly higher in the control rats for every measurement. Rats fed the low protein diet grew for a significantly longer period of time. The shape of the neurocranium was relatively conserved between diet groups; however, rats fed the low protein diet had shorter and relatively wider skulls than the controls. These results suggest that functional demands of the viscerocranium were greater after birth, and that growth in this area was faster. The viscerocranium reached functional adult proportions earlier and was therefore more susceptible to epigenetic perturbations such as dietary protein level. Protein malnutrition did not affect many aspects of adult size, but strongly altered the growth trajectory to achieve that size. J. Nutr. 129: 2061–2069, 1999.

KEY WORDS: • *protein malnutrition* • *craniofacial* • *skeleton* • *sexual dimorphism* • *rats*

The greatest nongenetic, environmental effect on the rate and timing of growth in humans is that of malnutrition, particularly protein malnutrition (Golden 1994, Malcolm 1979). In nature, growing neonates make the greatest demands for food that is rich in nitrogen for the synthesis of new protein (White 1993). Numerous adaptations exist that allow consumption of different species and ages of vegetation; the crucially limiting resource generating all of these adaptations is food that contains sufficient protein for successful reproduction. The major factor limiting the numbers of animals is the search for sufficient protein to sustain females through pregnancy and lactation, and the young through growth after weaning (White 1993). Protein is critical for growth; levels of 5–10% protein in food are marginal for an increase in body weight for laboratory rats, and growth accelerates with increasing dietary protein levels up to 25% (Edozien and Switzer 1978). Studies using body weight as a measure of growth show that protein malnutrition results in smaller-sized individuals (Cabak et al. 1963, Cameron and Eshelman 1996, Cotheran et al. 1985, Edozien and Switzer 1978, Elias and Samonds 1977, Fleagle et al. 1975, Pucciarelli 1981, Samonds and Hegsted 1978, Stewart et al. 1975, Yayha and Millward 1994).

The craniofacial skeleton is one portion of the body that is critically affected by malnutrition. Understanding how the mammalian skull develops is necessary for understanding the effect of malnutrition. The skull is not a single developing unit; rather, it has two distinct regions, the viscerocranium and the neurocranium (Cheverud 1982). The viscerocranium is used during feeding and breathing, and its growth is continuously subject to muscular loading (Cheverud 1982, Herring 1993), whereas the neurocranium houses the brain, and its growth is influenced primarily by brain expansion (Young 1959). The viscerocranium appears more susceptible to epigenetic factors than the neurocranium (Fields 1991, Pucciarelli 1980 and 1981). Stewart et al. (1975) found that changes in the shape of the head were attributable to the size of the facial bones, but that overall head length was less markedly affected. If the cranial bones had been restricted to the same extent as other bones, there would have been substantial pressure on the nearly full-size brain.

However, one overlooked issue in many of these studies is the dynamics of growth trajectories, or how malnourishment affects the rates and timing of developmental events. Much of this experimental work (Stewart et al. 1975, Yayha and Millward 1994) convincingly demonstrates the effect of protein malnourishment on growth. However, these studies share the problem of lacking complete growth trajectories of these individuals. Furthermore, there are no estimates of ultimate body size for malnourished individuals. It is not known how normal

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growth patterns are interrupted to produce variation in sizes and shapes throughout ontogeny as a result of malnutrition or whether these differences in size and shape are found in the ultimate body size of the individual.

This paper addresses the effect of protein malnutrition on growth and body size, on the craniofacial skeleton in particular. A longitudinal design permitted measurement and analysis of differences among individual growth trajectories. These data can provide a basis for understanding the specific effect of low protein on growth trajectories, those bones that are most are affected by protein deficiency, and whether males and females react similarly to protein malnutrition.

MATERIALS AND METHODS

Animals. Breeders of *Rattus norvegicus* were obtained from a colony of Zivic Miller: Sprague-Dawley strain of rats at the University of Cincinnati. All animals procedures were approved by the University of Cincinnati (IACUC # 91–05-27–01 approval). All rats (n = 37) used were from three litters that had equal amounts of relatedness.

Diets. The two treatments in this study were a control diet (CT) consisting of 24% protein and an experimental low protein diet (LPT) of 4% protein. The 24% CT fell within the range of the maximum growth rates associated with increases in protein intakes up to 25% (Edozien and Switzer 1978); the 4% LPT was deficient enough for differences in the rates of growth to be detected but still high enough to reduce health risks in the LPT rats (Anthony and Edozien 1975, Cameron and Eshelman 1996, Edozien and Switzer 1978, Elias and Samonds 1977, Fleagle et al. 1975, Samonds and Hegsted 1978, Yayha and Millward 1994). Both diets were based on the AIN-93G standard diet recommended to support growth (Reeves et al. 1993). The diets (Dyets, Bethlehem, PA) were isocaloric; thus the only dietary variable altered was protein (**Table 1**). Food consumption and spillage were measured to the nearest 0.1 g using a Fisher Scientific Model S-400 (Denver Instrument, Denver, CO) electronic scale.

Pregnant females were watched carefully so that the exact date of birth was known. At weaning, each litter was separated by sex and randomly assigned one of the two diets. There were four groups (as equal in size as possible), i.e., male control (CT, n = 8), female control (CT, n = 10), male experimental (LPT, n = 9) and female experimental (LPT, n = 10). The three litters were weaned at 22 d of age, placed in hanging basket cages and allowed to eat and drink ad libitum. Each rat was housed in a separate cage so that food consumption and body weight could be measured daily. Body weight was measured to the nearest gram using an Ohaus Lume-O-Gram Lo-Pro (Ohaus Scale, Florham Park, NJ) electronic scale. Daily weighing ensured that there were no health problems occurring in rats fed the low protein diet and provided data for subsequent analysis.

TABLE 1

Contents diet of low protein (LPT) and control (CPT) diets[1]

Ingredient	Low (4% protein)	Control (24% protein)
	g/kg	
Casein	46	276
Cornstarch	500.9	329.9
DYETROSE	167	110
Sucrose	100	100
Cellulose	50	50
Soybean oil	70	70
t-Butylhydroquinone	0.014	0.014
Salt mix #213266	35	35
Calcium phosphate dibasic	11.66	4.08
Calcium carbonate	3.91	9.49
Vitamin mix #310025	10	10
L-Cystine	0.7	4.1
Choline bitartrate	2.5	2.5
Blue dye	—	0.05

[1] Reeves et al. (1993).

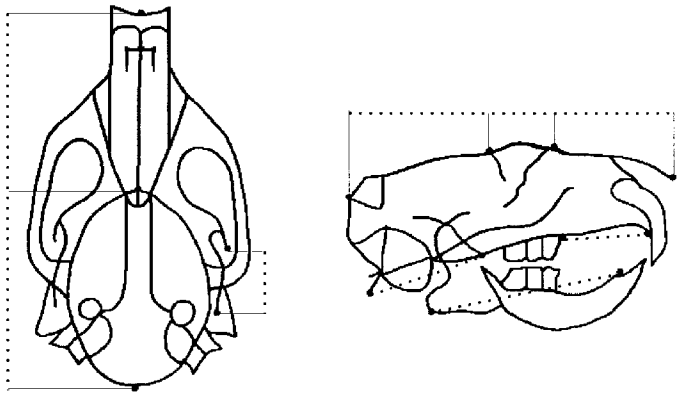

FIGURE 1 Adult rat skull with the length measurements from (*left*) dorsoventral and (*right*) lateral radiographs. Details of specific measurements are in Table 2.

Data collection. For data collection, the rats were lightly anesthetized in a small induction chamber using an Ohio 4000 Compact Anesthesia Machine with isoflurane gas (Anaquest, Liberty Corner, NJ) at 2–3.5% per liter of oxygen for ~5 min. Once the rats were sedated, they were hand positioned on a film cassette for radiographing. Two radiographs were taken of each rat, one in a dorsal-ventral plane and another in a lateral plane. We used Kodak MRM-film and low amounts of radiation from a Bennett Mammography Machine (Bennett X-Ray, Copiague, NY) set for 0.25 s at 75 mA and 44–47 kV, depending on the size of the rat. The rats awoke within minutes and suffered no ill effects. Rats were radiographed three times per week starting at 22 d of age, when growth was occurring at its fastest rate. The frequency of the radiography sessions decreased ultimately to once every 2 wk as the rate of growth slowed down and continued until an accurate estimate of the final size of the individual rat was determined. Previous studies indicated that there are no adverse growth effects from the radiography (Fiorello and German 1997).

The data on craniofacial dimensions were taken from radiographs using a Numonics AccuGrid Digitizing Tablet (Numonics, Montgomeryville, PA; accuracy of 0.127 mm). Radiographs were assessed for misalignment or poor resolution. In >1400 radiographs, only eight were removed because of bad resolution. Cartesian coordinates were obtained from landmarks on the skull bones that were both homologous and repeatable in all rats in the study. A total of 31 points on each radiograph were digitized, 19 points from each dorsoventral view and 12 points from each lateral view. The points were used to measure two-dimensional distances in millimeters in the different regions of the skull (**Figs. 1** and **2**). Points were identified (**Table 2**) from descriptions given by Lightfoot and German (1998) and Popesko et al. (1990).

The measurements in this study were both homologous and repeatable, and chosen to give the most accurate representation of the size and shape of each region. The 11 measurements for the viscerocranium included both the mandible and nasal regions. There were seven measurements for the neurocranium and an additional four measurements of total length, including both the viscerocranium and neurocranium. There were 22 skeletal measurements, plus body weight for a total of 23 measurements.

Statistical analysis. Mammalian growth is usually nonlinear, and we used the nonlinear Gompertz equation to model growth (Gille et al. 1996). This equation is recommended for modeling mammalian ontogeny because it provides one of the best empirical fits for the sigmoidal nature of mammalian growth (German et al. 1994, Lightfoot and German 1998, Laird 1965 and 1966, Laird et al. 1965, Maunz and German 1996). Furthermore, the Gompertz equation has biological meanings associated with the parameters of the equation (Gille et al. 1996). To analyze the data we used the NONLIN module

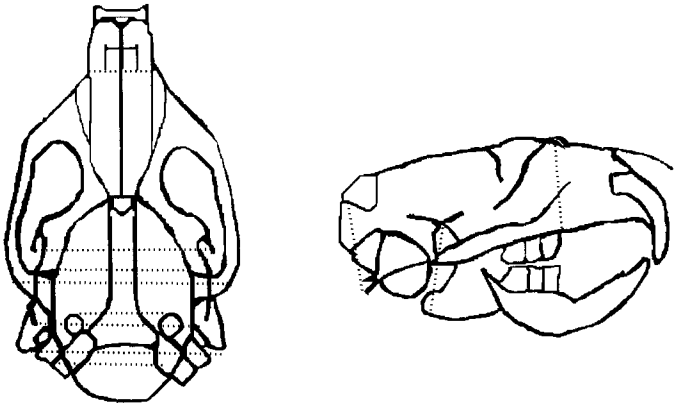

FIGURE 2 Adult rat skull with the width and height measurements from (*left*) dorsoventral (width) and (*right*) lateral (height) radiographs. Details of specific measurements are in Table 2.

of SYSTAT (Wilkinson 1997) with two algebraically equivalent forms of the Gompertz equation as follows:

$$y = Ae^{-be^{-kt}} \quad (1)$$

$$y = we^{(I/k(1-e^{-kt}))} \quad (2)$$

where y is the variable being measured, t is time and was measured in days, e is the base of the natural logarithm, and b is a parameter of limited biological importance describing initial growth (Laird et al. 1965). Parameter w is the value of y at $t = 0$ and is an estimate of the initial size, I is the initial slope of the line at $t = 0$ and is an estimate of the instantaneous initial rate of growth, k measures growth decay and is an estimate of how fast growth slows down, and A is the asymptote or an estimate of the final size of the measurement y. A, b, and k were obtained from nonlinear regressions, whereas values for w and I were calculated from the following relationships:

$$w = Ae^{-b} \quad (3)$$

$$I = bk \quad (4)$$

These equations can also provide an estimate for the time at which growth stops. The first derivative of the Gompertz equation gives the rate of growth over time. From the first derivative, we have the following:

$$dy/dt = Abke^{-be^{-kt}}e^{-kt} \quad (5)$$

We calculated the maximum rate (R_m) of growth and the time at which each measurement was increasing at 5% of its maximum rate T_f, and used this as an estimate for the duration of growth.

A Gompertz curve was fit to each rat's individual growth trajectory for all measurements; thus the individual rat was the unit of analysis. A three-way ANOVA was used to test for significant differences among diet, sex and litter for each of the seven Gompertz growth parameters. Litter was included in the ANOVA as a random factor so that the model was complete. This allowed us to partition any variation due to litter effect, although it reduced both the degrees of freedom and the amount of variation seen in the model due to error. In no case was the litter factor significant. By including litter as a factor, however, any variation in the data due to differences in litter would be excluded from our analysis of differences in diet and sex.

The full model also tested for an interaction between sex and diet. Corrected R^2 values were used to determine how much of the variation could be explained by the model. It is important to use the same model for both treatments to be able to compare differences in growth (Klingenberg 1998). Given the large number of comparisons and dependent or response variables, groups were considered significantly different if the P-value was < 0.001 and marginally different if the P-value was < 0.01. These values are in line with standard Bonferroni corrections for the calculation of multiple ANOVA (Neter et al. 1996).

Additional tests were computed using initial and final sizes. The data used were the measured weights at the earliest time (weaning), 0 d, or at the time of final measurement, which varied for different treatments. Thus, the raw values for weight were tested for treatment differences at the start of data collection with a three-way ANOVA, using litter, sex and diet as factors. The two measures of final size, the A parameter and the actual values for each measurement at the end of the study quantified slightly different things. The A parameter was a prediction, based on the growth trajectory, of ultimate final size. If the model predicted further growth, then A would be higher than the final measurements from the radiographs.

Linear regressions were used to determine the relative proportions of the different areas of the skull and to test for scaling or allometric differences between the diets (Klingenberg 1998). This method allowed determination of the change in relative shape over time, and

TABLE 2

Definitions of 22 craniofacial measurements taken from two radiographic views: dorso-ventral and lateral

Dorsoventral
Total skull
Skull length = Anterior tip of nasal bone − posterior edge of occipital bone
Skull width = Right zygomatic and temporal bone suture − left zygomatic and Temporal bone suture
Viscerocranium
Facial length = Anterior tip of nasal bone − anterior edge of cribiform plate
Nasal width = Anterior tip of right zygomatic arch − anterior tip of left Zygomatic arch
Distance between coronoids = Right coronoid process of mandible − left coronoid process of mandible
Distance between condyles = Right mandibular condyle − left mandibular condyle
Distance between angles = Right mandibular angle − left mandibular angle
Neurocranium
Neurocranial length = Anterior edge of cribiform plate − posterior edge of occipital bone
Neurocranial width = Right temporal line of the parietal bone − left temporal line of the Parietal bone
Distance between tympanic bulla = Anteromedial edge of right tympanic bulla − anteromedial edge of left tympanic bulla
Distance between mastoid processes = Right mastoid process − left mastoid process
Lateral
Total skull
Lateral skull length = Anterior tip of nasal bone − posterior edge of nuchal crest
Skull height = Suture between nasal and frontal bone − most posterior point of upper diastema
Viscerocranium
Nasal bone length = Anterior tip of nasal bone − suture between nasal and frontal bone
Frontal length = Suture between nasal and frontal bone − lateral ridge of frontal bone
Mandible length = Posterior most point of mandibular angle − most anterior point of lower diastema
Right mandibular notch length = Right coronoid process of mandible − right condylar process of mandible
Upper diastema length = Anterior most point of upper diastema − most posterior point of upper diastema
Mandible height = Posterior most point on mandibular angle − most superior point of condyle
Neurocranium
Lateral neurocranial length = Lateral ridge of frontal bone − posterior edge of nuchal crest
Basicranial length = Edge of occipital condyle − posterior edge of palatine bone
Neurocranial height = Posterior edge of nuchal crest − edge of occipital condyle

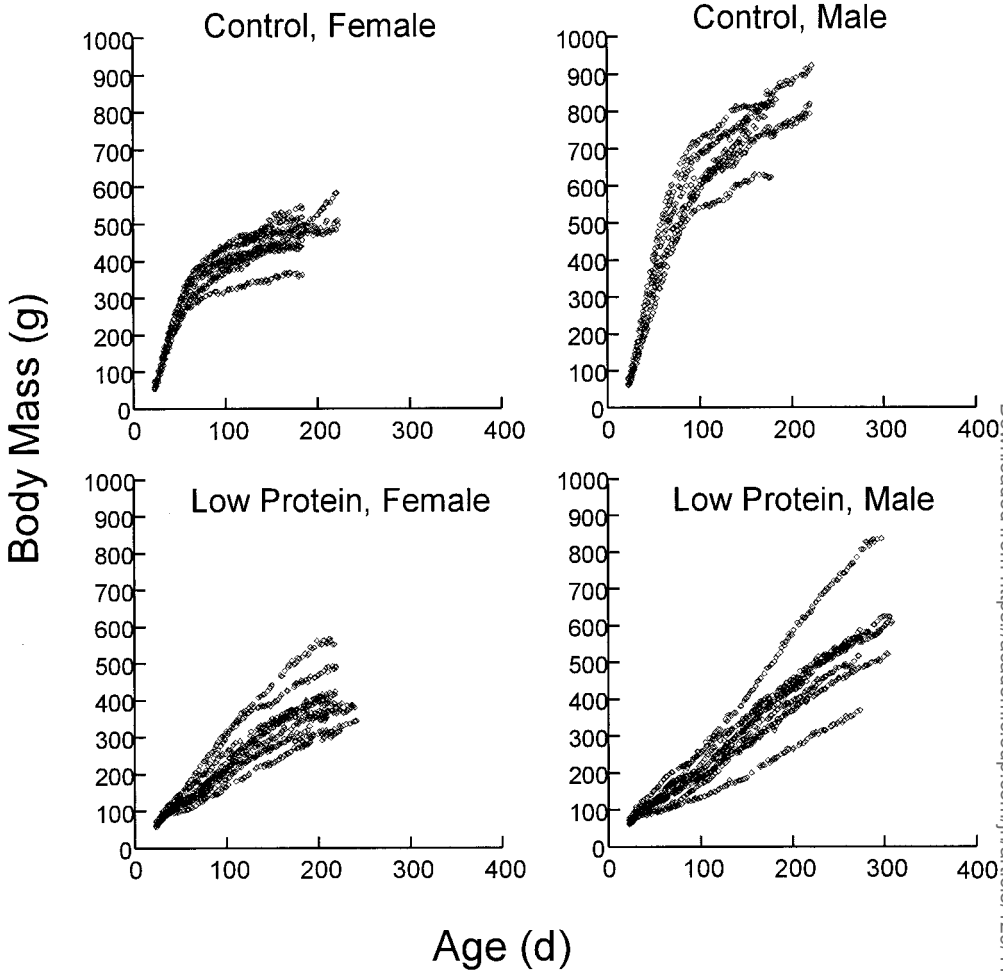

FIGURE 3 Growth in body mass as a function of time for male and female rats, fed a control diet (CT) or a low protein diet (LPT). Data are longitudinal for $n = 8$ CT males, 10 CT females, 9 LPT males and 10 LPT females.

provided information in addition to examining the individual measurements. It allowed testing of the hypothesis that variation in shape as a function of growth differed as a result of diet. These allometric tests measured proportionate shape change, beyond how each individual measure changed with time. Regressions were fit to the data for each individual rat for seven different relationships; thus the unit of analysis was again the individual. A linear slope was calculated for all of the following measurements: mandible length vs. distance between mandibular angles; mandible length vs. mandible height; nasal bone length vs. nasal width; lateral neurocranial length vs. neurocranial width; lateral neurocranial length vs. neurocranial height; lateral skull length vs. skull width; and lateral skull length vs. skull height. Each of the seven sets of slopes was tested for significant differences among the diets and sexes using a mixed-model three-way ANOVA, with sex and diet as fixed factors, and litter as a random factor. Finally, we tested for differences in food consumption by rats consuming the two diets using a repeated-measures ANOVA model with factors for sex, diet and litter.

RESULTS

Food consumption and weight. The amount of protein in the diet was a fixed factor for all rats in the study, but because they were allowed to eat and drink ad libitum, variation occurred in the amount of protein but not in the percentage of protein relative to total daily energy intake. CT rats consumed significantly more food than LPT rats at any given age ($P < 0.001$). At any given body mass, absolute consumption was greater in the LPT than in the CT group ($P < 0.001$). However, when corrected for body weight, total protein consumed relative to body weight was always greater in the CT group ($P < 0.001$)

Body weight. Initial body weight did not differ between the two groups ($P = 0.850$), although it did differ between sexes ($P < 0.001$). The patterns of growth in body weight were different between the two treatment groups (**Fig. 3**). Final body weight differed between diet groups and between sexes ($P < 0.001$). There was also a significant interaction between sex and diet, with the diet effect greater in males than in females. These results differed from the predictions of the Gompertz model in which there was no significant diet effect or interaction for final body weight predicted by the *A* parameter. All other Gompertz parameters that measured rates and duration of growth were overwhelmingly significant for differences due to diet and sex.

Sexual dimorphism. The Gompertz model fit the skeletal data well (**Fig. 4**). The mean corrected R^2 was 0.966 over all models. The minimum corrected R^2 for any single measurement was 0.873 for frontal length, and the maximum R^2 was 0.996 for skull width.

The final size estimate, *A*, was significantly different ($P < 0.001$) between males and females for all skeletal measurements, i.e., the males were consistently larger. All other parameters had consistent patterns, although for a few measurements there was no significant difference between males and females (**Fig. 5**). The initial size, *w*, at the time of weaning was larger for male than for female rats for all but five measurements. There were few differences found for the instantaneous

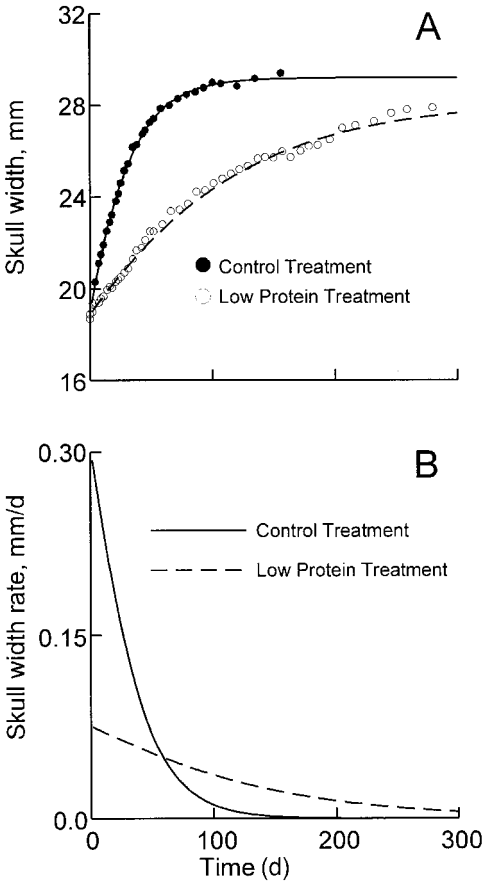

FIGURE 4 Examples of the Gompertz curve and first derivative. (*A*) Skull width vs. time for one rat fed the control diet (CT) and one rat fed the low protein diet (LPT), each fitted with the Gompertz model. (*B*) The first derivative for each model, indicating rates of growth with respect to time. The corrected R^2 for the two models were CT= 0.999 and LPT= 0.985.

growth rate, I, for which the growth curves of female rats were initially steeper. Female rats reached a significantly higher maximum rate of growth for 16 measurements, but after reaching that maximum rate, their growth slowed more quickly than that of male rats, as reflected by their higher k values for 19 measurements. T_f was significantly larger in male rats for 16 measurements. This indicated that male rats grew for a longer period of time, except in the neurocranium in which four of the six measurements were nonsignificant.

Dietary effects. Normal growth for craniofacial dimensions was markedly sigmoidal and nonlinear. The LPT rats had a more linear appearance to their growth trajectory; therefore it is more difficult to find a good empirical fit using the Gompertz model for this group (Fig. 5). The corrected R^2 were slightly lower for the LPT fits ($R^2 = 0.962$) compared with the CT ($R^2 = 0.968$). Furthermore, the initial size of the individual, w, was significantly different for some measurements. Statistical tests of the actual measurements at the beginning of the study showed no significant difference among diet groups ($P > 0.350$). The differences in the initial size of the rats suggested that the Gompertz equation was not providing an accurate measure of initial size. This is probably attributable to the linear appearance of the LPT curves compared with the more sigmoidal growth curves for CT rats.

The LPT rats had a lower initial instantaneous growth rate, a lower maximum rate of growth, a lower rate of decay of growth and a longer duration of growth; k, I, T_f, and R_m were significantly different between diet groups ($P < 0.001$) in all 22 skeletal measurements and body weight. The graphical interpretation of these patterns was evident in Figure 5. The first derivative plot clearly showed a lower initial absolute rate of growth for the LPT rats. The rate of growth in the CT rats slowed drastically as growth continued (k parameter); thus the LPT rats were at a higher rate of growth by ~80 d of age, and this continued until 300 d or longer.

Final size measured by the A parameter was different from the final values measured for most skeletal variables. For A, the final size was not significantly different between the CT and LPT rats for 12 of 22 skeletal measurements (**Table 3**). In four measurements of lengths, the CT rats had a marginally or

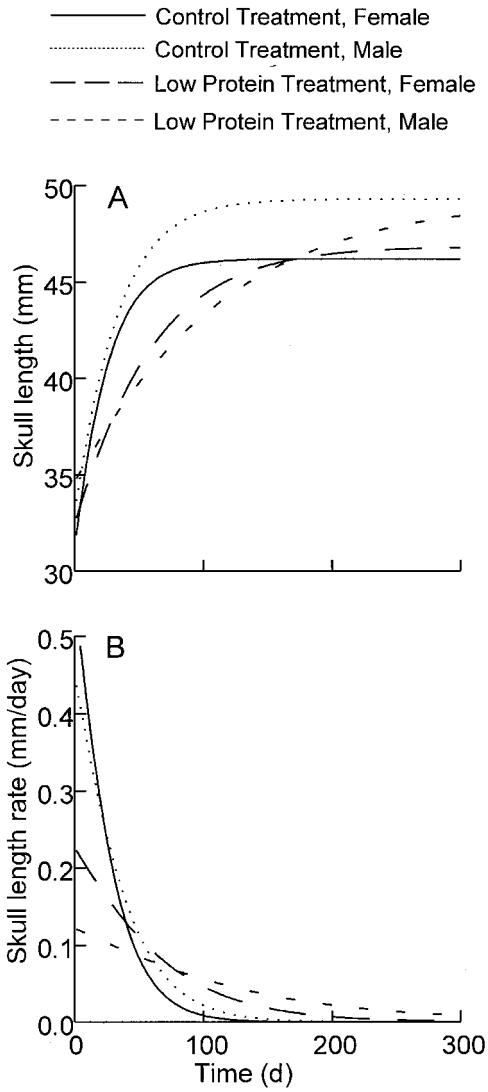

FIGURE 5 The fitted Gompertz model for skull length for the averages of male rats fed the control diet (CT), female rats fed the CT, male rats fed the low protein diet (LPT) and female rats fed the LPT. The lower graph is the first derivative for each of the four models, measuring rate of growth over time. The pattern observed in skull length is typical of all measurements in this study. The CT rats had a sigmoidal shape to the growth trajectory and that of the LPT rats appeared more linear. Female rats had higher maximum rates of growth early, but their growth slowed more quickly and resulted in a smaller final size. The CT rats had a high rate of growth that decreased quickly. By ~80 d of age, the LPT rats were at a higher rate of growth and continued their growth for a substantial period of time.

significantly larger value of A. The LPT rats had a larger A for five of the measurements for widths and heights.

In contrast, the final measured values showed no ($P > 0.1$) or marginally ($P > 0.01$) significant difference for 13 of 22 skeletal measurements for female rats. For male rats, only one measurement was not significantly different ($P = 0.92$). In the remaining measurements that were significantly different between diet groups ($P > 0.001$), the CT rats were larger than the LPT rats (**Table 4**). The average difference in final size of the measurements between the two diet groups was relatively small, 2.7% for female and 6.9% for male rats.

In the cases in which the A parameter predicted differences in final skeletal measurements for LPT and CT rats, a pattern of systematic covariation between the maximum rate of growth and the duration of growth was evident (Table 3). For areas in which CT rats were larger than LPT rats, we expected a relatively shorter duration of growth for the latter group and a relatively higher rate of growth for the former. However, the maximum rates of growth, R_m, were higher than in other measurements for both CT and LPT rats. The ratio of CT R_m to LPT R_m was 2.7, whereas in measurements in which the CT and LPT were of equal size, the ratio of CT R_m to LPT R_m was 3.1. This suggested that the LPT rats had a relatively higher than expected maximum rate for the measurements in which they were smaller. The important difference, then, was that for variables in which the CT rats were larger, the duration of growth for the LPT rats was significantly shorter than that for LPT rats in variables in which the two treatment groups were the same size.

In those cases in which the LPT rats were ultimately larger, with a higher A value than the CT rats, a somewhat more complex pattern existed. First, the duration of growth in the LPT rats was significantly longer and included the five longest durations, all of which were at least 40 d longer than the other measurements. The duration of growth in the CT rats, however, did not differ from the duration for all other measurements. The maximum rate of growth, R_m, was lower in this group of measurements than for the other measurements for both CT and LPT rats. The CT R_m was almost 30% lower, but the LPT group was at less than half the rate it achieved in other measurements.

Interaction. There were few interactions between sex and diet in any of the measurements. Thus, variation in the data were attributable to the main factors, i.e., sex and diet. The parameter with the greatest number of interactions was T_f, the duration of growth, with three significant and six marginal differences, equally in the neurocranium and viscerocranium. In these cases, there was a higher degree of sexual dimorphism between the LPT rats than between the CT rats.

Allometry and scaling. Differences among growth variables suggested that some scaling differences over time existed among the four groups. There were no significant interactions between sex and diet for any of the seven comparisons. For the

TABLE 3

Average duration (T_f) and average maximum rate (R_m) of growth for rats fed on a low protein diet (LPT) and rats fed on a control diet (CT), grouped by differences in A, *final size predicted by the Gompertz model for each measurement*

Significant differences in the *A* Parameter	Duration of growth		Maximum rate of growth	
	LPT	CT	LPT	CT
	d		*mm/d*	
CT ⩾ LPT[1]				
Lat. skull length	255	87	0.224	0.654
Frontal length	291	74	0.048	0.165
Mandible length	271	112	0.119	0.305
Diastema length	196	98	0.082	0.192
CT = LPT[1]				
Skull length	257	83	0.251	0.684
Skull height	329	99	0.051	0.160
Facial length	218	83	0.138	0.350
Nasal bone length	255	108	0.096	0.233
Rt. notch length	280	123	0.043	0.100
Coronoid width	332	80	0.075	0.270
Condyle width	354	62	0.071	0.353
Angle width	344	104	0.105	0.290
Mandible height	345	72	0.054	0.234
Neuro length	359	83	0.109	0.334
Lat. neuro length	305	67	0.076	0.298
Basicranial length	382	122	0.079	0.222
Mastoid process	268	88	0.091	0.259
C ⩽ LPT[1]				
Skull width	426	84	0.093	0.373
Nasal width	440	74	0.040	0.165
Neuro width	632	78	0.031	0.165
Tympanic bulla	648	81	0.022	0.138
Neuro height	579	97	0.032	0.135

[1] CT ⩾ LPT: rats fed control diet were larger ($P < 0.01$) in final size for this measurement than rats fed the low protein diet; CT = LPT: rats fed control diet did not differ in final size from rats fed the low protein diet; CT ⩽ LPT: rats fed control diet were smaller ($P < 0.01$) in final size for this measurement than rats fed the low protein diet.

TABLE 4

Percentage differences in final measured size between control diet (CT) and low protein diet (LPT) rats calculated separately for female and for male rats

	Percentage differences[1]	
	Females	Males
Total skull		
Skull length	0.026***	0.054***
Lat. skull length	0.000	0.080***
Skull width	0.017	0.049***
Skull height	0.036***	0.083***
Viscerocranium		
Facial length	0.037***	0.104***
Nasal bone length	0.034***	0.054***
Frontal length	0.068	0.107***
Mandible length	0.050***	0.103***
Rt. notch length	0.059	0.094***
Diastema length	0.055***	0.126***
Nasal width	0.004	−0.001
Coronoid width	0.020	0.064***
Condyle width	0.036***	0.064***
Angle width	0.019	0.044***
Mandible height	0.000	0.072***
Neurocranium		
Neuro length	0.018	0.051***
Lat. neuro length	0.012	0.052***
Basicranial length	0.009	0.066***
Neuro width	−0.004	0.031***
Tympanic bulla w.	0.044	0.083***
Mastoid process w.	0.016***	0.047***
Neuro height	0.020	0.070***

[1] Percentage differences calculated by (CT value − LPT value)/CT value. Positive values indicate CT > LPT and negative values LPT > CT. *** Indicates significant difference ($P < 0.001$) between CT diet and LPT diet groups.

scaling in the skull, the slopes of length vs. width for both the mandible and nasal measurements, along with total skull length vs. total skull width, had significantly different scaling relationships between the CT and LPT groups, and the mandible length vs. height was marginally different (**Table 5**). In all of these relationships, the CT rats had a larger slope, indicating that as width or height increased, *y* increased in length at the higher rate (**Fig. 6**). The mandible and skull length vs. width scaling were the only significant differences between males and females. There were no significant rate differences between the diet groups or sexes for neurocranium length vs. neurocranium width or neurocranium height, although the LPT rats had a smaller length at any given width (Fig. 6). The intercepts of the two diet group lines for neurocranium and skull scaling relationships were not significantly different, implying that the slopes were not different and that there were no shape differences between the rats fed the two diets in this area of the skull.

DISCUSSION

One concern with this project was that differences between the two dietary groups would be minimal because the LPT rats, having free access to food, could eat more food to obtain an adequate amount of protein. Although at any age the CT group consumed more than the LPT group, they were also larger at every age. This is consistent with the theory that consumption is more dependent on body mass than age (Cameron and Eshelman 1996, Edozien and Switzer 1978). The rats fed the low protein diet consumed significantly more food per gram of body weight than did the CT group. Cameron and Eshelman (1996) showed a similar increase in ingestion by hispid cotton rats to attempt to compensate for low levels of protein. The increased consumption had the effect of increasing energy intake, given that the two diets are isocaloric. However, the additional consumption did not fully compensate for the amount of protein in the diet. Edozien and Switzer (1978) found that rats fed a low protein diet are less energy efficient because they grew less, despite consuming more energy.

All of the measurements in this study showed ultimate body size sexual dimorphism. Three or more significant or marginal differences in Gompertz parameters led to the ultimate body size sexual dimorphism in 18 of the 23 measurements. In the significant differences between males and females, all six

TABLE 5

Differences in allometric scaling in the skull for sex (male/female) and diet (control/low protein) factors in analysis of covariance

	Significance of ANCOVA term	
	Sex	Diet
Mandible length vs. width	***	***
Mandible length vs. height	—	**
Nasal length vs. width	—	***
Neurocranial length vs. width	—	—
Neurocranial length vs. height	—	—
Skull length vs. width	***	***
Skull length vs. height	—	—

*** Significant difference ($P < 0.001$); ** marginal difference ($P < 0.01$); —, no difference. The interactions were never significant.

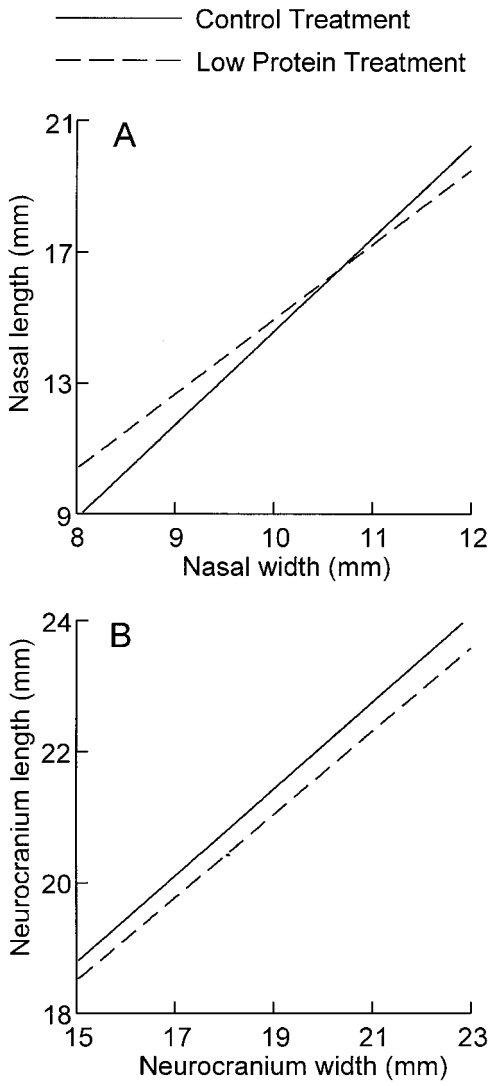

FIGURE 6 Allometric scaling of length to width measurements for rats fed the control diet (CT) or the low protein diet (LPT). *Upper panel*: allometric scaling between nasal length and nasal width for rats fed the two diets. The CT rats had a greater slope, indicating that as width increased, the LPT rats increased in length at a slower rate. This produced LPT rats with an increasingly shorter and wider skull over time. The slopes of the line were significantly different ($P < 0.001$). *Lower panel*: scaling relationship between neurocranium length and neurocranium width. There was no significant rate difference between the diet groups; thus the lines are parallel. The *y*-intercepts for these two lines were not significantly different from one another, indicating that the two lines were not different.

Gompertz parameters followed consistent trends. The smaller initial size, faster deceleration of growth and shorter duration of growth contributed to the smaller size of females, as is true in other species (Lightfoot and German 1998, Maunz and German 1996). The most surprising result was that females had a higher maximum rate of growth in 16 variables. In mammalian growth, the rate of growth slows with the age of an animal until it approaches adult size (Laird et al. 1965), with the maximum rate of growth often occurring perinatally in some aspects of growth, particularly these skeletal measurements (Fig. 4).

As an individual moves along its growth trajectory, the potential increases for processes outside of genetic control to act on growth (Edozien and Switzer 1978, Elias and Samonds

1977, Fleagle et al. 1975, Helm and German 1996, Laird et al. 1965, Samonds and Hegsted 1978). Helm and German (1996) suggested that early growth in miniature pigs is less susceptible to nongenetic perturbations, but as growth continues, so does the potential for the effect of epigenetic factors on body size. Their change in diet with weaning had less effect on early growth than on later growth. Edozien and Switzer (1978) found significant differences in growth rates of rats. These differences increased progressively with increasing levels of dietary protein. However, their study was not long enough to determine whether the differences in growth rate would be reflected in the ultimate body size.

The differences in final size between rats fed the two diets were either nonexistent (using the *A* parameter to predict final size) or small (using the final measurements). This suggested that low amounts of dietary protein in the diet did not necessarily result in smaller skulls. In every measurement, the CT rats had a higher decay of growth, a higher instantaneous initial rate of growth, a higher maximum rate of growth, and the LPT rats had a much longer duration of growth. The average duration of growth of all of the measurements for the LPT rats was four times the duration of growth of the CT rats (CT = 91.4 d, LPT = 365.1 d).

These results differed dramatically from those in the literature, which suggest a much larger effect of protein malnutrition on size. Previous studies did not include a sufficient duration of growth for accurate measurement of ultimate adult size (Edozien and Switzer 1978, Elias and Samonds 1977, Pucciarelli 1980 and 1981, Samonds and Hegsted 1978, Stewart et al. 1975, Yayha and Millward 1994). When we followed growth over time, final size was not significantly different, whereas the paths by which that size was achieved were significantly different.

A few exceptions to this pattern existed in which the CT and LPT rats did not reach the same final asymptote as predicted by *A*. Duration of growth was the parameter that best explained these differences in size. For variables in which the LPT rats were smaller than the CT rats, the LPT rats had a duration of growth marginally shorter than they did for other measurements. When the LPT rats were larger, they had durations of growth significantly longer than their average. For both the variables in which either the CT rats were larger or those in which they were smaller, the duration of growth for the CT rats was not different from that of measurements in which the CT and LPT rats were of equal size. However, the patterns of maximum rate of growth for variables with either the LPT rats larger or the CT rats larger were not as expected. When LPT rats were smaller, the maximum rates of growth for both groups were high, and the LPT rats had a proportionately higher maximum rate of growth. When the LPT rats were larger, the maximum rates of growth for both groups were low, and the LPT rats had proportionately lower maximum rates of growth. These patterns of maximum rate were the opposite of what was necessary to produce the final size effect for both sets of variables. This implied that the duration differences must have been of sufficient magnitude to offset this variable to produce the end effect of adult size differences between the two diet groups.

The measurements for which the LPT rats were smaller were all lengths in the viscerocranium and the entire skull. Previous work suggests that the viscerocranium grows at a faster rate and for a shorter time than does the neurocranium (Clark and Smith 1993, Dressino and Pucciarelli 1997). The lengths of the viscerocranium, in particular, are associated with the functional demands of weaning and tooth eruption (German and Crompton 1996, Maunz and German 1996). It is possible that there was less flexibility in the growth schedules of this region of the skull, and therefore growth cannot be extended in the LPT rats. By the time of weaning, the jaw must be functional for mastication and of sufficient length to accommodate the postcanine dentition. A delay in jaw development, particularly the length of the jaw, which is the functional lever arm during mastication (Hylander et al. 1987), could have a detrimental effect on normal function. Therefore, the sutures in these bones, and their growth, would be more resistant to epigenetic perturbations such as an extension of the duration of growth.

The measurements in which the LPT rats were larger are neurocranial and viscerocranial measurements of width. Again, the neurocranium grows more slowly and for a longer period of time (Clark and Smith 1993, Dressino and Pucciarelli 1997, Maunz and German 1996). Given that the neurocranium houses the brain and in fact grows in response to brain growth, timing constraints due to muscular function are not nearly as severe in the neurocranium as those in the viscerocranium. Thus, extending the growth of this region may not have had a high developmental or survival cost. The widths of the viscerocranium that fell into this group were those portions of the viscerocranium that were growing most slowly. They were also less important for the biomechanics of mastication.

The only significant interaction between the sex and diet factors that occurred consistently was in the duration of growth in which larger differences existed between the male rats fed the two diets than between the female rats. Either the LPT male rats were biologically more susceptible to the effect of low protein or the female rats had a biological protection against this problem. Our data did not permit a distinction between these two alternatives.

Few significant differences existed in scaling over time. The shape of the neurocranium was conserved in the two diet groups. These measurements were in an area of great stability, and they are less reactive to force in the postnatal skull (Fields 1991, Helm and German 1996, Zelditch et al. 1992). The significant differences were in the relationship between lengths and widths of the viscerocranium and of the total skull. In the mandible, nasal and total skull regions, all LPT rats had shorter and relatively wider skulls compared with the CT rats. These results support the work of Clark and Smith (1993), who found that at birth, the neurocranium has already completed the majority of its growth, and to attain proper adult proportions, the viscerocranium must grow faster than the neurocranium. This differential growth rate was due in part to the functional demands of the viscerocranium and the application of muscular forces on the facial skull (Lightfoot and German 1998). Evidence from this study supported the idea that functional demands of the viscerocranium are greater after birth and that to reach functional adult proportions, growth in this area occurred at a higher rate. Therefore, there was an increased chance of being affected by an epigenetic factor such as dietary protein.

LITERATURE CITED

Anthony, L. E. & Edozien, J. C. (1975) Experimental protein and energy deficiencies in the rat. J. Nutr. 105: 631–648.

Cabak, V., Dickerson, J.W.T. & Widdowson, E.M. (1963) Response of young rats to deprivation of protein or of calories. Br. J. Nutr. 17: 601–616.

Cameron, G. N. & Eshelman, B. D. (1996) Growth and reproduction of hispid cotton rats (*Sigmodon hispidus*) in response to naturally occurring levels of dietary protein. J. Mammal. 77: 220–231.

Cheverud, J. M. (1982) Phenotypic, genetic, and environmental morphological integration in the cranium. Evolution 36: 499–516.

Clark, C. T. & Smith, K. K. (1993) Cranial osteogenesis in *Monodelphis do-*

mestica (Didelphidae) and *Macropus eugenii* (Macropodidae). J. Morphol. 215: 119–149.
Cotheran, E. G., Aivaliotis, M. J. & Vandeberg, J. L. (1985) The effects of diet on growth and reproduction in the short-tailed opossums (*Monodelphis domestica*). J. Exp. Zool. 236: 103–114.
Dressino, V. & Pucciarelli, H. M. (1997) Cranial growth in *Saimiri sciureus* (Cebidae) and its alteration by nutritional factors: a longitudinal study. Am. J. Phys. Anthropol. 102: 545–554.
Edozien, J. C. & Switzer, B. R. (1978) Influence of diet on growth in the rat. J. Nutr. 108: 282–290.
Elias, M. F. & Samonds, K. W. (1977) Protein and calorie malnutrition in infant cebus monkeys: growth and behavioral development during deprivation and rehabilitation. Am. J. Clin. Nutr. 30: 355–366.
Fields, H. W. (1991) Craniofacial growth from infancy through adulthood. Pediatr. Clin. N. Am. 38: 1053–1088.
Fiorello, C. V. & German, R. Z. (1997) Comparison of growth trajectories among giant, dwarf, and standard-sized rabbits. Am. Zool. 32: 142A (abs.).
Fleagle, J. G. Samonds, K. W. & Hegsted, D. M. (1975) Physical growth of cebus monkeys, *Cebus albifrons*, during protein or calorie deficiency. Am. J. Clin. Nutr. 28: 246–253.
German, R. Z. & Crompton, A. W. (1996) Ontogeny of suckling mechanisms in opossum (*Didelphis virginiana*). Brain Behav. Evol. 48: 157–164.
German, R. Z., Hertweck, D. W., Sirianni, J. E. & Swindler, D. R. (1994) Heterochrony and sexual dimorphism in the pigtailed macaque (*Macaca nemestria*). Am. J. Phys. Anthropol. 93: 373–380.
Gille, U., Salomon, F., Rieck, O., Gericke, A. & Ludwig, B. (1996) Growth in rats (*Rattus norvegicus* Berkenhout): 1. Growth of body mass: a comparison of different models. J. Exp. Anim. Sci. 37: 190–199.
Golden, M.H.N. (1994) Is complete catch-up possible for stunted malnourished children? Eur. J. Clin. Nutr. 48: S58–S71.
Helm, J. W. & German, R. Z. (1996) Epigenetic impact of weaning on the craniofacial morphology during growth. J. Exp. Zool. 276: 243–253.
Herring, S. W. (1993) Formation of the vertebrate face: epigenetic and functional influences. Am. Zool. 33: 472–483.
Hylander, W. L., Johnson, K. R. & Crompton, A. W. (1987) Loading patterns and jaw movement during mastication in *Macaca fascicularis*: a bone-strain, electromyographic, and cineradiographic analysis. Am. J. Phys. Anthropol. 72: 287–314.
Klingenberg, C. P. (1998) Heterochrony and allometry: the analysis of evolutionary change in ontogeny. Biol. Rev. 73: 79–123.
Laird, A. K. (1965) Dynamics of relative growth. Growth 29: 249–263.
Laird, A. K. (1966) Dynamics of embryonic growth. Growth 30: 263–275.
Laird, A. K., Tyler, S. A. & Barton, A. D. (1965) Dynamics of normal growth. Growth 29: 233–248.
Lightfoot, P. S. & German, R. Z. (1998) The effects of muscular dystrophy on craniofacial growth in mice: a study of heterochrony and ontogenetic allometry. J. Morphol. 235: 1–16.
Malcolm, L. (1979) Protein-energy malnutrition and growth. In: Human Growth (Faulkner, F. & Tanner, J., eds.). Plenum Press, New York, NY.
Maunz, M. & German, R. Z. (1996) Craniofacial heterochrony and sexual dimorphism in the short-tailed opossum (*Monodelphis domestica).* J. Mammal. 77: 992–1005.
Neter, J., Kutner, M. H., Nachtsheim, C. J. & Wasserman, W. (1996) Applied Linear Statistical Models. Richard D. Irwin, Homewood, IL.
Popesko, P., Rajtova, V. & Horak, J. (1990) A Colour Atlas of Anatomy of Small Laboratory Animals, Vol. 2: Rat, Mouse, and Hamster, pp. 114–118. Wolfe Publishing, London, UK.
Pucciarelli, H. M. (1980) The effects of race, sex, and nutrition on craniofacial differentiation in rats. A multivariate analysis. Am. J. Phys. Anthropol. 53: 359–368.
Pucciarelli, H. M. (1981) Growth of the functional components of the rat skull and its alterations by nutritional effects. Am. J. Phys. Anthropol. 56: 33–41.
Reeves, P.G ., Nielsen, F. H. & Fahey, G. C., Jr. (1993) AIN-93 purified diets for laboratory rodents: final report of the American Institute of Nutrition ad hoc writing committee on the reformulation of the AIN-76A rodent diet. J. Nutr. 123: 1939–1951.
Samonds, K. W. & Hegsted, D. M. (1978) Protein deficiency and energy restriction in young cebus monkeys. Proc. Natl. Acad. Sci. U.S.A. 75: 1600–1604.
Stewart, R.J.C., Freece, R. F. & Sheppard, H. G. (1975) Twelve generations of marginal protein deficiency. Br. J. Nutr. 33: 233–253.
White, T.C.R. (1993) Herbivores in an inadequate environment. In: The Inadequate Environment; Nitrogen and the Abundance of Animals (Munzenmayer, R., ed.). Springer-Verlag, Berlin, Germany.
Wilkinson, L. (1997) SYSTAT 7.0: New Statistics. Chicago, IL.
Yayha, Z.A.H. & Millward, D. J. (1994) Dietary protein and the regulation of long-bone and muscle growth in the rat. Clin. Sci. (Lond.) 87: 213–224.
Young, R. W. (1959) The influences of cranial contents on postnatal growth of the skull in the rat. Am. J. Anat. 105: 383–409.
Zelditch, M. L., Bookstein, F. L. & Lundrigan, B. L. (1992) Ontogeny of integrated skull growth in the cotton rat *Sigmodon fulviventer*. Evolution 46: 1164–1180.