Journal of Developmental Origins of Health and Disease (2012), **3**(2), 92–102.

doi:10.1017/S2040174412000037

ORIGINAL ARTICLE

Accelerated growth without prepubertal obesity in nutritionally programmed microswine offspring

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Poor fetal growth and associated prepubertal growth acceleration are linked to increased risk of cardiometabolic dysfunction in later life, but whether obesity is integral to 'catch-up' growth and its ensuing risks are unknown. In microswine offspring exposed to perinatal maternal protein restriction (MPR), we measured body and organ sizes (during MPR); linear growth and weight gain (birth to 5 months of age); feed intake and utilization efficiency (5–14 weeks); and body composition at 6 and 11 weeks of age (by dual-energy X-ray absorptiometry, DEXA). During MPR, low protein offspring (LPO) showed asymmetric growth restriction with reduced body weight (Wt):length (Lth) at birth and elevated heart Wt:liver Wt ratio by 2 weeks of age. In LPO, after slow early postnatal growth (0–5 weeks), subsequent linear growth on *ad libitum* normal feed was absolutely accelerated (cm/week; $P < 0.001$) over 6–11 weeks but normal thereafter, whereas absolute weight gain (kg/week) was similar to controls but accelerated relative to lower LPO nadir weights. Concurrently, rates of fat and lean tissue accrual in LPO over 6–11 weeks were similar to normal protein offspring in absolute terms (g/5 weeks) but increased relative to lower mass at 6 weeks, yielding normal lean:Lth but reduced fat:Lth ratios at 11 weeks. LPO had higher relative feed intake (g/kg/meal) in both sexes and higher feed efficiency in females over 5–11 weeks of age. Findings suggest that postnatal linear growth acceleration preserved thinness in juvenile LPO. Given separately reported abnormalities of vascular (Bagby *et al.*, 2011) and adipocyte function in juvenile LPO, (DuPriest *et al.*, 2011) findings demonstrate that perinatal MPR programs catch-up growth and cardiovascular abnormalities independently of obesity.

Received 25 July 2011; Revised 11 January 2012; Accepted 12 January 2012

Key words: Appetite, catch-up growth, feed intake, linear growth, maternal protein restriction

Introduction

Poor fetal growth is associated with increased risk of metabolic and cardiovascular disease later in life.[1–6] Accelerated prepubertal growth amplifies these risks.[7,8] However, it is unclear from retrospective epidemiological studies whether accelerated prepubertal growth reflects nutritional programming alone or additionally requires enriched postnatal diets to promote metabolic or cardiovascular disease in adulthood. How accelerated prepubertal growth enhances disease risk also remains unclear, but excess adiposity accompanying accelerated growth has been often implicated.

Rodents and sheep exposed to maternal nutrient restriction often have increased adiposity in adulthood (see review).[9] These models typically exhibit low birthweight with normal or higher body weight later in life. However, rodents exhibit brief periods between weaning and sexual maturity, limiting study of prepubertal growth and development. In addition, both rodents and sheep consume distinctly different diets than those of humans and develop relatively little subcutaneous adipose tissue. The pig is an omnivorous nonruminant mammal with the capacity to store relatively large quantities of subcutaneous fat, and has an extended period between weaning (at 4 weeks of age) and onset of puberty (at 5–6 months of age), allowing study of metabolic processes operative during prepubertal development. We hypothesized that accelerated postnatal growth in nutritionally programmed microswine would be associated with preferential deposition of fat tissue as compared with accrual of lean tissue, resulting in increased prepubertal adiposity.

Using perinatal maternal protein restriction (MPR) in microswine, we generated offspring with asymmetric growth restriction. These offspring were studied serially from birth to prepuberty (3–5 months of age at harvest) to determine whether and how MPR programs postnatal growth, adiposity, adipose tissue structure/function, glucocorticoid system function and/or vascular function. The present report addresses basic model features at birth and postnatal patterns of growth, feed intake and body composition; a forthcoming report addresses

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structure and function of adipose tissue, as well as glucocorticoid status in the same offspring.[2]

Materials and methods

Ethical approval

Experiments were approved by the OHSU Institutional Animal Care and Use Committee under protocol No. A439 in June 2005. Requirements to justify utilization of animal models were fully met and approved; efforts to reduce animal numbers and to refine procedures to minimize pain and distress were also described in detail and approved.

Animal care and experimental design

Time-bred Yucatan microswine sows at 0.65 Gestation were obtained commercially (Charles Rivers Laboratories, later Sinclair Research Labs) and maintained in the OHSU Animal Care Facility until study. Sows and offspring were housed in a facility with 12:12-hour light–dark cycle. In pilot studies, piglets were not growth restricted by 3% (wt:wt) MPR but showed excess neonatal mortality during 0.5% MPR (S.P. Bagby, unpublished observations); thus, an isocaloric 1% protein diet was selected. With this diet, there was no difference in numbers of piglets born in low protein (LP) *v.* normal protein (NP) litters (6.18 ± 0.40 *v.* 6.20 ± 0.39, $n = 11$ LP litters and 10 NP litters; $P = 0.41$). Because there was no difference in litter size between groups, no culling was performed. Male:female ratio at birth was approximately 1:1, and was not affected by maternal diet. Runts, defined as normal protein offspring (NPO) having a birthweight more than 2 standard deviations below the littermates' mean, were excluded from the analysis; only two runts were born out of 10 NP litters. Longitudinal studies reported here are based on a total of 35 offspring, 17 NPO from 3 litters (9 males, 8 females) and 18 low protein offspring (LPO) from 5 litters (10 M, 8 F).

Sows were purchased in pairs and randomly assigned to either NP (14% wt/wt)/normal carbohydrate (75%) or isocaloric low-protein (1%)/high-carbohydrate (92%) custom-formulated diets (Purina, Inc.; for diet composition, see Table 1); diets were applied during the last fourth of gestation [beginning at 0.75 Gestation (gestational day, GD, 85 of 115)] plus 2 weeks postnatally. Sows were meal-fed twice per day. The window of MPR was chosen to encompass developmental events in the later-maturing piglet, which occur prenatally in humans. Specifically, piglets are born with minimal white adipose tissue and impaired thermoregulation (*v.* abundant fat in precocial species at term);[10–12] similarly, swine nephrogenesis continues until 1–2 weeks after birth[13] *v.* completion before birth in more precocial species. At 2 weeks postnatally, all sows were returned to a normal *ad libitum* diet (Purina Mills Lab Porcine Diet Grower). Offspring to be studied as juveniles were weaned at 4 weeks of age to juvenile piglet diet (PMI Nutrition International, LLC) containing (as % of calories) 19% protein, 9% fat and 71% carbohydrate, and were meal-fed twice per day (see Feed Intake methods, below). Animals generated for this study form the basis for several studies reported separately and addressing separate aspects of the model. The experiment was concluded by terminal harvest of selected organs and tissues from anesthetized well-perfused juvenile offspring, as described below for fetal and neonatal offspring, at 3–5 months of age in an age-matched manner. This harvest protocol is described in detail in a forthcoming manuscript.[2]

Table 1. *Maternal diet composition*

Ingredient	Low protein diet (1.0%), % (wt/wt)	Normal protein diet (14%), %(wt/wt)
Soybean meal (47.5% protein)	1.8	30.1
Alfalfa meal (15% protein)	1.0	1.0
Corn starch	67.9	46.6
Glucose monohydrate	23	16
Soybean oil	3.0	3.0
Limestone (grd)	1.0	1.0
Dicalcium PO_4	1.5	1.5
Salt	0.5	0.5
Vitamin/mineral supplement	0.25	0.25
Total	100	100

Body size measurements

Weight and crown-to-rump length (using a flexible measuring tape along the spine, with head raised approximately horizontal) were measured 5–7 days (d)/week for the first 6 weeks of age, then 3–5 d/week thereafter, up to and including at the time of terminal tissue harvest. For analysis, individual values were averaged to yield a single weekly estimate for each piglet. To easily visualize differences in rates of growth, LPO and NPO values for weight (Wt), length (Lth) and Wt:Lth ratios were converted to percent of the average sex-matched control and plotted against age.

Feed intake

After weaning at 4 weeks of age, piglets were housed separately (in adjacent wire-mesh cages permitting visual and physical contact between offspring) to facilitate feed measurement and avoid confounding by social hierarchy or aggressive behaviors. Piglets were fed two weighed meals per day in excess of appetite. For composition of piglet growth chow, see Table 2. Feed intake was measured by subtraction of residual from initial feed weight for both meals on each day, for 5 d/week during weeks 7–9 for the first set of litters studied and for 3 d/week during weeks 6–14 for subsequent litters. Feed intake (g) per meal was normalized to same-day body weight; weekly per-meal averages were determined for

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Table 2. *Dietary composition of piglet growth chow*

Ingredient	Percentage of feed (wt/wt)
Crude protein	20.0
Lysine (min)	0.065
Crude fat (min)	4.00
Calcium (min)	0.65
Calcium (max)	1.15
Phosphorus (min)	0.5
Salt (NaCl; min)	0.5
Salt (NaCl; max)	1.00
Zinc (min)	100 ppm
Selenium	0.3 ppm
Ash (max)	6.50

Piglets were fed Lab mini-pig chow: starter (5080; PMI Nutrition International, LLC Brentwood, MO, USA).

each piglet and results analyzed according to weeks of age. For calculation of feed utilization efficiency, total Wt gain over one week was divided by calculated total weekly feed intake (average per meal intake $\times$14 meals).

Body composition

At 6 weeks of age (when weights were most reduced in LPO *v.* NPO in pilot studies) and 11 weeks of age (when weights were statistically similar between LPO and NPO in pilot studies), body composition was assessed by dual-energy X-ray absorptiometry (DEXA). Piglets were anesthetized by inhalation of isoflurane (2.0–2.5% isoflurane, up to 3.0% during the first minutes of induction, with an oxygen flow rate of 2.0 l/min) and placed prone on the DEXA scanner (Hologic QDR-4500W). Fat mass and lean mass were measured by region – trunk, right leg and left leg. The head region was excluded from calculations because of the high lipid content of the brain; forelimbs were excluded because of anatomic constraints imposed by the use of pediatric software (Experimental Pediatric Whole Body v8.26 and v12.3). Adiposity was assessed both by normalizing fat mass to body Wt as % fat (g fat:body Wt $\times$ 100) and to body Lth (fat mass:body Lth ratio). Thinness was assessed using body Wt:Lth ratio and also via DEXA scan by lean mass:Lth ratio (as LPO Lth was least affected by MPR throughout the study period).

Organ harvest for assessment of growth asymmetry in near-term fetal and neonatal offspring

For collection of selected organs from near-term fetal offspring, a separate set of 6 sows [3 NP litters with 20 offspring (8 males, 12 females); 3 LP litters with 16 offspring (10 males, 6 females)] at GD 113 (term is GD 115) were optimally maintained under isoflurane anesthesia (2.0–2.5% inhaled isoflurane with an O_2 flow rate of 2.0 l/min). Piglets were sequentially delivered by Cesarean section, and each quickly euthanized by exsanguination via cardiac puncture. Organs were carefully dissected, cleaned and weighed; tissue aliquots were snap-frozen or fixed in 4% paraformaldehyde for use in other studies. For collection of organs from 2-week-old neonates, individual offspring from a separate set of 4 sows [2 NP litters with 9 offspring (5 males, 4 females); 2 LP litters with 8 offspring (5 males, 3 females)] were placed under isoflurane anesthesia and organs were harvested as described above except that animals were euthanized by removal of the heart at the end of organ harvest.

Statistical methods

Wt, Lth and Wt:Lth ratios were analyzed by nonlinear regression (Logistic model, SPSS v16.0 software) to compare rates and patterns of growth over time. As growth curves exhibited two distinct segments (slowed growth over 0–5 weeks of age, normal or increased growth over 5–14 weeks of age), GLM-univariate analysis (SPSS v16.0 for Windows, SPSS Inc., Chicago, IL, USA) was applied to each segment with maternal diet, sex and week of age as factors. Feed intake, feed utilization efficiency, body composition parameters and fetal and neonatal organ Wt:body Wt ratios were similarly analyzed, using sex and maternal diet as factors. Data are presented as means $\pm$ SEM. For all statistical tests, a P-value <0.05 was considered significant.

Results

Evidence of growth asymmetry during MPR in offspring studied at near-term fetal and neonatal stages

In near-term fetal pigs (sacrificed on GD 113 of 115), body Wt:Lth ratios were reduced in LPO compared with NPO controls ($P = 0.026$; Fig. 1 and Supplementary Table). This reflected a modest $\sim$10% reduction in body Wt ($P = 0.028$) with no change in body Lth. Body Wt:Lth ratios were more severely reduced in the neonatal LPO euthanized at 2 weeks of age compared with neonatal NPO ($P < 0.001$; Fig. 1 and Supplementary Table), at this stage reflecting a more severe ($\sim$30%) body Wt reduction ($P < 0.001$; Supplementary Table).

In addition to body size asymmetry, LPO at 2 weeks of age (but not at near-term) exhibited increased upper body:lower body organ Wt ratios compatible with organ growth asymmetry (Fig. 1; Supplementary Table). Specifically, the Heart Wt:Liver Wt ratio was significantly increased in 2-week LPO of both sexes (0.23 ± 0.01 g/g *v.* 0.19 ± 0.01 in NPO; $P = 0.02$; Fig. 1). This was supported by the transition from a significantly low Heart Wt:Body Wt ratio in near-term LPO ($P = 0.003$) to a numerically elevated Heart Wt:Body Wt ratio in neonatal LPO (Supplementary Table); concurrently, LPO liver Wt:Body Wt ratios trended in the opposite direction (normal to low) with duration of MPR.

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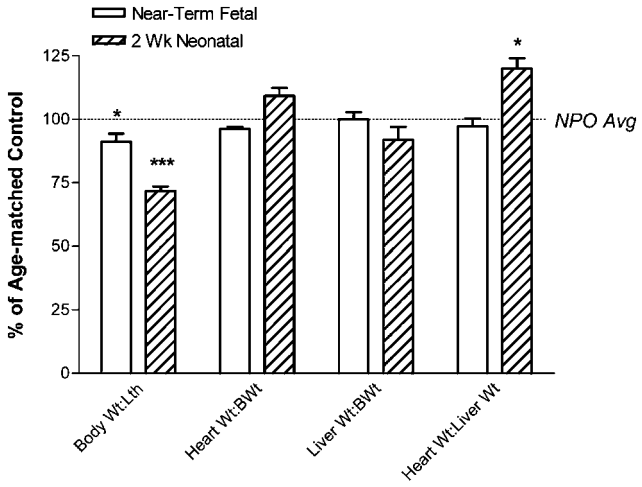

Fig. 1. Organ weight-to-body weight ratios. Body Wt:Lth, heart Wt:body Wt, liver Wt:body Wt and heart Wt:liver Wt ratios are shown for low protein offspring (LPO) at near-term fetal (GD113 of 115) and 2-week-old neonatal ages as a percent of normal protein offspring (NPO) controls. Open bars, near-term fetal offspring [15 LPO (4 males, 11 females); 14 NPO (8 males, 6 females)]; cross-hatched bars, 2-week-old neonatal offspring [7 LPO (4 males, 3 females); 8 NPO (4 males, 4 females)]. $^{*}P < 0.05$; $^{***}P < 0.001$.

Growth patterns in juvenile offspring studied serially from birth

At birth, body Wt (Fig. 2a and 2b) and Wt:Lth ratios (Fig. 2d and 2e) in LPO were modestly reduced to $93.7 \pm 0.7\%$ ($P < 0.01$) and $93.3 \pm 4.1\%$ ($P = 0.056$), respectively, of sex-matched NPO control averages. LPO Lth at birth did not differ from NPO ($99.3 \pm 1.5\%$ of NPO; Fig. 2c and 2d). There were no sex differences in body size indexes at birth. From birth to just post weaning (including the 2 weeks of continued MPR), growth in LPO slowed progressively (diet × time interaction $P < 0.001$; Fig. 2), so that by the 5th postnatal week, Wt, Lth and Wt:Lth ratios in LPO of both sexes were each more severely reduced ($P < 0.001$) compared with age/sex-matched NPO. For each parameter, early postnatal growth slowing in LPO was consistently greater in females than in males (maternal diet × sex interaction <0.001). Furthermore, postnatal growth slowing in LPO was asymmetric: nadir Wt at 5 weeks in LPO ($75.7 \pm 4.4\%$ of average NPO value; Fig. 2b) were lower proportionally than nadir Lth ($89.5 \pm 2.2\%$ of average NPO value; Fig. 2d), indicating a sustained and progressive body growth asymmetry during postnatal MPR (0–2 weeks) and its immediate aftermath (2–5 weeks).

In contrast, from 6 to 14 weeks of age, Wt gain and linear growth in LPO were each accelerated but with distinct patterns (Fig. 2a–2d). Rate of increase in body Wt in LPO, in absolute terms (kg mass accrued per week), was similar to that of NPO; however, relative to the lower LPO nadir weights at 5 weeks of age, rate of Wt gain was accelerated. LPO Wt did not fully reach average NPO levels during the period of serial study. However, after age adjustment of body Wt measured at harvest (spanning 12–20 weeks) to the group average age (at harvest) of 16.5 weeks, LPO Wt (25.7 ± 2.6 kg, $n = 12$) was $94.6 \pm 4.3\%$ of NPO (27.2 ± 2.8 kg, $n = 9$) and did not differ significantly from NPO ($P = 0.18$), suggesting that by 17 weeks, LPO are approaching full catch-up of body Wt. (See regression inset and boxed data points at 16.5 weeks in Fig. 2a)

Linear growth in LPO, by contrast, was accelerated in both relative and absolute terms (increased cm/week) over 6–11 weeks of age (Fig. 2c and 2d), attaining sex-matched NPO levels by 11 weeks of age and slowing to match NPO rates thereafter. Rates of absolute Lth gain over the 6 weeks preceding full catch-up (from 5 to 11 weeks of age) were 3.76 ± 0.14 cm/week in LPO ($n = 17$) *v.* 2.94 ± 0.14 cm/week in NPO ($n = 16$; $P < 0.001$).

As expected from the Wt and Lth growth patterns, the Wt:Lth ratio remained lower in LPO throughout the 6–14-week period (Figs. 2e and 2f, $P < 0.001$); this difference was not affected by age over this time period. Moreover, LPO females exhibited a more severe reduction in Wt:Lth ratio than LPO males (each relative to sex-matched NPO) throughout the rapid growth phase (maternal diet × sex: $P < 0.001$; Fig. 2f). Overall, in both LPO and NPO, male Wt:Lth ratio exceeded that of females ($P < 0.001$).

Feed intake

In absolute terms (g feed consumed per animal per meal), male LPO consumed the same amount of feed as male NPO (Fig. 3a), whereas female LPO consumed about 20% less feed compared with female NPO ($P < 0.001$; Fig. 3b). However, when feed intake was normalized to same-day body Wt, LPO of both sexes consistently consumed more feed over the 5–12-week time period (LPO: 32.2 ± 0.4 g/kg/meal; NPO: 28.8 ± 0.5 g/kg/meal; $P < 0.001$; Fig. 3c and 3d). Overall, females consumed more feed/kg/meal than males ($P < 0.01$).

Feed utilization efficiency (conversion of ingested feed to tissue mass) over 6–12 weeks of age was increased in female LPO, averaging 0.456 ± 0.135 g Wt gain/g feed consumed *v.* 0.372 ± 0.125 g in female NPO ($P < 0.001$; Fig. 3f). Male LPO did not differ from male NPO (maternal diet by sex interaction: $P = 0.05$; Fig. 3e). A steady age-related decrease in feed utilization efficiency was observed in all groups ($P < 0.0001$; Fig. 3e and 3f). Sex did not influence feed utilization efficiency in NPO.

Body composition

At 6 weeks of age, when Wt was lowest in LPO as a percent of age- and sex-matched NPO control average, percent body fat in LPO was decreased compared with NPO ($P = 0.0005$); percent body fat at 6 weeks of age was unrelated to body Wt.

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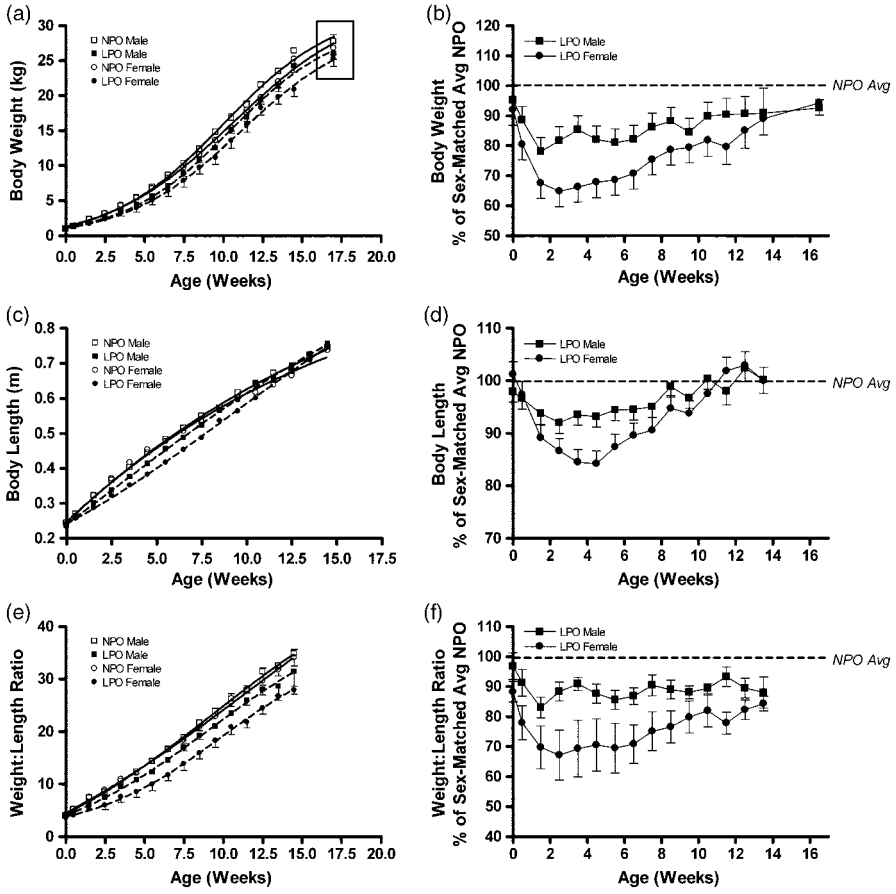

Fig. 2. Weight and length growth rates. For each group, average weekly litter-adjusted values are shown for Wt (*a*), Lth (*c*) and Wt:Lth ratio (*e*); values were also converted to percent of sex-matched controls and plotted for Wt (*b*), Lth (*d*) and Wt:Lth ratio (*f*). Boxed data point in (*a*) represents body weights at harvest of all animals age-adjusted to group average of 16.5 weeks. All data are shown as means ± S.E.M. □, male NPO ($n = 8$); ○, female NPO ($n = 10$); ■, male LPO ($n = 9$); ●, female LPO ($n = 8$). Statistical details are provided in the text. LPO, low protein offspring; NPO, normal protein offspring.

Normalized lean mass (lean mass:Lth ratio, an index of thinness) was also reduced in LPO at 6 weeks of age compared with NPO ($P = 0.034$; Fig. 4d). In both groups, females had higher percent body fat than males ($P = 0.037$; Fig. 4a).

At 11 weeks of age (late in the rapid growth period), although percent body fat in LPO was numerically lower *v.* NPO, it did not reach significance ($P = 0.06$; Fig. 4b). Percent body fat did not correlate with body Wt. However, when body fat mass (g) was normalized to body Lth (which was no longer different in LPO *v.* NPO by 11 weeks of age, see Fig. 2d), body fat was significantly lower in LPO of both the sexes: 43.7 ± 2.6 g fat/cm in LPO *v.* 55.9 ± 3.5 in NPO ($P < 0.02$). Lean mass normalized to body Lth, in 11-week-old LPO, was not significantly different from NPO ($P = 0.12$; Fig. 4e). Fat:lean ratios were also normal in 11-week-old LPO. Thus, LPO were 'thin' primarily because of a lower fat mass *relative to length*. In addition, as expected, 11-week-old female offspring overall had higher percent body fat ($P = 0.012$; Fig. 4b) and higher Lth-normalized fat mass ($P = 0.04$; Fig. 4c) compared with males; male offspring exhibited higher Lth-normalized lean mass compared with females ($P = 0.003$; Fig. 4e).

Accrual of fat and lean mass

The fold increase in fat mass from the 6-week to the 11-week scan (g fat at 11 weeks/g fat at 6 weeks; Fig. 5b) was greater in LPO than in NPO ($P = 0.003$), thus a relatively accelerated fat tissue accrual. Fold increase in lean mass over the same period (g lean at 11 weeks/g lean at 6 weeks; Fig. 5d) was also greater in LPO than in NPO ($P = 0.02$). However, cumulative *absolute* increase in fat mass and in lean mass over 6–11 weeks of age in LPO did not differ from NPO (Fig. 5a and 5c), and the ratio of increase in fat to increase in lean (g/g) was also not different between LPO and NPO; this indicates a balanced accrual of fat relative to lean mass during the period of accelerated growth (Fig. 5e). Thus, LPO remained thin (low body Wt:Lth ratio) at 11 weeks

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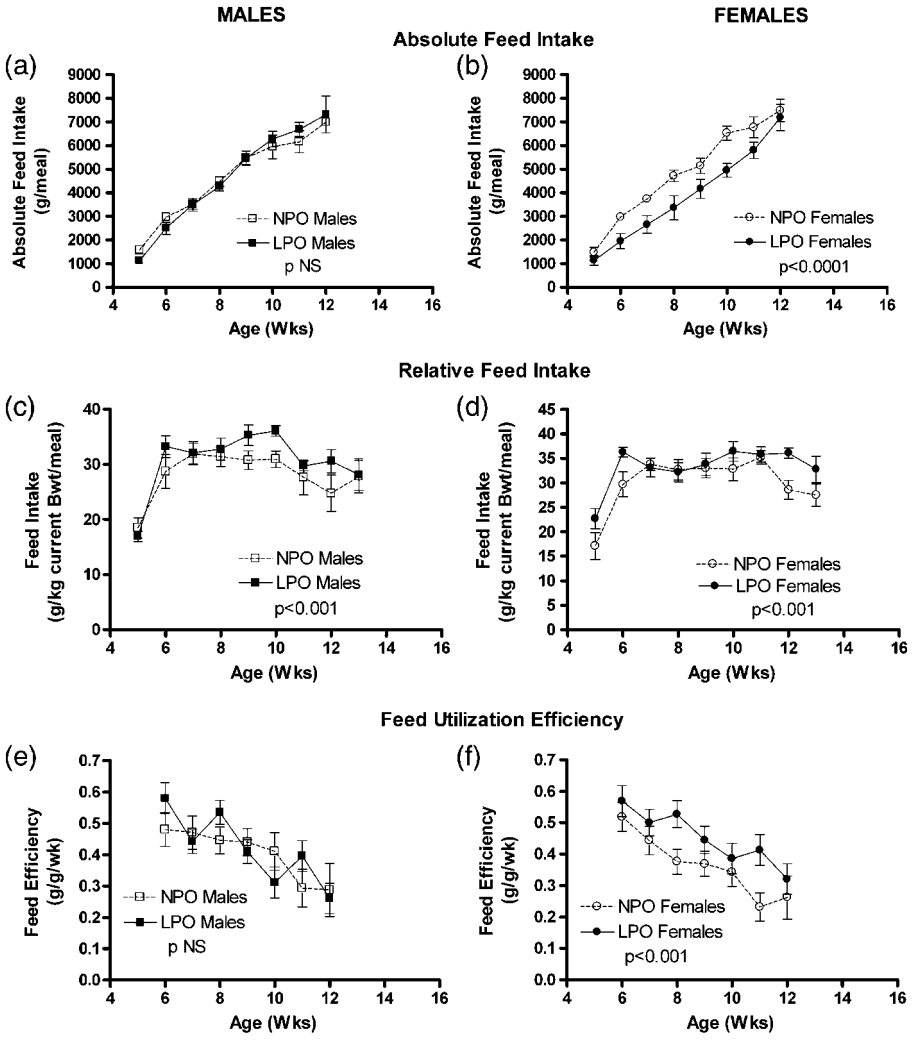

Fig. 3. Feed intake and feed utilization efficiency. Absolute feed intake (males, *a*; females, *b*); relative feed intake (males, *c*; females, *d*); and feed utilization efficiency (g of Wt gain per g of feed consumed) (males, *e*; females, *f*) were measured serially and are shown as weekly means for low protein offspring (LPO) and normal protein offspring (NPO) over ages 6–12 weeks. □, male NPO ($n = 4$–8); ○, female NPO ($n = 7$–9); ■, male LPO ($n = 6$–10); ●, female LPO ($n = 6$–8).

of age, not because of absolutely less fat or lean mass accumulation, but because the accelerated linear growth restored Lth to control levels without (prior to) full restoration of non-skeletal mass components. Overall, males and females accrued equal absolute amounts of fat, whereas males accrued greater g of lean mass ($P = 0.03$; Fig. 5c). Females accrued more g fat relative to g lean as compared with males ($P = 0.006$; Fig. 5e).

Discussion

Growth patterns during early life, both prenatal and postnatal, have been shown to influence risk of cardiovascular and metabolic disease later in life,[3–6] but the mechanisms involved are not yet clear. It has been suggested that poor perinatal growth – with or without low birthweight – is fundamentally tied to later accelerated growth with development of obesity, and consequent increased disease risk. To investigate this in the present study, we generated asymmetrically growth-restricted offspring using a perinatal maternal low-protein/high-carbohydrate isocaloric diet in microswine, a large and long-lived omnivore chosen for its similarity to humans in cardiovascular, digestive, metabolic and renal anatomy and physiology[14] and in its genome.[15] Moreover, the extended period between weaning and puberty in swine provides optimum conditions for study of prepubertal growth and development. To encompass key developmental events that are complete at birth in more precocial species (human, sheep),[11] but not in piglets, we extended MPR to include the first 2 weeks postnatally. At this age, nephrogenesis is complete in swine,[13] whereas ovine and human nephrogenesis is complete before birth. In addition, piglets are born with insufficient body fat to sustain body temperature (~1% body fat), whereas by

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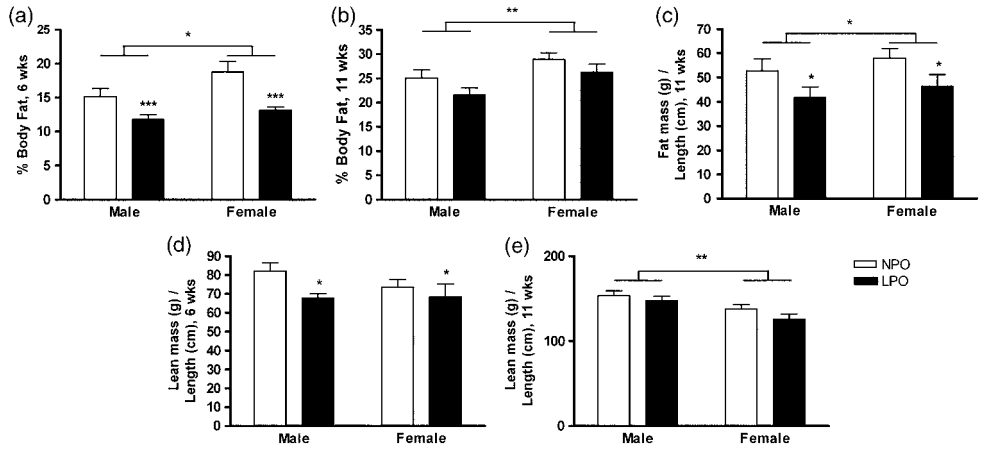

Fig. 4. Body composition at 6 and 11 weeks of age. Percent body fat at 6 weeks of age (*a*) and at 11 weeks of age (*b*); Lth-normalized fat mass at 11 weeks of age (*c*); and Lth-normalized lean mass at 6 weeks (*d*) and 11 weeks of age (*e*) are shown as means ± S.E.M. White bars, NPO ($n = 18$: 9 males, 9 females); black bars, LPO ($n = 16$: 10 males, 6 females). $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$. LPO, low protein offspring; NPO, normal protein offspring.

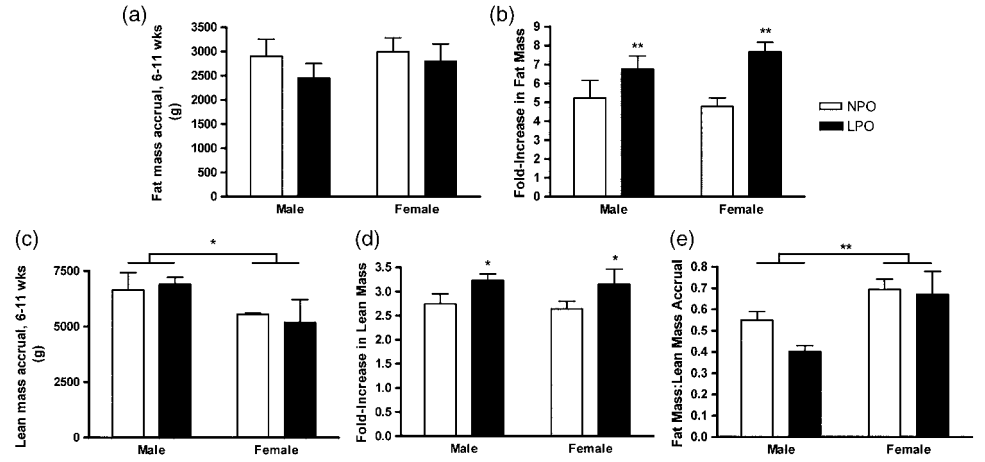

Fig. 5. Rates of tissue accrual over 6–11 weeks of age. Absolute accrual of fat (*a*) and lean (*c*) tissue in g; fold increases of fat (*b*) and lean (*d*) tissue; and the ratio of accrual of fat:lean tissue (*e*) over 6–11 weeks of age are shown as means ± S.E.M. White bars, NPO ($n = 15$: 6 males, 9 females); black bars, LPO ($n = 14$: 8 males, 6 females). $^{*}P < 0.05$; $^{**}P < 0.01$. LPO, low protein offspring; NPO, normal protein offspring.

2 weeks of age they have accumulated the 12–15% body fat typical of precocial species at birth.[10–12]

This report describes the basic features of the model, addressing changes in body and organ growth, appetite and serially measured body composition in response to perinatal MPR in microswine offspring. We are unable to discern whether abnormalities observed[2] are due to protein deficit, carbohydrate excess or an interaction between the two. Of potential relevance, the experimental diet reflects a 93% reduction in protein content but only a 22% increase in carbohydrate content. In addition, protein and fat contents in both LP and NP diets are largely soy-based (Table 1) and may not mimic effects of lard-based or casein-based diets.

Major findings in LPO offspring exposed to MPR include poor intrauterine and early postnatal growth, an asymmetric pattern of organ and whole-body growth restriction during MPR, accelerated post-weaning growth with unexpected predominance of linear growth over body mass growth in both sexes, and sex-specific patterns of increase in feed intake and feed utilization efficiency. Moreover, serial measurements of fat and lean mass at early and late points during rapid growth suggest that the predominance of linear growth acceleration, not less absolute fat or lean tissue accruals, accounted for persistent thinness (reduced Wt:Lth ratio at 11 weeks) during catch-up growth in LPO. Relative to the fully restored Lth at 11 weeks of age, thinness in LPO reflected mainly lower fat mass relative to Lth, with more effective preservation of lean mass. Results document that perinatal MPR induces early slowing and 'programs' late acceleration of growth in offspring on *ad libitum* normal low-fat (9%) diet

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post weaning, and further that prepubertal obesity is not an invariable feature of postnatal catch-up growth following intrauterine growth restriction (IUGR). Finally, on the basis of programmed abnormalities of vascular and adipose tissues reported separately in these juvenile LPO,[1,2] obesity is also not essential to the nutritional pathways by which MPR programs the vascular contractile hyperreactivity to pressors or the adipocyte dysfunction observed in juvenile offspring.

Patterns of growth during MPR

LPO grew slowly before birth (in Wt but not Lth) and progressively more slowly early after birth (in both Wt and Lth) during continued MPR. The 0–2-week growth slowing may additionally reflect in part a superimposed global caloric deficit due to reduced milk production by LP dams (S.P. Bagby, unpublished).

Concurrently with the slowed prenatal and early postnatal (0–2 weeks of age) growth *during* MPR, an asymmetric pattern of both body and organ growth restriction was observed in LPO. Although body Wt:Lth ratio was lower in LPO at both near-term and 2 weeks postnatally (the end of MPR), upper body *v.* lower body organ growth asymmetry was apparent only at the postnatal time point, where liver growth had slowed to a greater degree than heart growth, thus a relative sparing of the heart. This relative increase in heart Wt:liver Wt ratio is consistent with the asymmetry observed in human IUGR following mid–late gestation placental insufficiency, suggesting a commonality of offspring adaptive responses to deficits despite different stressors. The failure to see an asymmetric pattern of organ growth restriction in LPO at birth may be due to the short intrauterine phase of perinatal MPR (last 1/4 of gestation) coupled with the innate protein utilization efficiency of the pregnant sow. (e.g. Rippel *et al.*[16] found that isocaloric 5% protein restriction in pregnant sows produced no difference in litter number, average birthweight or survivability *v.* 16% protein). It is tempting to speculate that, as described for fetal blood flow redistribution during placental insufficiency,[17] activated sympathetic vasoconstrictive and/or nitric oxide vasodilatory systems[18] may be activated (or persist) postnatally in LPO to differentially modify organ nutrient flow rates during MPR. Persistence of slowed growth beyond 2 weeks of age, despite resumption of normal maternal feed plus weaning, may in part reflect the well-documented delay in gastrointestinal mucosal maturation in growth-restricted piglets.[19,20]

Patterns of post-weaning growth in offspring on ad libitum *normal diet*

LPO subsequently underwent accelerated growth in the post-weaning period with distinct patterns for linear *v.* total mass accruals. Acceleration of linear growth was unexpectedly predominant, with an *absolute* increase in rate (cm/week) in LPO over NPO. In contrast, body Wt gain progressed at an absolute rate (kg/week) similar to NPO controls, and thus was accelerated only in relation to the lower initial body Wt in LPO. Also unexpectedly, rapid growth was accompanied not by increased adiposity but by a normally balanced accrual of fat and lean tissue; this yielded body composition at 11 weeks of age that was normal for lean mass and either low–normal or low for fat mass depending on the normalization method. Adiposity as assessed by percent body fat was marginally (but not significantly) low in LPO at 11 weeks of age. However, because LPO were accruing length faster than mass, body fat expressed relative to body Lth was significantly reduced as compared with NPO. Thus, LPO at 11 weeks of age remained thin, primarily because of absolutely accelerated linear growth rather than because of failure of either lean or fat mass accrual. Moreover, lean mass accrual was preserved more efficiently than fat mass accrual, as lean:Lth ratio was maintained. Most importantly, body fat was not increased in LPO by any measure at 11 weeks of age. Of note, the normal commercially formulated postnatal pig feed was low in fat (9% of calories); our findings do not preclude development of prepubertal obesity in LPO on a Western-type diet with higher fat content and raise the question of whether a low-fat diet with adequate nutrients/calories could attenuate prepubertal obesity in children programmed by poor early nutrition.

Between 11 and 14 weeks of age, linear growth rate in LPO slowed to match that of NPO with no indication of overshoot; thus linear catch-up reached 100% and was sustained at that level. Age-adjusted body Wt at harvest (age adjusted to the overall group average 16.5 weeks) in LPO averaged $94.6 \pm 2.4\%$ of sex-matched NPO ($100 \pm 2.4\%$) and was not statistically different. This suggests not only that non-skeletal body mass catch-up was progressing but also that LPO did not progress to become obese between DEXA scanning at 11 weeks of age and at the time of harvest. As late as 14.5 weeks in the serial age-matched data, Wt:Lth ratio remained low in LPO (Fig. 2f). We recognize, however, that as DEXA scans cannot differentiate subcutaneous *v.* intra-abdominal components of truncal fat, we cannot formally exclude differences in LPO intra-abdominal fat depots despite the normal truncal fat mass.

Relevance to developmental programming in human populations

In children born of mothers pregnant during the Dutch Hunger Winter of 1944–1945, obesity risk was enhanced after maternal famine exposure in early gestation, but reduced after exposure in late gestation and first few months of life;[21] the perinatal MPR exposure period applied in microswine LPO may thus be too late to trigger the altered postnatal fat deposition observed after early gestation undernutrition.

The postnatal patterns of growth observed in microswine LPO also exhibit similarities to findings reported in prepubertal Finnish children who went on to develop hypertension in adulthood[7,22]: prepubertal obesity was not a feature of the

https://doi.org/10.1017/S2040174412000037 Published online by Cambridge University Press

accelerated childhood growth, appearing only in later life. However, although linear growth was also accelerated in these Finnish children,[7] it was less pronounced than their growth in mass, leading to a rising BMI between 5–12 years of age. Our swine LPO differ in exhibiting a clear predominance of linear growth acceleration and persistence of thinness throughout the rapid growth period. The dietary fat content of the Finnish children is not known; it would be of interest to learn whether dietary macronutrients have differential effects on mass *v.* linear growth rates. As noted above, prepubertal thinness does not preclude the possibility that increased adiposity develops later in life. Of relevance, Poore and Fowden[23] showed that spontaneous low-birthweight (runt) pigs have normal fat pad thickness at 3 months of age, but increased back fat depth by 12 months of age.

Failed linear growth is a major issue in childhood growth following early insults. It is interesting to consider that, if linear and nonlinear growth components are separately regulated and/or programmed, then understanding how to preserve optimum linear growth may provide a strategy for preventing obesity in children programmed for acceleration of postnatal mass accrual. Importantly, we cannot currently determine whether the pattern of accelerated linear growth with persistence of prepubertal thinness in microswine LPO will have a favorable or unfavorable impact on future risk of obesity and/or other chronic developmentally programmed disorders of adulthood. Pludowski *et al.*, have reported that rapid skeletal maturation in children, regardless of BMI, is linked with increased risk of primary hypertension.[24] As in these children, acceleration of linear growth rate in microswine LPO was at no point linked with excess height or weight *per se*. It is thus conceivable that, as suggested by Pludowski *et al.*, it is the excessively rapid biological maturation that connects hypertension (and potentially other cardiometabolic risks) accompanying accelerated prepubertal growth. Our swine results would also support the conclusion of Pludowski that obesity is not an essential mediator of these relationships.

Although patterns of growth in microswine LPO are similar to some human studies, they are generally distinct from those observed in other animal models. In rodents, depending upon severity and duration of maternal nutrient restriction, either Wt never normalizes or it does so before weaning.[25–28] Moreover, accelerated growth in rodents has been typically accompanied by excess central adiposity even without full Wt catch-up.[25] Such differences may reflect the relatively longer intrauterine exposure window, the use of global caloric *v.* protein restriction or species differences such as a differential allocation of subcutaneous *v.* intra-abdominal adipose tissue.

Feed intake and feed utilization efficiency following MPR

The accelerated growth in LPO was accompanied both by changes in feed intake (both sexes) and, in females only, by increased feed utilization efficiency (i.e. the amount of Wt gained per g feed consumed). As neither male nor female LPO ate absolutely more feed per meal than their sex-matched controls, it is not clear whether the feeding behaviors qualify as true hyperphagia; potentially 'appetite' is normal but dissociated from body mass cues. The reduced plasma leptin levels observed at 2 weeks of age in LPO (reported separately)[2] may have played a role in programming altered neural appetite regulation observed over 6–12 weeks of age.[25,29,30] In any case, feed intake was significantly increased relative to body Wt. There were also sex differences in the feeding responses: male LPO relied solely on the relative increase in feed intake to sustain accelerated growth, consuming the same absolute amounts per meal as their larger male NPO counterparts; in contrast, female LPO utilized both a relative increase in feed intake (but consuming only 80% of the female NPO absolute g feed/meal intake) and a significant increase in feed utilization efficiency to support enhanced growth. As females were more severely affected during MPR in terms of impaired growth in Wt and Lth, the apparent male–female differences may reflect severity of IUGR rather than sex-specific responses.

Possible mechanisms conveying MPR effects

The present report does not shed light on specific mechanisms through which perinatal maternal low-protein/high-carbohydrate diet effects growth restriction. It does, however, highlight the complex sequence of events that evolve during and beyond the window of exposure, suggesting at least four phases during which direct and indirect pathways of developmental programming may take place: (1) during MPR *in utero*; (2) during postnatal MPR (0–2 weeks of age); (3) over 2–5 weeks postnatally wherein normal nutrients are available but growth slowing persists; and (4) during accelerated prepubertal growth (5–20 weeks of age). Of relevance to mechanisms operative during MPR we have recently reported molecular studies in microswine MPR demonstrating a marked epigenetically mediated global reduction in transcriptional activity in kidney cortex of both near-term and 2-week-old neonatal LPO, accompanied by a 20–25% reduction in *per cell* RNA (including ribosomal, transfer and messenger RNA);[31] comparable per cell RNA reductions were also shown in fetal kidneys from an established ovine placental insufficiency model, suggesting a common pathway operative in two distinct IUGR models in two species.[31] If such a profound global change in transcription rate is reflected in protein synthesis, this would substantially contribute to organ growth slowing.

Also relevant to mechanisms potentially active during the *in utero* phase of MPR, studies by others provide strong evidence that maternal nutrient restriction, and specifically MPR, have major detrimental effects on placental growth, vascularization and transport function.[17] In humans[32–34] and animals,[35,36] protein restriction has been linked with reduced placental amino acid transport; in the rat, reduced placental

https://doi.org/10.1017/S2040174412000037 Published online by Cambridge University Press

amino acid transport preceded onset of fetal growth restriction,[37] suggesting that placental dysfunction is a major mediator of slowed fetal growth *in utero*. Specifically in swine, MPR (0.5% isocaloric) reduces placental and endometrial nitric oxide synthase and ornithine decarboxylase activities, factors critical for both placental growth and vascular development.[38]

Finally, studies now under analysis in the microswine MPR model address the fourth potential phase of programming: contribution of accelerated prepubertal growth to vascular and metabolic outcomes in juvenile LPO. Results of global feed restriction suggest that prevention of accelerated growth attenuates mesenteric vascular hyperreactivity to norepinephrine[1] but fails to modify abnormal adipokine regulation.[39]

In summary, we describe a microswine model of perinatal MPR, which results in progressive asymmetric growth restriction during MPR, subsequent acceleration of linear (absolute) and mass (relative to body Wt) growth during prepubertal development, sex-specific alterations in feeding behaviors (relatively increased appetite in all with enhanced feed utilization efficiency in females), and yielding at 11 weeks normal length, normal lean body composition (lean:Lth) with reduced indexes of adiposity (low fat:Lth ratio). The remarkable acceleration of linear growth, despite normal accruals of absolute fat and lean tissue mass on a lower initial body mass, effectively preserved thinness (low Wt:Lth), the latter based largely on low adiposity (low fat:Lth) with protection of lean mass (lean:Lth). The microswine model may be especially useful for investigating how nutritionally programmed growth acceleration, independently of obesity, interacts with and contributes to adverse cardiovascular and metabolic outcomes. Moreover, the model permits testing whether differential regulation of linear *v.* mass tissue accruals might offer new therapeutic strategies to prevent prepubertal obesity.

Acknowledgments

Work was supported by NIH/NICHD 1P01 HD34430, R01 HD042570, Medical Research Foundation of Oregon and the Eagles Foundation of Spokane, WA, USA. E.A. DuPriest was supported by American Heart Association Pacific Mountain Affiliate Predoctoral Fellowship Award No. 0415530Z and by the Oregon Health & Science University Tartar Trust Fellowship.

Supplementary materials

For Supplementary material referred to in this article, please visit http://dx.doi.org/doi:10.1017/S2040174412000037

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