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Gender-specific early postnatal catch-up growth after intrauterine growth retardation by food restriction in swine with obesity/leptin resistance

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Abstract

The effects of undernutrition during pregnancy on prenatal and postnatal development of the offspring were evaluated in sows with obesity/leptin resistance. Females were fed, from day 35 of pregnancy onwards, a diet fulfilling either 100% (group control, $n=10$) or 50% of the nutritional requirements (group underfed, $n=10$). In the control group, maternal body weight increased during pregnancy ($P<0.05$) while it decreased or remained steady in the underfed group. At days 75 and 100 of gestation, plasma triglycerides were lower but urea levels were higher in restricted than in control sows ($P<0.05$ for both). Assessment of the offspring indicated that the trunk diameter was always smaller in the restricted group ($P<0.01$ at day 50, $P<0.005$ at days 75 and 100 and $P<0.0001$ at birth) while head measurements were similar through pregnancy, although smaller in the restricted than in the control group at birth ($P<0.05$). Newborns from restricted sows were also lighter than offspring from control females ($P<0.01$) and had higher incidence of growth retardation ($P<0.01$). Afterwards, during lactation, early postnatal growth in restricted piglets was modulated by gender. At weaning, males from restricted sows were still lighter than their control counterparts ($P<0.05$), while females from control and underfed sows were similar. Thus, the current study indicates a gender-related differential effect in the growth patterns of the piglets, with females from restricted sows evidencing catch-up growth to neutralise prenatal retardation and reaching similar development than control counterparts.
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Introduction

The pandemic increase in the prevalence of common obesity and metabolic alterations in human beings has been related to an interaction between genetic and environmental factors (Gonzalez-Bulnes *et al*. 2011). There is increasing evidence that environmental factors influencing obesity and associated diseases are especially critical during prenatal and early postnatal stages (Gonzalez-Bulnes & Ovilo 2012). Prenatal development, both in human and animal species, is dependant on an adequate placental supply of oxygen and nutrients (Wu *et al*. 2006, Vuguin 2007). Placental supply of nutrients is directly related to the nutritional status of the mother. Females with undernutrition will induce undernutrition of the conceptuses, causing deficiencies in their growth leading to intrauterine growth retardation (IUGR) and reduced birth weight. Individuals with IUGR, depending on the diet during the infantile and juvenile stages of life, will continue to be small at maturity or will become obese (Gonzalez-Bulnes & Ovilo 2012).

A large set of interventional studies, based on epidemiological evidences in human beings, have been developed in both laboratory and farm animals. Both rodents and swine are commonly used in obesity studies. Pig has the advantage of sharing several similarities with humans: omnivorous habits, propensity to sedentary behaviour and obesity, similar characteristics of metabolism and cardiovascular system and proportional organ sizes (Douglas 1972, Mahley *et al*. 1975, Lunney 2007, Spurlock & Gabler 2008). Among the different swine genotypes, there are ancient breeds that, conversely to modern lean pigs, have a huge tendency towards fat accumulation. Main examples of fatty pigs are Iberian and Mangalica breeds, which have developed a syndrome of leptin resistance similar to that described in human beings (Martin *et al*. 2008,

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Mizuta *et al*. 2008, Myers *et al*. 2008), with higher plasma leptin levels but higher voluntary food intake and higher trend towards storing excess fat than lean swine breeds (Nieto *et al*. 2002, Ovilo *et al*. 2005, Brüssow *et al*. 2008, Muñoz *et al*. 2009).

Moreover, research on swine has a dual purpose, not only from the viewpoint of biomedicine model but also from the viewpoint of animal production and welfare. Occurrence of IUGR is common for swine, induced either by maternal diets or, mainly, by intrauterine crowding and subsequent placental insufficiency in high-prolific lines (Ashworth *et al*. 2001, Foxcroft *et al*. 2006, Wu *et al*. 2006). From a productive viewpoint, IUGR has a negative impact on profitability. Several studies on lean breeds have demonstrated that low-birth weight offspring have reduced growth potential, poor meat quality and a longer period for achieving market weight than their littermates (Quiniou *et al*. 2002, Bee 2004, Rehfeldt & Kuhn 2006). However, there are no similar studies on fatty pigs. At the same time, recent studies of our group characterise the Iberian pig as a robust, amenable and reliable translational model for studies on obesity, metabolic syndrome and nutrition-associated diseases in humans (Torres-Rovira *et al*. 2012); hence, studies on pregnant Iberian sows could be extrapolated to humans and other animal species.

Thus, the main objective of the current experiment was to test the hypothesis that undernutrition in mothers with leptin resistance would modify their metabolic status and would affect prenatal and postnatal development of their offspring. There are no dynamic and sequential studies of the prenatal development of offspring from females with nutritional restriction, in any species of large animals. Hence, an additional objective was to compare the developmental dynamics of normal and restricted foetuses in swine by means of real-time ultrasonography through screening of the same conceptuses in successive days throughout pregnancy.

Results

Changes in body weight, fatness and metabolic status of the sows

At day 35 of pregnancy (first day of the experimental treatment), both body weight and back-fat depth were similar among sows assigned subsequently to control and underfed groups (Fig. 1). Afterwards, in control females, mean body weight increased between days 35 and 100 of gestation ($P<0.05$). In restricted sows, mean body weight decreased between days 35 and 50 and remained steady until the end of the study; the differences in mean body weight between the groups only reached statistical significance at day 100 ($P<0.05$).

In both control and restricted sows, back-fat depth remained constant between days 35 and 50. Thereafter, values increased between days 50 and 75 ($P<0.05$) and remained steady between days 75 and 100 in control females. In the restricted sows, back-fat depth decreased from days 50 to 100 ($P<0.05$).

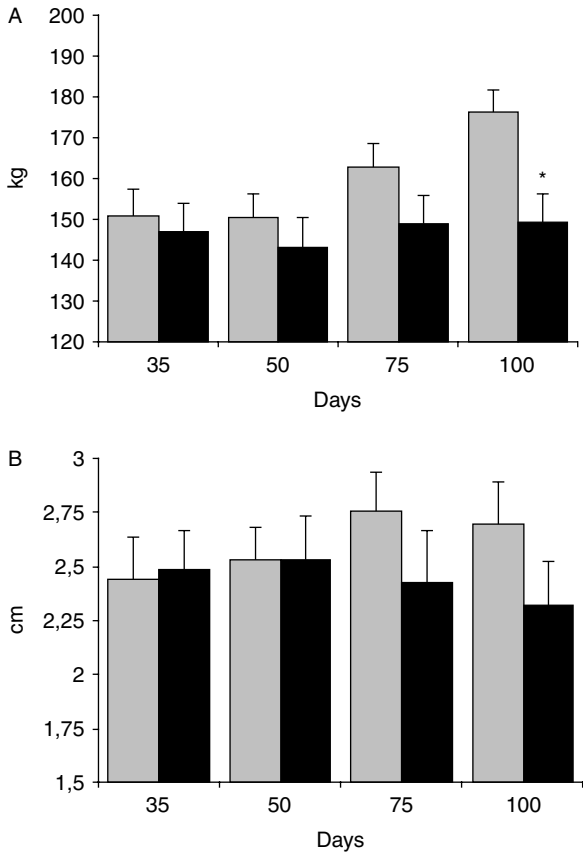

Figure 1 Changes in mean (±S.E.M.) live weight (A) and back-fat depth (B) over time of pregnancy in control sows (grey bars) and sows with restricted intake (black bar). Asterisks indicate significant differences.

The profile of secretion of leptin was similar throughout pregnancy in both groups (Fig. 2), but values were always lower in the underfed females from day 50 of gestation ($P<0.05$). The study of the metabolic features at day 35 showed no significant differences in plasma indexes of the metabolism of carbohydrates, lipids and proteins between animals that were subsequently allocated to the control or the restricted group (Fig. 3). Afterwards, all the sows showed a linear decrease in plasma insulin levels throughout pregnancy ($P<0.05$), without differences between treatments excepting a remarkable decrease in the control group at day 50 ($P<0.01$). On the other hand, comparison of the indexes of lipids metabolism between groups showed that plasma triglycerides were lower in restricted than in control sows at days 75 and 100 ($P<0.05$ for both). Conversely, plasma urea concentrations were higher in restricted sows than in control animals at days 75 and 100 ($P<0.05$).

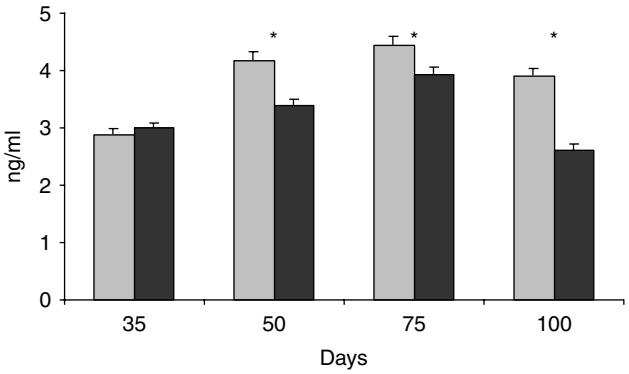

Figure 2 Changes in mean (±S.E.M.) plasma leptin concentrations over time of pregnancy in control sows (grey bars) and sows with restricted intake (black bar). Asterisks indicate significant differences.

Ultrasonographic measurements of conceptus development

There were no significant differences, among sows assigned subsequently to control and restricted groups, in any of the mean values of the transversal diameter of the embryo vesicle, the cranial–rump length, the trunk diameter, the biparietal diameter and the occipito-nasal length at the first ultrasonographic observation at day 35 of pregnancy, when the experimental treatment started.

In successive observations (Fig. 4), there was a significant effect of both time of pregnancy and maternal nutrition on the values for trunk diameter of the foetuses. These values were always higher in the foetuses from control sows than in conceptuses from restricted sows ($P<0.01$ at day 50, $P<0.005$ at days 75 and 100). On the other hand, biparietal diameter and occipito-nasal length were affected by time but not by nutritional treatment throughout pregnancy; the values for head measurements were similar during pregnancy, excepting the biparietal diameter at day 50 (2.1 ± 0.1 vs 1.9 ± 0.1 mm, $P<0.005$). At delivery, the biparietal diameter, the occipito-nasal length and trunk diameter were significantly larger in the piglets born from control females ($P<0.05$ for head measurements and $P<0.0001$ for trunk size) than in piglets from restricted sows.

Evaluation of weight and body measurement of the newborns

There were no significant differences in litter size between control and underfed sows (8.4 ± 0.5 vs 7.6 ± 0.8 piglets). The retrospective analysis of the ultrasonographic scanning performed throughout gestation showed that the litter size was mainly established before day 35 of pregnancy, without remarkable influences of the diet afterwards.

Overall, at farrowing, offspring from control sows were heavier and larger than piglets from restricted females ($P<0.01$). Incidence of IUGR was higher in restricted than in control piglets (43.4 vs 19.3% when considering 1 S.D. and 18.4 vs 1.2% when considering 2 S.D.s, $P<0.01$, in both cases). Asymmetric IUGR (measured through the ratio between head and trunk diameters) was observed in 68.7 and 81.8% of the control and restricted piglets with growth retardation above 1 S.D.

There were no effects of maternal food restriction on gender distribution of the newborns. Comparison of phenotype between genders within treatment showed that control males and females had similar weights and measurements (Table 1). On the other hand, in the restricted group, males were significantly heavier than females ($P<0.01$) but having similar body measurements. The comparison of males and females between treatments showed that all the variables (weight, body length and abdominal and thoracic circumferences) were significantly larger in control males and females than in their restricted counterparts, with the exception of the abdominal circumference in males, in which the difference was not statistically significant. The analysis of newborns with IUGR showed that 31.6% of the female piglets from control and 59.4% of the female piglets from restricted sows evidenced growth retardation on the basis of 1 S.D. below the mean value ($P<0.05$). On the other hand, 9.5% of the male piglets from control and 32.5% of the male piglets from restricted sows evidenced growth retardation ($P<0.005$). Thus, incidence of IUGR was higher in female than in male newborns ($P<0.01$ in both groups).

Thereafter, the assessment of early postnatal growth showed that, overall, control piglets remained heavier and larger than restricted piglets at 21 days old ($P<0.005$). However, differences were determined by the gender of the piglets, as represented in Fig. 5. Values for body weight and length were higher in control than in restricted males ($P<0.05$), as well as the values for thoracic and abdominal circumferences ($P<0.05$ and $P<0.01$ respectively). Conversely, there were no significant differences in body weight and length between control and restricted females, but only in corpulence; control females had larger thoracic and abdominal circumferences ($P<0.05$ for both). This lack of differences between control and restricted females, while males continued to be different between treatments, was caused by a different growth pattern in restricted males and females. As a result, restricted females had similar weight and body measurements than their male littermates at 21 days old.

At 28 days old, control piglets continued to be significantly heavier and more corpulent than restricted piglets ($P<0.001$), but differences in body length had been lost. Effects of gender were still maintained (Fig. 4), as control males were heavier than restricted males ($P<0.05$) and had larger thoracic and abdominal circumferences ($P<0.005$ and $P<0.01$ respectively). In females, only the abdominal circumference remained

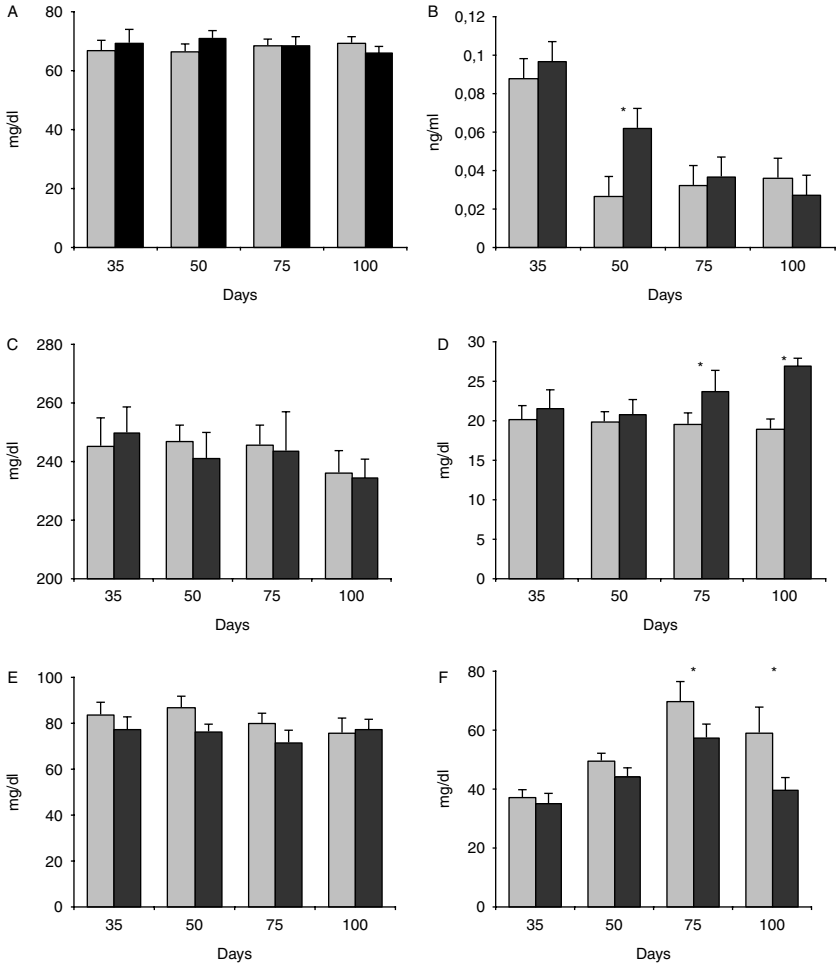

Figure 3 Changes in mean (±S.E.M.) plasma concentrations of glucose (A), insulin (B), fructosamine (C), urea (D), cholesterol (E) and triglycerides (F) over time of pregnancy in control sows (grey bars) and sows with restricted intake (black bar). Asterisks indicate significant differences.

larger in control than in restricted piglets ($P<0.01$). Surprisingly, at 28 days old, restricted females had significantly larger body length and thoracic circumferences than their male littermates ($P<0.05$ for both) and showed a trend towards a larger abdominal circumference ($P=0.09$). Moreover, restricted females were heavier than males (8.1±0.3 vs 7.3±0.4 kg), with differences close to statistical significance ($P=0.08$).

In the same way, the growth of the piglets born with IUGR was also affected by gender. At weaning, only 3.5% of the female piglets from control and 5.9% of the female piglets from restricted sows evidenced growth retardation on the basis of 1 S.D. below the mean value, percentages that were not statistically significant. On the other hand, 12.5% of the male piglets from control and 48% of the male piglets from restricted sows evidenced growth retardation ($P<0.005$ for treatments, $P<0.05$ for gender in the control group and $P<0.005$ for gender in the restricted group).

Discussion

In the present experiment, food restriction in pregnant sows with leptin resistance was found to be related to alterations in the normal pregnancy-related increase in body weight, with mobilisation of fat reserves and even with catabolic metabolism of endogenous protein mass in some of the females. In spite of these changes aiming to protect the developing conceptus, foetal growth was affected in food-restricted females and, in consequence, their newborns have lower body weight and measurements. Afterwards, there was a gender-related differential effect in the growth patterns of these piglets during their early postnatal life and, while growth remained hampered in males from restricted sows, their sisters evidenced catch-up growth and reached similar weight and size to their control counterparts.

The control sows of the current study showed an expected and sustained increase in body weight throughout pregnancy. Increases in body weight during early pregnancy in mammals are caused by fat accumulation, associated with both hyperphagia and increased lipogenesis (Herrera 2000). Increases in body weight during late pregnancy are related to increases in the weight of the foetuses and gestational annexes, while there is an accelerated breakdown of the previously accumulated fat depots for an adequate foetal development (Herrera 2000). In the control females of the

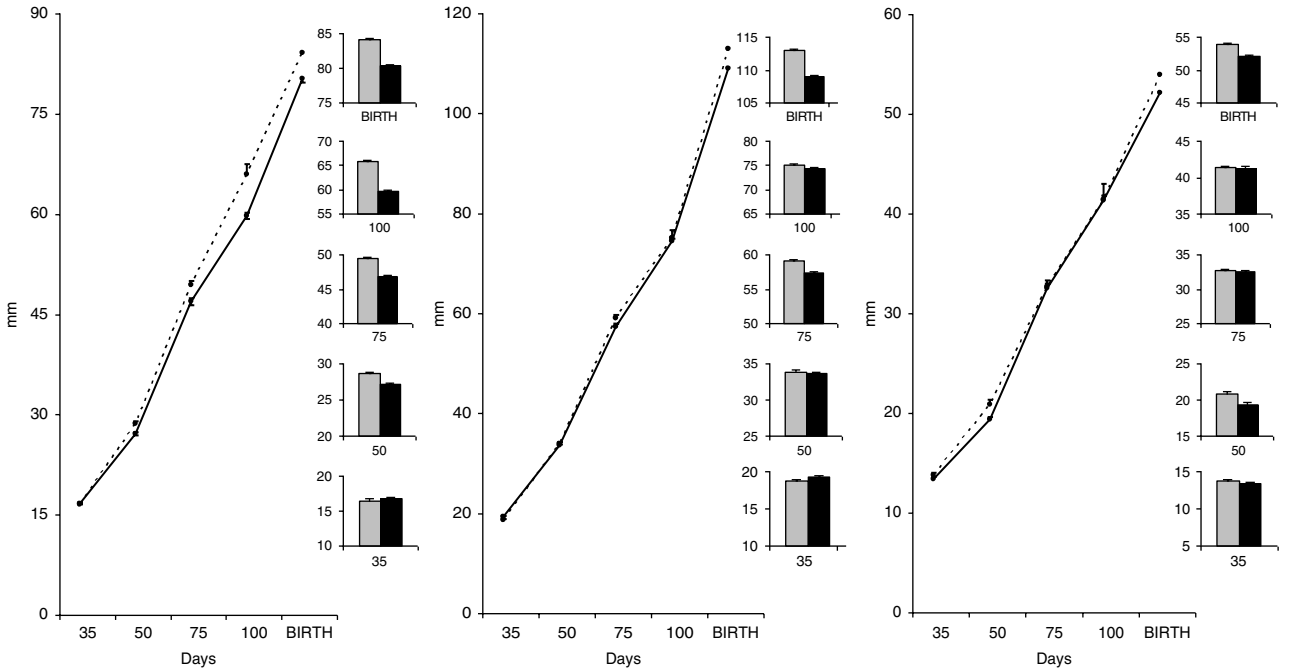

Figure 4 Changes in mean values (±S.E.M.) of trunk diameter (left panel), occipito-nasal length (middle panel) and biparietal diameter (right panel) of embryos/foetuses/newborns over time of pregnancy in control sows (discontinuous line) and sows with restricted intake (continuous line). The figures in the inset represent the comparison of mean values (±S.E.M.) at each time-point.

present experiment, food intake was sufficient for meeting the energy demands for maintenance of the sow and growth of its litter and there was no tissue mobilisation. On the opposite, food intake was clearly insufficient in the group of sows exposed to feed restriction. There were no significant increases in body weight throughout pregnancy in any of the undernourished females (i.e. there was a severe mobilisation of body reserves). All the underfed females performed an intense use of fat depots, as indicated by a clear decrease in back-fat depth throughout pregnancy. At this point, we have to draw attention to the fact that, in this study, we have only measured subcutaneous fat depots. It is known that the Iberian swine is characterised by a high predisposition for accumulating visceral fat (evidenced by magnetic resonance imaging; Gonzalez-Bulnes *et al.* (2011)). A possible employment of visceral fat for catabolism was not possible to be determined in the conditions of our study. In any case, most of the restricted females had a so pressing need for fulfilling energy necessities of pregnancy that used protein mass, as evidenced by higher plasma urea concentrations. Urea is the final product of nitrogen metabolism in all the terrestrial vertebrates (Mahler & Cordes 1966); hence, increases in plasma urea levels of pregnant swine indicate increases in protein catabolism for supplying carbohydrate intermediates and covering gestation requirements (Atinmo *et al.* 1974).

Thus, the undernourished sows were able to compensate energy and carbohydrate requirements of their litters using their fat and even protein reserves. Hence, plasma levels of glucose, the most abundant nutrient crossing the placenta (Shelley *et al.* 1975), were not significantly different between control and restricted females throughout gestation. The same was found when analysing fructosamine, which indicates the amount of glycated protein and is a good index of glucose levels over a precedent period of 2–3 weeks. The analysis of carbohydrate metabolism in the current study also showed a classical pregnancy-associated diabetogenic effect in all the sows, for assuring foetal exposure to high glucose levels, by decreasing insulin secretion throughout pregnancy, an effect early described in lean swine (George *et al.* 1978).

The increasing plasma levels of triglycerides and cholesterol found in control sows of the current experiment is also a characteristic feature during

Table 1 Mean values, at birth, of body weight, body length and thoracic and abdominal circumferences of male and female piglets from control sows and sows with restricted intake.

	Weight (kg)	**Body length** (cm)	**Thoracic circumference** (cm)	**Abdominal circumference** (cm)
Control males	1.48 ± 0.03^{a}	40.0 ± 0.4^{c}	26.7 ± 0.2^{g}	22.9 ± 0.3
Restricted males	1.38 ± 0.03^{b}	38.7 ± 0.3^{d}	25.4 ± 0.3^{h}	22.2 ± 0.3
Control females	1.42 ± 0.02^{g}	39.0 ± 0.3^{a}	26.2 ± 0.2^{e}	22.7 ± 0.2^{a}
Restricted females	1.20 ± 0.04^{h}	38.0 ± 0.4^{b}	25.0 ± 0.3^{f}	21.8 ± 0.4^{b}

$^{a\neq b}P<0.05$; $^{c\neq d}P<0.01$; $^{e\neq f}P<0.005$; $^{g\neq h}P<0.001$.

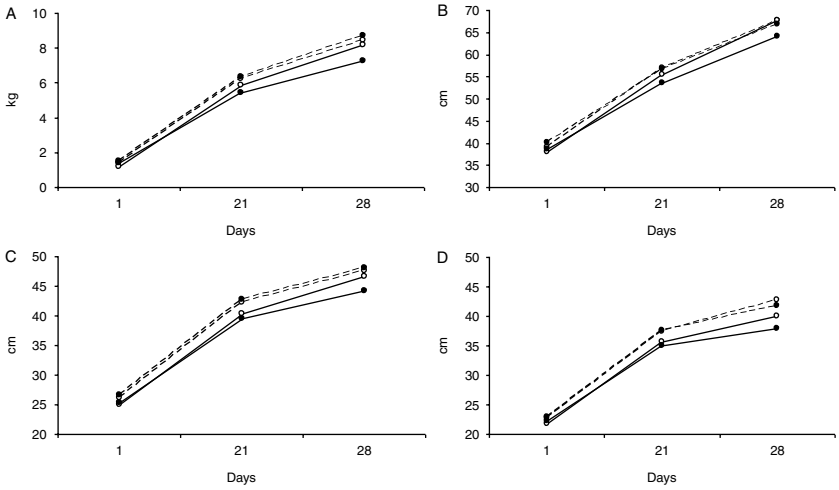

Figure 5 Changes in mean values of body weight (A), body length (B) and thoracic and abdominal circumferences (C and D respectively) of male (black dots) and female (white dots) piglets from control sows (discontinuous line) and sows with restricted intake (continuous line). Values for S.E.M. have been omitted for clarity of the figure.

pregnancy of mammals. Triglycerides are the major source of energy for the developing foetus (Herrera 2002*a*). Maternal hypertriglyceridemia, although triglycerides do not cross the placental barrier and need hydrolysation, assures the release to the foetus of polyunsaturated fatty acids (Szabo *et al*. 1973, Coleman & Haynes 1984, Herrera 2002*a*). A high availability of cholesterol is also essential for the viability and development of the foetus (Wise *et al*. 1993, 1997), as cholesterol is a key constituent of cell membranes and the precursor of hormones and metabolic regulators (Woollett 2001, Palinski 2009). Placental and foetal tissues have the capacity for *de novo* cholesterol synthesis (Wadsack *et al*. 2007); however, the high cholesterol demand in the foetal tissues makes necessary the transport of maternal cholesterol through the placenta (Herrera 2002*b*, Herrera *et al*. 2006).

The importance of cholesterol and triglycerides, like glucose, in the viability and development of conceptuses from sows with leptin resistance has been previously evidenced by our group (Gonzalez-Bulnes *et al*. 2012). In this study, plasma concentration and, by extrapolation, foetal availability of triglycerides was lower in restricted than in control sows.

In this scenario, appearance of IUGR in undernourished sows was predictable. IUGR uses to be classified clinically on the basis of a birth weight below the 10th percentile or under 1 S.D. of the mean of the relevant population or, in experimental studies, as a body weight below 2 S.D.s of the mean of the relevant study population (Anthony *et al*. 2003, Wadsack *et al*. 2007, Blomberg *et al*. 2010). IUGR, both in humans and in animal models, may be classified as 'symmetrical' or 'asymmetrical' (Anthony *et al*. 2003). Symmetrical IUGR is characterised by a uniform reduction of the foetus and its organs from early pregnancy and is associated with genetic and infectious factors. Asymmetrical IUGR is characterised by a reduction in size in some organs, while the remaining organs are normal; the brain is usually less affected by undernutrition and maintains its mass in comparison with the other organs, both in humans and domestic mammals (Yudkin *et al*. 1987, McMillen *et al*. 2001), including swine (Town *et al*. 2004). This is caused by a redistribution of foetal blood flow to protect a key organ like the brain (which is known as the 'brain sparing effect'; Rudolph (1984)) at other organ expenses. Asymmetrical IUGR is often associated with inadequate supply of oxygen and nutrients to the foetus by maternal or placental factors (Wu *et al*. 2006, Vuguin 2007). In swine, like in other species, IUGR is mainly related to metabolic energy deficit (Metges *et al*. 2012). Occurrence of IUGR has also been associated with deficiencies in the physiological gestation-associated hyperlipidemia; in such case, the supply of cholesterol and triglycerides necessary for the adequate development of foetus is not fulfilled (Ruwe *et al*. 1991, Sattar *et al*. 1999, Cetin *et al*. 2002, Metges *et al*. 2012). Surprisingly, the excess of cholesterol and triglycerides also causes alterations in foetal growth (Woollett 2005, 2011). In the present experiment, IUGR in the foetuses from underfed mothers was clearly asymmetric during the entire pregnancy; cranial length and diameter were similar to control foetuses while trunk diameter was significantly lower. At delivery, the mean of these measurements were significantly lower in restricted than in control newborns, which may be related to the fact that the critical changes in size and weight of swine foetuses are occurring at the last 25th day of gestation (Ma & Lindemann 2011) and nutritional supply of our undernourished sows was clearly insufficient at this stage. The individual analysis of the appearance of IUGR, and of the type of IUGR, showed clearly a higher incidence of asymmetrical IUGR in the piglets born from restricted sows.

Thus, the results of the current experiment offer interesting data on metabolic changes in pregnant restricted sows and developmental dynamics of IUGR in their litters to be added to previous information in

lean swine. Occurrence of foetal retardation in swine is largely known (Waldorf *et al*. 1957) and intensively studied and reviewed (Ashworth *et al*. 2001, Bauer *et al*. 2003, Foxcroft *et al*. 2006, Rehfeldt & Kuhn 2006, Wu *et al*. 2006, Foxcroft *et al*. 2009) due to its economical and biomedical importance. Previous studies on lean swine evidence that piglets with lower birth weight have later on lower postnatal weight gains (Quiniou *et al*. 2002, Bee 2004, Rehfeldt & Kuhn 2006).

The most outstanding result of the current experiment was the existence, in piglets with foetal growth retardation, of compensatory postnatal growth and the existence of different patterns in such growth between males and females. Such catch-up growth pattern was developed without food excess. Sows were supplemented but not overfed during lactation; thus, it is expected that piglets were well fed but not overfed. Males continued being smaller in size and weight than control counterparts, while there was appearance of catch-up growth in the females. Catch-up growth is a mechanism beneficial in the short term for counteracting restricted intrauterine growth of the newborn and increasing its possibilities of survival (Gonzalez-Bulnes & Ovilo 2012). However, catch-up growth may also be detrimental in case of exposition to an obesogenic environment, of continued overnutrition, and it would have adverse effects like obesity, hyperleptinemia, hyperinsulinism and hypertension during adult life (Breier *et al*. 2001, Hales & Ozanne 2003, Ross & Desai 2005, Eriksson 2006, Ibañez *et al*. 2006).

Gender-specific differences in compensatory postnatal growth in swine have not been reported or, conversely, data indicate higher growth performance in males (Bee 2004, Bérard *et al*. 2010). The gender-related differences in catch-up growth pattern found in this study have been previously described in laboratory rodents (Oyhenart *et al*. 2003) and human beings (Amador-Licona *et al*. 2007). Overall, girls normalise weight and body mass index (BMI) from the age of 2 years whereas boys remained at a lower BMI than standard (Gohlke *et al*. 2009). Causes for these differences remain to be elucidated and constitutes an interesting field of study. A recent work of Wright *et al*. (2011) in rats indicates the existence of different feeding behaviour between female and male offspring, with females having an increased eating frequency and a delayed transition from feeding to resting. These authors hypothesise that such differences may be driven by changes at hypothalamic level of neurotransmitters controlling food intake; however, further studies are necessary. In any case, it is also interesting to highlight that the course of alterations in metabolic, cardiovascular and renal function in young adult offspring caused by rapid postnatal catch-up growth following IUGR are different in males and females, consequences being usually less harmful in females (O'Regan *et al*. 2004, Boubred *et al*. 2009).

In summary, this study indicates that food restriction during pregnancy in sows with leptin resistance causes IUGR, although sows mobilise fat and even protein depots for minimising the impact on the developing conceptuses. There was a gender-related differential effect in the growth patterns of the suckling piglets, with females evidencing catch-up growth for counteracting IUGR and reaching similar weight and size to control counterparts. Possible causes may be related to differential programming effects on intake/satiety mechanisms that need to be studied. These findings may have wider significance in the context of current controversies on pre- and postnatal nutrition, both in domestic animals and in humans. Results obtained in the Iberian sows of the current study may be extrapolated to other breeds and species, even human beings, and may set the basis for constituting an experimental model for future genomic, epigenomic and metabolomic studies on IUGR.

Materials and Methods

Animals and experimental procedures

The current study was carried out at the facilities of the CIA Deheson del Encinar (Toledo, Spain), under a Project License from the INIA Scientific Ethic Committee. Animal manipulations were performed according to the Spanish Policy for Animal Protection RD1201/05, which meets the European Union Directive 86/609 about the protection of animals used in experimentation.

A total of 20 multiparous (two to four parities) Iberian sows from the Torbiscal strain of this breed, maintained at the CIA, were used for this study. These females were selected, after pregnancy diagnosis at day 35, from a group of animals that were mated after weaning. From weaning to day 35 of pregnancy, sows were fed with 2 kg standard grain-based food diet with mean values of 13.0% of crude protein, 2.8% of fat and 3.00 Mcal/kg metabolisable energy.

At day 35 of pregnancy, the 20 selected experimental sows were weighted and pair matched according to age, number of previous deliveries and body weight. Immediately, each sow was housed in a single pen of 5.49 m^2 until the end of the experiment, when her piglets were weaned. Thus, each female had her own diet individually adjusted to her own weight; every day, each individual food ration was weighted and gave to each sow in her pen.

From day 35 of pregnancy to farrowing, sows were fed with the same standard diet but fulfilling either their daily maintenance requirements for pregnancy (control group, $n=10$) or only the 50% of such quantity (underfed group, $n=10$). From farrowing to weaning, sows were fed with 3 kg standard grain-based food diet with 15.0% of crude protein, 3.1% of fat and 3.10 Mcal/kg metabolisable energy.

Evaluation of changes in body weight, back-fat depth and metabolic state of the sows and changes in phenotypic parameters of the developing embryos/foetuses was performed at four points over time: 35, 50, 75 and 100 days of pregnancy. At farrowing, number and phenotype (body weight and measurements) of all the piglets (live and stillborn)

were determined. Number and causes of deceases from birth to 28 days later were assessed and living piglets were weighed and measured again at 21 and 28 days old.

Evaluation of weight, fatness and metabolic status of the sows

At each observation, the sows were weighed and measured for back-fat depth. Fat depth was determined at P2 point, at the level of the head of the last rib, with a SonoSite S-Series ultrasound machine equipped with a multifrequency (5–8 MHz) lineal array probe (SonoSite, Inc., Bothell, WA, USA). Concurrently, blood samples were drawn, by puncture of the orbital sinus (Huhn *et al*. 1969), in 5 ml sterile heparin blood vacuum tubes (Vacutainer Systems Europe, Meylan, France). Immediately after recovery, the blood was centrifuged at 1500 ***g*** for 15 min and the plasma was separated and stored into polypropylene vials at −20 °C until assayed for determination of leptin and parameters of carbohydrate, protein and lipid metabolism.

Concentrations of leptin were determined in a single analysis using the Multi-species Leptin RIA kit (Demeditec Diagnostics GmbH, Kiel-Wellsee, Germany). The assay sensitivity was 1.0 ng/ml; the intra-assay variation coefficient was 3.1%.

Insulin concentrations were measured with a Porcine Insulin ELISA kit (Mercodia AB, Uppsala, Sweden). The assay sensitivity was 0.26 UI/l; the intra-assay variation coefficient was 3.5%.

Glucose, fructosamine, urea, triglycerides, total cholesterol, high-density lipoprotein cholesterol (HDL-c) and low-density lipoprotein cholesterol (LDL-c) were measured with a clinical chemistry analyser (Screen Point, Hospitex Diagnostics, Sesto Fiorentino, Italy). Plasma HDL-c ratio and LDL-c ratio was calculated by dividing HDL-c and LDL-c concentrations, respectively, by total cholesterol; plasma LDL-c/HDL-c ratio was obtained by dividing LDL-c levels by HDL-c concentrations.

Screening of conceptus development

The development of embryos and later foetuses was determined by measuring trunk diameter and head size (biparietal diameter and occipito-nasal length). At day 35 of pregnancy, it was also possible to measure the transversal diameter of the embryo vesicle and the cranial–rump length.

Observations were performed with a SonoSite S-Series ultrasound machine equipped with a multifrequency (2–5 MHz) sectorial array probe (SonoSite, Inc.). For viewing the uterine horns and conceptuses in transverse, frontal or sagittal planes, ultrasound scans were performed by placing the transducer on one abdominal flank and moving it to the opposite flank. After identifying the image of the foetuses, the probe was slowly moved over each foetus for obtaining the largest measurement of its vesicle, head and trunk. Scans were recorded, using the machine's cine-loop option. Thereafter, the size of the structures of interest (transversal diameter of the embryo vesicle and the cranial–rump length, trunk diameter and head biparietal diameter and occipito-nasal length) was measured with built-in electronic callipers on the cine-loop recordings. To avoid distortions resulting from the corpulence of individual embryo/foetus, measurements were taken from all the conceptuses observed in each scanning.

Evaluation of phenotype and early postnatal development of the piglets

All the piglets (live and stillborns) were sexed and identified with earrings at birth. Immediately, all of them were weighed. Concurrently, trunk diameter and head size (biparietal diameter and occipito-nasal length) were assessed with a hand calliper, while body length and thoracic and abdominal circumferences were measured with a measure-tape. Piglets remained with their mothers in their individual pens until weaning. Body measurements of the piglets (weight, body length and thoracic and abdominal circumferences) were assessed again at 21 days old (the usual data for weaning piglets under intensive conditions) and 28 days old (when weaning was performed, according to traditional management of Iberian sows).

Statistical analyses

Effects of diet on body weight, fat content and metabolic features of the sows were assessed by ANOVA for repeated measures (split-plot ANOVA), while changes over time were measured by Pearson correlation procedures. Ultrasonographic data were grouped according to the day of gestation and a statistical study was carried out using standard linear and quadratic analyses. The scale for determining IUGR was adapted on the basis of a birth weight both below 1 or 2 S.D.s of the mean of the control values (Anthony *et al*. 2003, Wadsack *et al*. 2007, Blomberg *et al*. 2010); asymmetric IUGR was considered when the ratio between head and trunk diameters exceeded 2 S.D.s of the mean of the control values. The effects of the maternal diet on number and phenotype of embryos, foetuses, newborns and later growing piglets were also tested by within-litter split-plot ANOVA for avoiding effects of litter size. All the results were expressed as mean ±S.E.M. and statistical significance was accepted for $P \leq 0.05$.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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