

Impaired insulin secretion in perfused pancreases isolated from offspring of protein malnourished rats

M.P.G. BARNETT¹, A.R.J. PHILLIPS, P.M. HARRIS² AND G.J.S. COOPER

School of Biological Sciences, The University of Auckland, Private Bag 92019, Auckland, New Zealand.

ABSTRACT

Insufficient maternal protein intake may contribute to changes in carbohydrate metabolism and subsequent diabetes mellitus in adult mammalian progeny. Here, female rats (a model of mammalian metabolism) were fed throughout pregnancy and lactation with otherwise-complete isocaloric diets sufficient (20% whey protein, control (C)) or insufficient (5% whey protein, low-protein (LP)) in protein. From weaning, all offspring ate diet C. Weight gain of LP mothers during gestation was less than C mothers (C: 117 g vs. LP: 78 g, SED = 9, $P < 0.001$). There was no difference in offspring birth weight, total weight gain or food consumption. Total insulin secretion from perfused pancreases isolated from LP offspring was decreased (C: 162 pmol vs. LP: 64 pmol, SED = 28, $P < 0.001$), while studies in skeletal muscle demonstrated no difference in insulin sensitivity between the two groups. We conclude that dietary protein insufficiency in female rats during pregnancy and lactation can evoke a persistent functional abnormality in the endocrine pancreas of the progeny independent of bodyweight. This result is compared to previously published data, and the relevance to animal production is discussed.

Keywords: protein; programming; insulin; endocrine pancreas; rat.

INTRODUCTION

Programming (Bertram & Hanson, 2001) is a process whereby poor early nutrition leads to permanent changes in an organism (Barker, 2001). Early adaptations to nutritional stress permanently alter the physiology and metabolism of an organ such that these changes continue to be expressed in the absence of the original causative events (Patel & Srinivasan, 2002). In humans, such changes are postulated to contribute to later disease, including type 2 diabetes mellitus (Bertram & Hanson, 2001), cardiovascular disease (Barker *et al.*, 1993) and hypertension (Godfrey *et al.*, 1993).

Timing of nutritional stress appears to be critical, with earlier events more likely to cause permanent changes (McCance & Widdowson, 1974). Therefore, foetal and early neonatal life are of particular interest. Numerous studies in rats have demonstrated that low maternal protein intake during gestation can result in low birth weight (Snoeck *et al.*, 1990; Corstius *et al.*, 2005) and subsequent metabolic disturbances in adulthood, including high blood pressure (McMullen & Langley-Evans, 2005), altered glucose metabolism (Burns *et al.*, 1997) and insulin resistance (Ozanne, 1999). Similarly, in sheep, food restriction in late gestation affects insulin-glucose homeostasis in adult offspring (Gardner *et al.*, 2005). Additional studies are warranted to explore the relationships linking impaired early nutrition to the postnatal development of disease.

Using a rat model of animal metabolism, we tested the hypothesis that decreased maternal protein intake during pregnancy and lactation results in a metabolic insult sufficient to cause impaired pancreatic function and altered glucose metabolism in adult offspring. Female rats were fed diets either sufficient or insufficient in protein through gestation and lactation, and pancreatic function and muscle glucose metabolism were studied in the offspring.

MATERIALS AND METHODS

Animals

Virgin female Sprague-Dawley rats were kept at $21 \pm 1^\circ\text{C}$, subjected to a 12:12 hour light:dark cycle. Standard laboratory chow (NRM diet 86, 18.5% crude protein, 5% fat) was supplied *ad libitum* prior to mating. Females in oestrus were randomly selected and mated with one of three males. A positive pregnancy was determined by the appearance of a vaginal plug, at which stage rats were transferred to individual cages and alternately assigned one of two isocaloric semi-synthetic diets containing either 20% ("control", C), or 5% ("low-protein", LP) whey protein. Dams were assigned such that offspring of each male would be represented in both of the diet groups, to ensure any observed results were not due to a simple genetic effect. Diets were prepared in the laboratory from commercially available ingredients, and freeze-dried into pellet form.

¹Metabolism & Microbial Genomics, AgResearch Limited, Grasslands Research Centre, Palmerston North, New Zealand.

²AgResearch Limited, Ruakura Research Centre, Private Bag 3123, Hamilton, New Zealand.

Compositional analyses were performed on the diets (Table 1). Throughout gestation, food intake was restricted to 95% of *ad libitum* feeding (as determined in a previous feeding trial, data not shown) to prevent compensatory feeding in the LP group, which has been observed (Rogers, 1979). All animals had free access to water.

Following spontaneous delivery of pups (day 22-23 of gestation), mothers were maintained *ad libitum* on the same diet as supplied during gestation. At 21 days of age, all pups were weaned onto diet C, supplied *ad libitum* for the remainder of the trial.

TABLE 1: Composition and analysis of experimental diets. Diets were prepared by the authors using commercially available ingredients listed in the table.

	Diet C	Diet LP
Ingredient (g/100 g diet)		
Cellulose	5.0	5.0
Cornflour	47.8	62.8
DL-methionine	0.2	0.2
Mineral mix	3.9	3.9
Whey protein	20.0	5.0
Vegetable oil	7.0	7.0
Sucrose	15.0	15.0
Vitamin mix	1.1	1.1
Analysis (g/100 g diet)		
Nitrogen	3.0	0.9
Total protein	19.1	5.7
Lipid	7.7	7.3
Carbohydrate	73.4	87.0
NME (MJ / kg diet)	16.9	17.1

Food intake and body weight

Food intake and bodyweight of mothers and offspring were measured daily. Gestation was divided into two equal periods (equivalent to the first and second trimesters of human pregnancy (Qiang *et al.*, 2002)) to determine if any observations in the offspring were due to differences in maternal intake or bodyweight during a specific part of gestation.

For offspring (housed as litters), daily food intake per rat was calculated by dividing total litter intake by the number of rats in the litter. This data was summarized as cumulative food intake per rat from weaning to 50 days of age.

Isolation and perfusion of the pancreas

Fed offspring (male and female, 42 ± 2 d) were used as pancreas donors. A total of 5 ($n = 2$ litters) and 8 ($n = 3$ litters) animals were used from the C and LP groups respectively. Pancreases were prepared according to the method of Grodsky and Fanska (1975). Perfusion was performed

simultaneously through the coeliac and superior mesenteric arteries (flow rate 1.2 mL/min) and effluent was collected without recycling from the cannulated portal vein. Perfusion medium was modified Krebs-Henseleit buffer (KHB, final concentrations (mM): NaCl 112.8, KCl 4.4, KH_2PO_4 1.5, MgSO_4 1.2, CaCl_2 2.3, NaHCO_3 2.93, D-glucose 3), supplemented with Dextran (4% (w/v), MW 71,400; industrial grade, Sigma) and albumin (0.5% (w/v); bovine fraction V, Sigma A-6793). Medium was sterile filtered (0.2 μM Vacuap, Gelman, Ann Arbor, Michigan, USA) before equilibration against 95% O_2 / 5% CO_2 (BOC Gases NZ Ltd., Auckland, New Zealand), and had a final pH of 7.4 at 37°C.

Stimulation of the pancreas and sample collection

Organs were equilibrated with perfusion medium for 20 minutes. For the following 90 minutes, portal vein effluent fractions were collected at one-minute intervals and stored on ice. Gland stimulation was by side arm infusions (0.05 mL/min) of glucose (final concentration 21.7 mM), arginine (final concentration 10.85 mM), or a mixture of the two. Glucose and lactate were measured simultaneously on every second sample immediately after collection using a YSI 2300 Stat Plus glucose/lactate analyser (Yellow Springs Instrument Corporation, Yellow Springs, OH); glucose to ensure that final perfusate concentration was as expected, lactate to confirm pancreas viability (Gedulin *et al.*, 1991). Samples were stored at -80°C until analysis for insulin content.

Insulin radio immunoassay

Perfusate insulin concentration was determined in duplicate for each sample using a double antibody radio immunoassay (RIA). Samples were counted for one minute in a gamma counter (Wizard TM, Wallace, Finland). The minimum detectable concentration was 16 pM, linear range 140-1800 pM, and mean within-assay coefficient of variation (CV) 19% at the EC_{50} .

Insulin-stimulated uptake of glucose into muscle glycogen

Male and female offspring (C: $n = 13$ animals from 3 litters; LP: $n = 20$ animals from 4 litters, 50 ± 2 days) were fasted overnight (16-18 hours) with free access to water. Following anaesthesia, animals were sacrificed by cervical dislocation and the soleus muscle removed from each leg under KHB (Hasegawa *et al.*, 2000) saturated with 95% O_2 / 5% CO_2 . Each muscle was split longitudinally into 2 or 3 strips of approximately equal diameter and equilibrated in

95% O₂ / 5% CO₂-saturated KHB. Up to 5 strips were incubated for 2 hours at 30°C in 10 mL of normal DMEM containing 0.5 µCi of [U-¹⁴C]D-glucose (American Radiolabelled Chemicals Inc., St Louis, Missouri, USA) and one of the following concentrations of insulin: 0, 0.71, 2.37, 7.1, 23.7, 71.0, 237.0 nM. Each muscle strip was snap-frozen in liquid N₂ and freeze-dried for a minimum of 24 hours.

Glycogen extraction and scintillation counting

Freeze-dried muscle was digested in 60% (w/v) KOH. Glycogen was precipitated by addition of cold 95% ethanol, washed twice, dried at 60°C for 2 hours, redissolved in 200 µL deionized water, then mixed with 1.8 mL scintillation fluid and counted for 5 minutes (LS 3801 beta-counter, Beckman Coulter Inc., Fullerton, California, USA).

Statistical methods

Statistical analyses were performed using GenStat® for Windows 5th edition (Lawes Agricultural Trust, 2001) or 6th edition (Lawes Agricultural Trust, 2002). Unless otherwise stated, analysis was a linear mixed model Residual Maximum Likelihood (REML). Diet was the fixed effect, and litter the random effect, with litter size used as a covariate in the analysis of birth weight and offspring growth rates. In all cases, the experimental unit was the mother, thus the n value was determined by the number of litters investigated, rather than the total number of offspring.

For insulin-stimulated uptake of glucose into glycogen, a four-parameter logistic curve was fitted to the data with the equation:

$$y = (a - b) / (1 + (\text{insulin}/c)^d) + b$$

with parameters defined as follows:

- a = asymptote as x → 0 for all d > 0 (i.e., the minimum value)
- b = asymptote as x diverges (i.e., the maximum value)
- c = predicted response midway between the asymptotes (i.e., the effective insulin concentration at which the half-maximal response occurs (EC₅₀))
- d = a function of the rate of change (or slope) of the fitted curve at the point of inflection

Ethical approval

All work was approved by the University of Auckland Animal Ethics Committee, applications N633 and N857.

RESULTS

Table 2 summarizes food intake and body weight data of mothers and offspring.

Food intake of LP mothers was significantly lower than C mothers during the first half of gestation (P<0.01), while there was no difference during the second half of gestation.

Total weight gain of LP mothers through gestation was significantly lower (C: 117 g vs. LP: 78 g, SED = 9, P<0.001) than C mothers primarily due to a significant difference during the first half of gestation (P<0.01). There was no difference between the two groups with respect to weight change during the second half of gestation, or lactation.

In the offspring, there was no significant difference in birth weight, total weight gain, rate of weight gain (either pre- or post-weaning) or food intake post-weaning between the two groups.

TABLE 2: Food intake and body weight data for female Sprague-Dawley rats and their offspring. Mothers were fed isocaloric diets containing either 20% (C) or 5% (LP) whey protein through gestation and lactation. All offspring were weaned onto diet C. Data represent predicted mean values (Residual maximum likelihood, REML), standard error of the difference (SED) and probability (P) for comparisons between C and LP groups. BW = body weight, LWG = liveweight gain. Total number of observations was n = 6 and 8 for the C and LP groups respectively. This applied to offspring as well as dams because the litter was the experimental unit for statistical purposes.

		C	LP	SED	P
Maternal LWG (g)	Gestation (1 st half)	40	10	11	**
	Gestation (2 nd half)	77	68	8	NS
	Lactation	-23	-18	15	NS
Maternal food intake (g)	Gestation (1 st half)	248	219	11	**
	Gestation (2 nd half)	206	191	14	NS
	Lactation	619	530	48	NS
Offspring BW (g)	Birth	5.4	5.4	0.4	NS
Offspring LWG (g/day)	Pre-weaning	1.2	1.0	0.2	NS
	Post-weaning	2.7	2.8	0.8	NS
Offspring food intake (g)	Post-weaning	225	200	17	NS

TABLE 3: Insulin secretion in perfused pancreases isolated from offspring of mothers fed a control (20% whey; C) or low-protein (5% whey; LP) diet during gestation and lactation. Data represent predicted mean values (REML), standard error of the difference (SED) and probability (P) for comparisons between C (5 animals from 2 litters) and LP (8 animals from 3 litters) groups.

		C	LP	SED	P
Insulin secretion (pmol)	Glucose-stimulated	57	20	9	***
	Arginine-stimulated	13	5	4	*
	Glucose + Arginine	87	38	20	*
	Total	162	64	28	***
Mean basal insulin prior to stimulation (nmol/L)		0.16	0.05	0.04	*
First phase secretion (%)	Glucose-stimulated	48	43	6	NS
	Arginine-stimulated	56	51	7	NS
	Glucose + Arginine	40	30	2	***

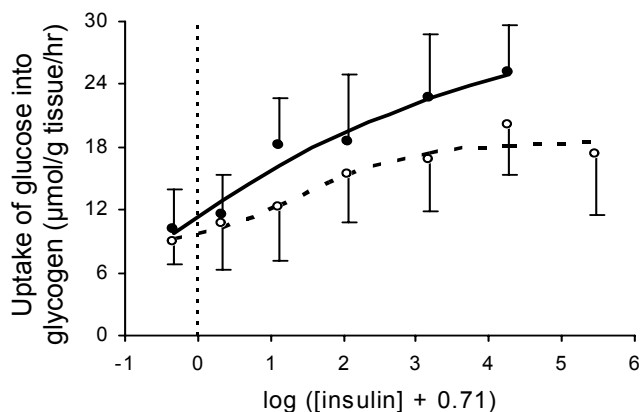
Pancreatic hormone secretion

Total insulin production ($P < 0.001$), mean pre-stimulation insulin secretion ($P < 0.01$) and secretion in response to glucose ($P < 0.001$), arginine ($P < 0.05$) and glucose + arginine ($P < 0.05$) stimulation were significantly reduced in LP offspring (Table 3). In the case of stimulation by glucose + arginine, LP offspring showed a loss of first-phase insulin secretion as a percentage of total secretion during this phase ($P < 0.001$).

Insulin-stimulated uptake of glucose into muscle glycogen

There was no significant difference between the two groups at any insulin concentration, or with respect to the EC_{50} , minimum, maximum or the slope (Fig. 1).

FIGURE 1: Incorporation of ^{14}C -glucose into soleus muscle glycogen in offspring of mothers fed a control (20% whey; C; ●) or low-protein (5% whey; LP; ○) diet during gestation and lactation. Measurements were performed after an overnight (16-18hr) fast. Data points represent the mean value \pm SD for $n = 13$ animals from 3 litters for C group, $n = 20$ animals from 4 litters for LP group. Curves represent a four-parameter logistic model. There was no significant difference between the two groups at any insulin concentration.



DISCUSSION

The pattern of food intake and bodyweight observed in LP mothers was as previously reported, with a decline in intake prior to parturition reflected in the slower weight gain during this period (Petry *et al.*, 2001), while low intake during lactation caused a loss of weight (Kanarek *et al.*, 1986). LP food intake during the first half of gestation was lower than in the C group, contrary to our previous data, where LP mothers consumed less in the latter half of gestation (Barnett *et al.*, 2003).

The decline in food intake prior to parturition and the low intake throughout lactation in C mothers (similar to LP mothers) was at variance with our own studies using standard laboratory chow (data not shown) and commercially prepared diets of the same composition (Barnett *et al.*, 2003), and with reported data (Shirley, 1984). This suggests that protein content of the diet is not the only factor controlling food intake. Diets in the current study were prepared in the laboratory and freeze-dried into pellet form, whereas those from the previous study were commercially prepared and fed as a powder. It is therefore possible that the freeze-drying influenced intake in the control mice. The pattern of maternal bodyweight in the C group reflected the pattern of food intake, with slower growth at the end of gestation and weight loss through lactation, neither of which have been observed in previous studies using a similar diet (Kang *et al.*, 2002).

Offspring in both diet groups (all weaned onto diet C) consumed significantly less than we observed in animals fed standard laboratory chow (data not shown). Bodyweight in both groups was also less than previously reported values (Taconic Technical Library, 1998). The fact that there was

no difference between the two groups in terms of birth weight was at variance with our previous observations (Barnett *et al.*, 2003) using the same diet composition. A number of other studies have reported a lack of difference in birth weight (Zambrano *et al.*, 2005; McMullen & Langley-Evans, 2005).

In the isolated perfused pancreas, LP offspring showed evidence of impaired pancreatic function in response to food (glucose) and surrogate food (arginine) stimuli. The loss of glucose-induced insulin secretion is in agreement with previous studies showing decreased insulin secretion both *in vivo* and *in vitro* (Latorraca *et al.*, 1998). There was also impaired first-phase insulin secretion in response to a combined glucose / arginine stimulation. A loss of first phase glucose-induced insulin secretion is a feature of early type 2 diabetes (Porte & Kahn, 2001) and could be due to either exhaustion of the labile pool of insulin granules or time-dependent inhibition of the secretion response (Nesher & Cerasi, 2002). The lower total insulin secretion observed in LP offspring may be due to one or more factors, including pancreatic islet size, proliferative capacity and vascularisation (Snoeck *et al.*, 1990), beta cell numbers within each islet, or percentage of islet tissue and beta cells within the pancreas (Berney *et al.*, 1997). Studies of the pancreatic morphology of these animals would be required to resolve this issue.

Several studies suggest that an increase in peripheral insulin sensitivity in the offspring of protein-restricted rats may be an adaptive response to reduced insulin secretion (Moura *et al.*, 1997; Latorraca *et al.*, 1998). In these studies, LP offspring were also of significantly lower bodyweight. In the current study, we found no difference between C and LP offspring bodyweight, and no evidence of increased insulin sensitivity in the LP offspring. Programming of muscle insulin sensitivity may therefore be related to programming of bodyweight, while that of the pancreas may be independent of factors influencing bodyweight. Alternatively, programming of the pancreas may be susceptible to the LP diet *per se*, whereas muscle glucose-insulin metabolism may be programmed by reduced total maternal food consumption in the latter half of gestation; data from a sheep model suggest that glucose-insulin homeostasis may be particularly susceptible to gestational undernutrition (Gardner *et al.*, 2005). Further studies in an appropriate ruminant model are required to clarify these issues.

CONCLUSION

These results show a decrease in pancreatic insulin secretion in offspring of female rats fed an LP diet through gestation and lactation, a factor associated with increased susceptibility to type 2 diabetes in adult life. This observation is in accordance with the hypothesis that the foetal / neonatal environment plays a role in programming type 2 diabetes. The change occurred in the absence of any effect of the LP diet on offspring birth weight.

More generally, these results demonstrate the benefits of an adequate maternal diet during pregnancy for the optimal long-term health and well-being of the offspring; this may have important implications for animal production.

ACKNOWLEDGEMENTS

For their assistance the authors thank Jenny Rains (animal handling and timed matings), Dr. Christina Buchanan (perfused pancreas and radioimmunoassay), Drs. Bernard Choong and Mirjana Stokjovic (iodination of hormones), Harold Henderson (statistical analyses and fitting of the four-parameter logistic model), and Drs Nicole Roy and Warren McNabb (constructive criticism of the manuscript). This research was funded by Endocore Research Trust. M. Barnett was funded by AGMARDT, The University of Auckland and Endocore Research Trust.

REFERENCES

- Barker, D.J.P. 2001: The malnourished baby and infant. *British medical bulletin* 60: 69-88.
- Barker, D.J.P.; Osmond, C.; Simmonds, S.J.; Wield, G.A. 1993: The relation of small head circumference and thinness at birth to death from cardiovascular disease in adult life. *British medical journal* 306: 422-426.
- Barnett, M.P.G.; Harris, P.M.; Cooper, G.J.S. 2003: Protein concentration in the rat maternal diet programs appetite and glucose metabolism in the offspring. *Proceedings of the New Zealand society of animal production* 63: 45-48.
- Berney, D.M.; Desai, M.; Palmer, D.J.; Greenwald, S.; Brown, A.; Hales, C.N.; Berry, C.L. 1997: The effects of maternal protein deprivation on the fetal rat pancreas: major structural changes and their recuperation. *The journal of pathology* 183: 109-115.
- Bertram, C.E.; Hanson, M.A. 2001: Animal models and programming of the metabolic syndrome. *British medical bulletin* 60: 103-121.
- Burns, S.P.; Desai, M.; Cohen, R.D.; Hales, C.N.; Iles, R.A.; Germain, J.P.; Going, T.C.H.; Bailey, R.A. 1997: Gluconeogenesis, glucose handling,

- and structural changes in livers of the adult offspring of rats partially deprived of protein during pregnancy and lactation. *Journal of clinical investigation* 100: 1768-1774.
- Corstius, H.B.; Zimanyi, M.A.; Maka, N.; Herath, T.; Thomas, W.; van der Laarse, A.; Wreford, N.G.; Black, M.J. 2005: Effect of intrauterine growth restriction on the number of cardiomyocytes in rat hearts. *Pediatric research* 57(6): 796-800.
- Gardner, D.S.; Tingey, K.; Van Bon, B.W.M.; Ozanne, S.E.; Wilson, V.; Dandrea, J.; Keisler, D.H.; Stephenson, T.; Symonds, M. E. 2005: Programming of glucose-insulin metabolism in adult sheep after maternal undernutrition. *The American journal of physiology* 289: R947-R954.
- Gedulin, B.; Cooper, G.J.S.; Young, A.A. 1991: Amylin secretion from the isolated perfused pancreas: dissociation from insulin and abnormal elevation in insulin-resistant diabetic rats. *Biochemical and biophysical research communications* 180: 782-789.
- Godfrey, K.M.; Barker, D.J.P.; Peace, J.; Cloke, J.; Osmond, C. 1993: Relation of fingerprints and shape of the palm to fetal growth and adult blood pressure [see comments]. *British medical journal* 307: 405-409.
- Grodsky, G.M.; Fanska, R.E. 1975: The in vitro perfused pancreas. *Methods in enzymology* 39: 364-372.
- Hasegawa, T.; Miura, T.; Tsuchida, A.; Miki, T.; Nakano, A.; Kuno, A.; Shimamoto, K. 2000: Endothelium-dependent coronary response is impaired in the myocardium at an early phase of post-infarct remodeling. *Japanese heart journal* 41:743-55.
- Kanarek, R.; Schoenfeld, P.; Morgane, P. 1986: Maternal malnutrition in the rat: effects on food intake and body weight. *Physiology and behaviour* 38: 509-515.
- Kang, K.S.; Che, J.H.; Lee, Y.S. 2002: Lack of adverse effects in the F1 offspring maternally exposed to genistein at human intake dose level. *Food and chemical toxicology: an international journal published for the British industrial biological research association* 40: 43-51.
- Latorraca, M.Q.; Carneiro, E.M.; Boschero, A.C.; Mello, M.A. 1998: Protein deficiency during pregnancy and lactation impairs glucose-induced insulin secretion but increases the sensitivity to insulin in weaned rats. *The British journal of nutrition* 80: 291-297.
- Lawes Agricultural Trust 2001: GenStat for Windows, Release 4.22 (PC/Windows XP)
- Lawes Agricultural Trust 2002: GenStat for Windows, Version 6.1.0.210.
- McCance, R.A.; Widdowson, E.M. 1974: The determinants of growth and form. *Proceedings of the Royal Society of London. Series B, Containing papers of a Biological character. Royal Society (Great Britain)* 185: 1-17.
- McMullen, S.; Langley-Evans, S.C. 2005: Maternal low-protein diet in rat pregnancy programs blood pressure through sex-specific mechanisms. *The American journal of physiology* 288: R85-R90.
- Moura, A.S.; Caldeira Filho, J.S.; de Freitas Mathias, P.C.; de Sa, C.C. 1997: Insulin secretion impairment and insulin sensitivity improvement in adult rats undernourished during early lactation. *Research communications in molecular pathology and pharmacology* 96: 179-192.
- Nesher, R.; Cerasi, E. 2002: Modeling phasic insulin release: immediate and time-dependent effects of glucose. *Diabetes* 51: S53-59.
- Ozanne, S.E. 1999: Programming of hepatic and peripheral tissue insulin sensitivity by maternal protein restriction. *Biochemical society transactions* 27(2):94-97.
- Patel, M.S.; Srinivasan, M. 2002: Metabolic programming: causes and consequences. *The journal of biological chemistry* 277: 1629-1632.
- Petry, C.J.; Ozanne, S.E.; Hales, C.N. 2001: Programming of intermediary metabolism. *Molecular and cellular endocrinology* 185: 81-91.
- Porte, D. Jr.; Kahn, S.E. 2001: Beta-cell dysfunction and failure in type 2 diabetes: potential mechanisms. *Diabetes* 50 supplement 1: S160-163
- Qiang, M.; Wang, M.W.; Elberger, A.J. 2002: Second trimester prenatal alcohol exposure alters development of rat corpus callosum. *Neurotoxicology and teratology* 24: 719-732.
- Rogers, A.E. 1979: Nutrition. In: Baker, H.J.; Lindsey, J.R.; Weisbroth, S.H. ed. *Biology and diseases, volume 2 of the series 'The laboratory rat'*. New York, Academic Press. pp 123-152.
- Shirley, B. 1984. The food intake of rats during pregnancy and lactation. *Laboratory animal science* 34: 169-172
- Snoeck, A.; Remacle, C.; Reusens, B.; Hoet, J.J. 1990: Effect of a low protein diet during pregnancy on the fetal rat endocrine pancreas. *Biology of the neonate* 57: 107-118.
- 'Taconic Technical Library'. 1998: SPRAGUE DAWLEY RATS (Tac:N(SD)fBR) Average control group body weight in grams [Online]. Taconic Farms, Inc. <http://www.taconic.com/addinfo/sdweight.htm> [1998].
- Zambrano, E.; Rodríguez-González, G.L.; Guzmán, C.; García-Becerra, R.; Boeck, L.; Díaz, L.; Menjivar, M.; Larrea, F.; Nathanielsz, P.W. 2005: A maternal low protein diet during pregnancy and lactation in the rat impairs male reproductive development. *The journal of physiology* 563: 275-284.