The Journal of Physiology

# Uteroplacental insufficiency reduces rat plasma leptin concentrations and alters placental leptin transporters: ameliorated with enhanced milk intake and nutrition

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**Key points**

- Uteroplacental insufficiency compromises maternal mammary development, milk production and pup organ development; this is ameliorated by cross-fostering, which improves pup growth and organ development and prevents adult diseases in growth-restricted (Restricted) offspring by enhancing postnatal nutrition.
- Leptin is transported to the fetus from the mother by the placenta; we report reduced plasma leptin concentrations in Restricted fetuses associated with sex-specific alterations in placental leptin transporter expression.
- Pup plasma leptin concentrations were also reduced during suckling, which may suggest reduced milk leptin transport or leptin reabsorption.
- Mothers suckled by Restricted pups had impaired mammary development and changes in milk fatty acid composition with no alterations in milk leptin; cross-fostering restored pup plasma leptin concentrations, which may be correlated to improved milk composition and intake.
- Increased plasma leptin and altered milk fatty acid composition in Restricted pups suckling mothers with normal lactation may improve postnatal growth and prevent adult diseases.

**Abstract** Uteroplacental insufficiency reduces birth weight and adversely affects fetal organ development, increasing adult disease risk. Cross-fostering improves postnatal nutrition and restores these deficits. Mothers with growth-restricted pups have compromised milk production and composition; however, the impact cross-fostering has on milk production and composition is unknown. Plasma leptin concentrations peak during the completion of organogenesis, which occurs postnatally in rats. Leptin is transferred to the fetus via the placenta and to the pup via the lactating mammary gland. This study investigated the effect of uteroplacental insufficiency on pup plasma leptin concentrations and placental leptin transporters. We additionally examined whether cross-fostering improves mammary development, milk composition and pup plasma leptin concentrations. Fetal growth restriction was induced by bilateral uterine vessel ligation surgery on gestation day 18 in Wistar Kyoto rats (termed uteroplacental insufficiency surgery mothers). Growth-restricted (Restricted) fetuses had reduced plasma leptin concentrations, persisting throughout lactation, and sex-specific alterations in placental leptin transporters. Mothers suckled by Restricted pups had impaired mammary development, altered milk fatty acid composition and increased plasma leptin concentrations, despite no changes in

D. H. Hryciw and M. E. Wlodek share senior authorship.

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DOI: 10.1113/JP273825

milk leptin. Milk intake was reduced in Restricted pups suckling uteroplacental insufficiency surgery mothers compared to Restricted pups suckling sham-operated mothers. Cross-fostering Restricted pups onto a sham-operated mother improved postnatal growth and restored plasma leptin concentrations compared to Restricted pups suckling uteroplacental insufficiency surgery mothers. Uteroplacental insufficiency alters leptin homeostasis. This is ameliorated with cross-fostering and enhanced milk fatty acid composition and consumption, which may protect the pups from developing adverse health conditions in adulthood.

(Received 23 November 2016; accepted after revision 8 February 2017)

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**Abbreviations** Akt, protein kinase B; AMPK, AMP-activated protein kinase; E, embryonic day; ELISA, enzyme-linked immunosorbent assay; FAMES, fatty acid methyl esters; FIL, feedback inhibitor of lactation; JAK, Janus kinase; LC-MUFA, long chain monounsaturated fatty acids; LC-PUFA, long chain polyunsaturated fatty acids; mTOR, mammalian target of rapamycin; ObR, leptin receptor; PI3K, phosphoinositide-3-kinase; PN, postnatal; PTHrP, parathyroid hormone related peptide; SOCS, suppressor of cytokine signalling; SRY, sex-determining region Y; STAT, signal transducers and activators of transcription; WKY, Wistar Kyoto (rats).

## Introduction

Being born small is a complication of 10% of pregnancies and results from a reduction in the delivery of nutrients and oxygen across the placenta to the growing fetus, which in Western cultures is commonly due to uteroplacental insufficiency (Barker *et al.* 1989). Exposure to adverse conditions during critical periods of development alters the trajectory and growth of organs, resulting in structural organ changes (McMillen & Robinson, 2005; Barker, 2007; Gluckman *et al.* 2008; Morton *et al.* 2016). Both epidemiological and experimental studies have identified that growth restriction impairs nephron (Hughson *et al.* 2003; Wlodek *et al.* 2007), cardiac (Black *et al.* 2012) and pancreas development (Siebel *et al.* 2010) and programmes male adult onset hypertension (Koklu *et al.* 2007; Wlodek *et al.* 2007) and glucose intolerance (Siebel *et al.* 2008), which is prevented with early accelerated growth (Lucas *et al.* 1997; Eriksson *et al.* 2000; Wlodek *et al.* 2007).

The adipokine leptin plays a role in normal pregnancy adaptations and fetal development (Attig *et al.* 2011; Briffa *et al.* 2015*b*). Leptin is predominantly secreted by white adipose tissue, the placenta (to a lesser extend in rodents) and mammary epithelium during lactation (Landt *et al.* 2003; Hama *et al.* 2004), and is transported from the mother to the fetus via the placenta (Smith & Waddell, 2003). After parturition it is not known whether the main source of offspring leptin is due to offspring leptin production from adipose stores or from the maternal milk supply. Research identified that milk leptin concentrations are elevated during peak lactation, which correlates with the period of rapid infant weight gain and height (Doneray *et al.* 2009), suggesting that leptin may play a role in regulating infant growth. Humans born growth restricted have reduced plasma leptin concentrations at birth (Koklu *et al.* 2007; Nezar *et al.* 2009), suggesting that there may be reduced placental leptin transportation. Leptin transportation and signalling occurs via the leptin receptor (ObR), which activates the Janus kinase–signal transducers and activators of transcription (JAK-STAT) pathway (Banks *et al.* 2000), or the scavenger receptor megalin, which activates the phosphoinositide-3-kinase (PI3K) pathway (Ahima *et al.* 1996; Briffa *et al.* 2014, 2015*a*). The mammalian target of rapamycin (mTOR) is downstream of the PI3K pathway and, under low glucose conditions, is inhibited by AMP-activated protein kinase (AMPK), which promotes autophagy (Kim *et al.* 2011) and we have previously demonstrated that leptin can modulate AMPK expression in the renal proximal tubule (opossum kidney cells) (Ahima *et al.* 1996; Briffa *et al.* 2014, 2015*a*). Critically, both of these signalling pathways regulate cellular growth, proliferation and differentiation.

In humans and most mammalian species there is a plasma leptin surge that is associated with the completion of organ maturation and development, which occurs postnatally in the rat (Ahima *et al.* 1998). Leptin antagonism in rats during the leptin surge (postnatal day (PN) 2–PN13) reduces glomerular number and size, and alters pancreatic, ovarian and thymic development (Attig *et al.* 2011). Therefore, it is possible that the impaired organogenesis in pups born small may be causally associated with their reduced plasma leptin concentrations. Interestingly, in rodent models of maternal undernutrition there is a reduction in the postnatal plasma leptin surge (Delahaye *et al.* 2008), as well as reductions in placental ObR (Nüsken *et al.* 2011), protein kinase B (Akt), mTOR and STAT3 (Rosario *et al.* 2011) expression, which is not observed in a model of bilateral uterine vessel ligation (Nüsken *et al.* 2011). This highlights that different models of growth restriction influence fetal leptin

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concentrations and placental leptin signalling targets in varying ways. A major limitation of the studies described above is that they pooled the placentae and did not determine differences between fetal sex, which have been shown to be important by Cuffe *et al.* (2012, 2014). Changes in placental leptin signalling through ObR and megalin following uteroplacental insufficiency, the major cause of growth restriction in the Western world, are unknown.

Postnatal growth rate and development is directly proportional to the quality and quantity of milk produced by the mammary gland, which has a major influence on disease risk. Adequate fatty acid intake during pregnancy and lactation is essential for normal pup growth and development (Mennitti *et al.* 2015). Uteroplacental insufficiency in rat dams results in lower circulating progesterone concentrations and dams experience premature lactogenesis II, characterised by an increase in $\alpha$-lactalbumin in late pregnancy (O'Dowd *et al.* 2008). Additionally, uteroplacental insufficiency dams have reduced mammary parathyroid hormone related protein (PTHrP) (O'Dowd *et al.* 2008), which appears to have important roles in mammary branching morphogenesis, calcium transport from the maternal plasma to milk and regulation of mammary blood flow (Philbrick *et al.* 1996). As a consequence, mammary development during pregnancy and mammary gland function after birth are impaired, further slowing postnatal growth (O'Dowd *et al.* 2008). We have previously demonstrated that improving early lactational nutrition, by cross-fostering growth restricted pups onto a mother with normal lactation, restores nephron (Wlodek *et al.* 2007) and cardiomyocyte numbers (Black *et al.* 2012) and pancreas morphology (Siebel *et al.* 2010), and prevents hypertension (Wlodek *et al.* 2007) and glucose intolerance (Siebel *et al.* 2008). As leptin is transferred to the pup by the milk (Dundar *et al.* 2005; Doneray *et al.* 2009), it is possible that this restoration of organogenesis following cross-fostering is due to enhanced leptin delivery as a result of improved postnatal nutrition.

Therefore, the aim of this study was to characterise the effect uteroplacental insufficiency has on placental leptin transporter expression. We additionally investigated the effect uteroplacental insufficiency has on maternal and offspring plasma leptin concentrations and milk leptin concentrations, and whether this is restored by improving pup postnatal nutritional intake through cross-fostering. Finally, we characterised the effect cross-fostering has on milk consumption and mammary development. As there are known sex-specific disease programming effects (Moritz *et al.* 2009; Tare *et al.* 2012; Wadley *et al.* 2013), a further aim was to establish whether sex-specific differences existed.

## Methods

### Ethics approval

All experiments were approved by The University of Melbourne's animal experimentation ethics subcommittee (AEC: 0004138 and 02081) and the La Trobe animal ethics committee (AEC: 12-42) following the National Health and Medical Research Councils (NHMRC) Australian code for the care and use of animals for scientific purposes. The investigators understand the ethical principles under which the journal operates and affirm that our work complies with the animal ethics checklist. Female Wistar Kyoto (WKY) rats (9–13 weeks of age) were obtained from the Animal Resources Centre (Canning Vale, WA, Australia) and provided with a 12 h light–dark cycle at 19–22°C with *ad libitum* access to food and water. Rats were mated and surgery was performed on day 18 of gestation (term = 22 days) as described previously (Wlodek *et al.* 2005). Briefly, F0 pregnant rats were randomly allocated to a sham (offspring termed Control) or uteroplacental insufficiency (offspring termed Restricted) group and were anaesthetised with 4% isoflurane and 650 ml min$^{-1}$ oxygen flow (reduced to 3.2% isoflurane and 250 ml min$^{-1}$ oxygen flow when suturing to aid in the animals' recovery). Uteroplacental insufficiency was induced by bilateral uterine vessel (artery and vein) ligation, which restricts blood supply and nutrient delivery to the fetuses. Sham surgery for the control group was performed in the same manner, except uterine vessels were not ligated.

### Study 1 – developmental timeline

At embryonic day (E) 20 one cohort of pregnant rats were anaesthetised (ketamine (50 mg kg$^{-1}$; Parnell Laboratories, Alexandria, NSW, Australia) and Ilium Xylazil-20 (10 mg kg$^{-1}$; Troy Laboratories, Glendenning, NSW, Australia)), each animal's uterus was exposed and amniotic fluid collected ($n$ = 10–20 mothers per group). Fetuses and pups at PN1 and PN7 (in separate cohorts) were weighed, sex visually determined by the anogenital distance and were killed by decapitation. In a final cohort at PN35, offspring were weighed, anaesthetised (ketamine (50 mg kg$^{-1}$) and Ilium Xylazil-20 (10 mg kg$^{-1}$)) and killed via cardiac puncture. Blood was collected immediately, which was pooled in each litter at E20 or separated by sex in the postnatal ages. The placentae at E20 were weighed, frozen immediately in liquid nitrogen and stored at −80°C for subsequent analysis. For tissue extraction, the placentae chosen were associated with one male and one female from each litter with body weights that were the closest to the litter average, with each sample representing a single animal (i.e. $n$ = 1). Additionally, maternal rats were weighed, anesthetised (ketamine (50 mg kg$^{-1}$) and Ilium

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Xylazil-20 (10 mg kg$^{-1}$)) and killed via cardiac puncture, and blood was collected for subsequent analysis.

To ensure accurate sex recording for pups at E20 a portion of the placenta (labyrinth zone; fetal interface) was incubated in proteinase K (Life Technologies; Mulgrave, VIC, Australia) overnight at 55°C to extract DNA, as described previously (Cuffe *et al.* 2012). 'Real-time' polymerase chain reaction (PCR) was then performed for sex-determining region Y (SRY) using a commercially available TaqMan probe (Rn04224592_u1; NM_012772.1) (Life Technologies).

## Placenta 'real-time' PCR

RNA was extracted from labyrinth and junctional zone placental regions using the Precellys 24 homogeniser (Bertin Technologies; Aix-en-Provence, France) with CK14 ceramic beads and TRIzol (Life Technologies) using a commercially available kit (Norgen; Cambridge, ON, Canada). First strand cDNA was generated from 400 ng RNA using the RT$^2$ HT First Strand Kit (Qiagen; Chadstone, VIC, Australia), according to the manufactures instructions. 'Real-time' PCR was then conducted using SYBR green as the fluorescent agent. Custom RT$^2$ Profiler PCR Arrays were designed and manufactured by Qiagen for the following leptin transporters and signalling targets of interest: *leptin* (NM_013076), *ObR* (NM_012596), *JAK2* (NM_031514), *STAT3* (NM_012747), *STAT5a* (NM_017064), suppressor of cytokine signalling 3 (*SOCS3*; NM_053565), *megalin* (NM_030827), *PI3K* (NM_001106723), *Akt3* (NM_031575), *mTOR* (NM_019906), *AMPK*$\alpha$ (NM_019142) and *AMPK*$\beta$ (NM_022627). To compensate for variations in RNA input amounts and reverse transcriptase efficiency, mRNA abundance of the genes of interest were normalised to the housekeeping gene succinate dehydrogenase subunit A (*SDHA*; NM_130428). HotStart DNA Taq Polymerase was activated by heating the mixture to 95°C for 10 min, then 'real-time' PCR reactions were run for 40 cycles of 95°C for 15 s and 60°C for 60 s. Relative changes in mRNA abundance were quantified using the $2^{-\Delta\Delta CT}$ method and reported in arbitrary units normalised to Control male values for the junctional zone and labyrinth. *SDHA* values were not different between sexes and treatments.

## Placental protein extraction and Western blot analysis

Membrane fractions of protein were extracted from 50 mg of placental labyrinth tissue (Gallo *et al.* 2016). Twenty micrograms of membrane lysate were separated on a 5% Tris-glycine eXtended (TGX) stain-free gel (Bio-Rad Laboratories; Gladesville, NSW, Australia) and transferred onto a nitrocellulose membrane (Bio-Rad Laboratories). Membranes were probed with megalin antibody (1:1000, Santa Cruz Biotechnology; Dallas, TX, USA). Densitometric analysis of the banding was performed using ChemiDoc MP with ImageLab Software (Bio-Rad Laboratories). Protein expression bands of interest were normalised relative to stain-free total protein (Parviainen *et al.* 2013) and expressed as values relative to Control males.

## Study 2 – cross-fostering protocol

In a separate cohort, Restricted and Control pups were cross-fostered 1 day after birth (PN1) onto separate mothers (sham-operated or uteroplacental insufficiency surgery mothers) to yield four treatment groups: Control-on-Control (Control pups suckling sham-operated mothers); Control-on-Restricted (Control pups suckling uteroplacental insufficiency surgery mothers); Restricted-on-Control (Restricted pups suckling a sham-operated mother); Restricted-on-Restricted (Restricted pups suckling a uteroplacental insufficiency surgery mother); $n$ = 12–18 mothers per group as previously described (Wlodek *et al.* 2007; Dickinson *et al.* 2016). Each experimental group had an even pup sex ratio. On the morning of PN6 (mammary development/function, milk composition and calcium studies) and PN7 (plasma leptin analysis), pups were removed from their mother and weighed. Pups were killed via decapitation and blood collected (pooled at PN6 and separated by sex PN7). Aprotinin (Sigma-Aldrich; Castle Hill, NSW, Australia) was added to the tubes used for plasma PTHrP analysis (PN6) (Wlodek *et al.* 2000). One pup per litter was frozen whole and stored at −20°C for body calcium analysis (PN6). Pup stomach contents were collected (PN6) for fatty acid analysis. Mothers were anaesthetised 4–6 h after removal of the pup to allow milk to accumulate and milk was collected following gentle massage of the left mammary gland and teats without the need for hormonal stimulation (Wlodek *et al.* 2003). Mothers were anesthetised (ketamine (50 mg kg$^{-1}$) and Ilium Xylazil-20 (10 mg kg$^{-1}$)) and killed by cardiac puncture with blood collected for subsequent analysis. The mammary glands were dissected and weighed and the right mammary gland snap frozen in liquid nitrogen or fixed in 10% neutral buffered formalin (Perrigo; Balcatta, WA, Australia) for histological analysis.

In another cohort, pups were weighed from birth to weaning (PN35) to determine pup growth and milk intake. Pups were weighed during a 1 h maternal separation period followed by a 3 h re-feeding period. Milk intake was calculated as a percentage of pup body weight (O'Dowd *et al.* 2008) and the area under the milk intake curve was calculated between PN3 and PN17 as an index of milk consumption.

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### Mammary 'real-time' PCR and histology

'Real-time' PCR was used to quantitate milk protein gene expression on PN6 as described previously (O'Dowd *et al.* 2008). RNA was extracted from frozen mammary tissue using the Qiagen RNeasy kit. 'Real-time' PCR was performed using TaqMan as the fluorescent agent against the milk protein genes *PTHrP* (forward primer: 5′-GAG GGC AGA TAC CTA ACT CAG GAA-3′; reverse primer: 5′-TTC CCG GGC GTC TTG A-3′; probe: VIC 5′-AAC AAG GTG GAG ACG TAC AAA GAG CAG CC-3′; NM_012636.1) (Life Technologies), *β-casein* (Rn00567460_m1; J00711.1) (Life Technologies) and *α-lactalbumin* (Rn00561447_m1; NM_012594.1) (Life Technologies). Ribosomal *18S* (forward primer: 5′-CGG CTA CCA CAT CCA AGG AA-3′; reverse primer: 5′-GCT GGA ATT ACC GCG GCT-3′; probe: VIC 5′-TGC TGG CAC CAG ACT TGC CCT C-3′; NM_012636.1) (Life Technologies) was used as an endogenous control and multiplexed with the milk protein genes. For the relative quantification of gene expression, a multiplex comparative threshold cycle method was employed as described previously (Wlodek *et al.* 2005). *18S* values were not different between treatments.

Fixed mammary tissue was processed into paraffin blocks, sectioned at 5 $\mu$m and stained with Haematoxylin and Eosin ($n$ = 4–5 per group). Five sections per sample were analysed for alveolar area and number using ImagePro Software (Medai Cybernetics; Warrendale, PA, USA) (Wlodek *et al.* 2003).

### PTHrP, corticosterone, calcium and electrolyte measurements

Plasma, milk and mammary tissue concentrations of PTHrP were quantified by a N-terminal radioimmunoassay with a minimum detection limit of 2 pmol $l^{-1}$, and intra- and inter-assay coefficients of variation of 4.8% and 13.6%, respectively (Wlodek *et al.* 1995). Plasma corticosterone was measured by enzyme immunoassay validated for direct measurements in diluted plasma following the manufacturer's protocol (Cayman Chemical; Ann Arbor, MI, USA) with a minimum detection limit of 30 pg $ml^{-1}$, and intra- and inter-assay coefficients of variation of 7.4% and 7%, respectively. Total calcium concentrations were determined using colorimetric spectrometry using the Synchron CX-5 Clinical System (Beckman Coulter; Lane Cove, NSW, Australia) and ionic calcium (active or free calcium; regulated by PTHrP), sodium and potassium concentrations were determined using ion selective electrodes correcting for pH (Ciba-Corning model 644; Cambridge, MA, USA) from milk as well as from pup and maternal plasma (Wlodek *et al.* 2000, 2009). Total calcium concentration in the pup body was determined after ashing using the CX-5 Analyser (Wlodek *et al.* 2003; O'Dowd *et al.* 2008). Total protein and lactose concentrations were analysed as described previously (O'Dowd *et al.* 2008).

### Fatty acid analysis

Total fatty acid composition of the milk was determined by the direct trans-esterification method of Lepage & Roy (1986). Briefly, 50–100 $\mu$L of milk was placed into a screw-capped Teflon-lined tube containing C23:0 as an internal standard. After the 1 h trans-esterification procedure and recovery of the fatty acid methyl esters (FAMEs) in the benzene phase, the FAMEs were analysed by capillary gas liquid chromatography. FAMEs were then separated and measured on a Shimadzu (17A) gas chromatograph with flame ionisation detection. A 50 mm $\times$ 0.25 mm BPX-70 fused silica capillary column (SGE Scientific; Ringwood, VIC, Australia) with a film thickness of 0.25 $\mu$m was used in conjunction with a Shimadzu on-column auto-injector. Ultrahigh purity hydrogen was used as a carrier gas at a flowrate of 2 ml $min^{-1}$. A temperature gradient programme was used with an initial temperature of 170°C, increasing at 3°C $min^{-1}$ to 218°C. Identification of the FAMEs was made by comparison with the retention times of chromatography reference standard mixtures (Nu-Chek Prep; Elysian, MN, USA) (Weisinger *et al.* 1999).

### Plasma leptin analysis

Leptin concentrations were determined from the developmental timeline study (E20, PN1, PN7 and PN35) and cross-foster study (PN7) from maternal and pup plasma, amniotic fluid (E20) and maternal milk (cross-foster study; PN7) using an enzyme-linked immunosorbent assay (ELISA) (R&D Systems; Minneapolis, MN, USA) as described previously (Romano *et al.* 2015; Tran *et al.* 2015) with a minimum detection limit of 22 pg $ml^{-1}$, and intra- and inter-assay coefficients of variation of 3.8% and 5.8%, respectively.

### Statistical analysis

For the placental genes, a two-way ANOVA was conducted to determine differences between treatment and sex for each placental region (labyrinth and junctional zone). For amniotic and offspring leptin concentrations (PN1, PN7 and PN35) a two-way ANOVA was performed to identify if there were changes across treatment groups and between sexes. Student's $t$ test was used if an interaction existed in the two-way ANOVA and to identify changes in body and placental weights, as well as maternal and fetal (E20) leptin concentrations.

For the cross-fostering groups, data were analysed with a two-way ANOVA (pup and mother) that provided the

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main effect for statistical differences. If an interaction was present in the two-way ANOVA, the data were split to determine where the difference existed using Student's *t* tests. ANOVA statistical analysis was performed using SPSS Statistics 22 (IBM; St Leonards, NSW, Australia) and Student's *t* tests were performed using Excel (Microsoft; North Ryde, NSW, Australia). All data are presented as means ± SEM and a $P < 0.05$ was assessed as being statistically significant.

## Results

### Study 1 – developmental timeline

**Body and organ weights and leptin concentrations.** There were no differences between treatment groups in maternal body weight at any developmental ages (data not shown). Mothers who underwent uteroplacental insufficiency surgery had lighter fetuses (E20 males 1.78 ± 0.05 g *vs.* 1.40 ± 0.06 g and females 1.73 ± 0.03 g *vs.* 1.40 ± 0.03 g, respectively for Control and Restricted; $P < 0.0001$, Student's *t* test) and gave birth to smaller pups (PN1 males 4.29 ± 0.07 g *vs.* 3.45 ± 0.11 g and females 4.06 ± 0.05 g *vs.* 3.47 ± 0.12 g, respectively for Control and Restricted; $P < 0.0004$, Student's *t* test) which remained smaller than Controls at all ages (PN35 males 78.32 ± 1.88 g *vs.* 68.63 ± 2.99 g and females 69.13 ± 1.92 g *vs.* 63.35 ± 2.46 g, respectively, for Control and Restricted; $P < 0.034$, Student's *t* test). Despite no changes in placental weight (data not shown), fetal–placental ratio was reduced in Restricted fetuses (male 5.87 ± 0.15 *vs.* 4.71 ± 0.20 and female 5.76 ± 0.14 *vs.* 4.52 ± 0.10, respectively, for Control and Restricted; $P = 0.001$, Student's *t* test). Mothers that underwent uteroplacental insufficiency surgery (Restricted) had no changes in plasma leptin concentrations at E20 (Fig. 1*A*), increased plasma leptin concentrations at PN1 and PN7 (Fig. 1*B*, $P < 0.014$, Student's *t* test), but were not different at PN35 (Fig. 1*D*) compared to sham-operated (Control) mothers. Uteroplacental insufficiency reduced fetal plasma leptin concentrations (Fig. 1*A*; $P = 0.045$, Student's *t* test) and postnatal plasma leptin concentrations at all ages (expect PN35 males) (Fig. 1*B*–*D*; $P < 0.0001$, two-way ANOVA main effect (PN1 and PN7) and $P = 0.0045$ Student's *t* test (PN35)). Control females had elevated plasma leptin concentrations at PN35 compared to Control males (Fig. 1*D*; $P = 0.011$, Student's *t* test). Despite the reductions in fetal leptin, amniotic fluid leptin concentrations were unaltered (Fig. 1*A*).

**Placental leptin transporter and signalling expression.** Uteroplacental insufficiency surgery reduced *ObR* expression in the junctional zone and tended to reduce labyrinth *ObR* expression, but this did not reach statistical significance ($P = 0.057$, two-way ANOVA) in either males or females (Fig. 2*A*). *Megalin* mRNA was increased in the placentae of Restricted fetuses in the labyrinth and was reduced in the junctional zone in Restricted females (Fig. 2*B*; $P = 0.044$, Student's *t* test). In the labyrinth of females, *megalin* mRNA expression was higher compared to males (Fig. 2*B*). Megalin protein expression in the labyrinth was increased in females following uteroplacental insufficiency surgery (Fig. 2*C*; $P = 0.004$, Student's *t* test), but not in males. Control females had lower megalin protein expression than Control males (Fig. 2*C*; $P = 0.039$, Student's *t* test). The molecular weight of megalin we observed is similar to those reported in several studies (Lebeau *et al.* 2001; Zou *et al.* 2004; Briffa *et al.* 2015*a*), including in placental BeWo cells (Akour *et al.* 2015).

Uteroplacental insufficiency increased junctional zone *leptin* expression in both male and female fetuses (Table 1). Downstream signalling mediators of ObR and megalin were altered in the labyrinth and junctional zone in response to uteroplacental insufficiency (see Table 1). Specifically, uteroplacental insufficiency alters *JAK2*, *STAT3*, *STAT5a*, *AMPKα* and *PI3K* (Restricted females only) (Table 1). Whereas in the junctional zone, following uteroplacental insufficiency surgery, there was a reduction in *SOCS3* and increase in *AMPKα* in Restricted males and females (Table 1). In the junctional zone of females, *STAT5a*, *PI3K*, *Akt3* and *AMPKβ* expression was higher compared to males (Table 1). As shown in Table 1, no treatment effect was observed in the junctional zone expression of *JAK2*, *STAT3*, *STAT5a*, *PI3K*, *Akt3*, *mTOR*, and *AMPKβ* or in the labyrinth expression of *SOCS3*, *Akt3*, *mTOR* and *AMPKβ*.

### Study 2 – cross-fostering

**Body weight.** We have previously demonstrated that uteroplacental insufficiency reduces litter size (Wlodek *et al.* 2007; O'Dowd *et al.* 2008). Consistent with this, cross-fostering litter size was reduced in mothers suckled by Restricted pups at PN6 (Table 2). Restricted pups had reduced body weight at PN1 (Table 2). Restricted pups suckling sham-operated (Control) mothers (Restricted-on-Control) increased their body weight compared to Restricted pups suckling uteroplacental insufficiency surgery (Restricted) mothers (Restricted-on-Restricted) after PN1 in males and after PN10 in females (Table 2). Male and female Restricted pups suckling sham-operated (Control) mothers (Restricted-on-Control) remained smaller than Control pups suckling sham-operated (Control) mothers (Control-on-Control) throughout lactation (Table 2). Cross-fostering a Control pup onto a uteroplacental insufficiency surgery (Restricted) mother (Control-on-Restricted) reduced postnatal body weight after PN1 in males and after PN10 in female offspring

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compared to Control pups suckling sham-operated (Control) mothers (Control-on-Control). Table 2 reports pup growth from birth to weaning.

**Mammary structure and maternal plasma analysis.** Cross-fostering did not alter maternal body weight at PN6 (Table 3). Despite no changes in alveolar number (data not shown), mammary alveolar area and absolute mammary weight were reduced in mothers suckled by Restricted pups (Fig. 3*A* and *B*). Mammary *β-casein* mRNA expression was reduced in uteroplacental insufficiency surgery (Restricted) mothers and in mothers suckled by Restricted pups (Table 3). There were no changes in mammary *α-lactalbumin* and *PTHrP* mRNA expression (Table 3). Mammary PTHrP protein was reduced in mothers suckled by Restricted pups (Fig. 3*C*); however, no changes were observed in maternal plasma and milk PTHrP concentrations (Table 3). Despite a significant interaction in maternal corticosterone concentration (Table 3), *post hoc* analysis did not identify any significant differences, which may be due to the natural variability in corticosterone concentrations.

**Pup plasma analysis and milk consumption.** Milk ionic calcium concentrations were increased in Restricted pups suckling sham-operated (Control) mothers (Restricted-on-Control) compared to Control pups suckling sham-operated (Control) mothers (Control-on-Control) as well as Restricted pups suckling uteroplacental insufficiency surgery (Restricted) mothers (Restricted-on-Restricted) (Table 3; $P = 0.044$ and $P = 0.005$ respectively, Student's $t$ test). Milk total calcium concentrations were reduced in mothers suckled by Restricted pups (Table 3). No changes were

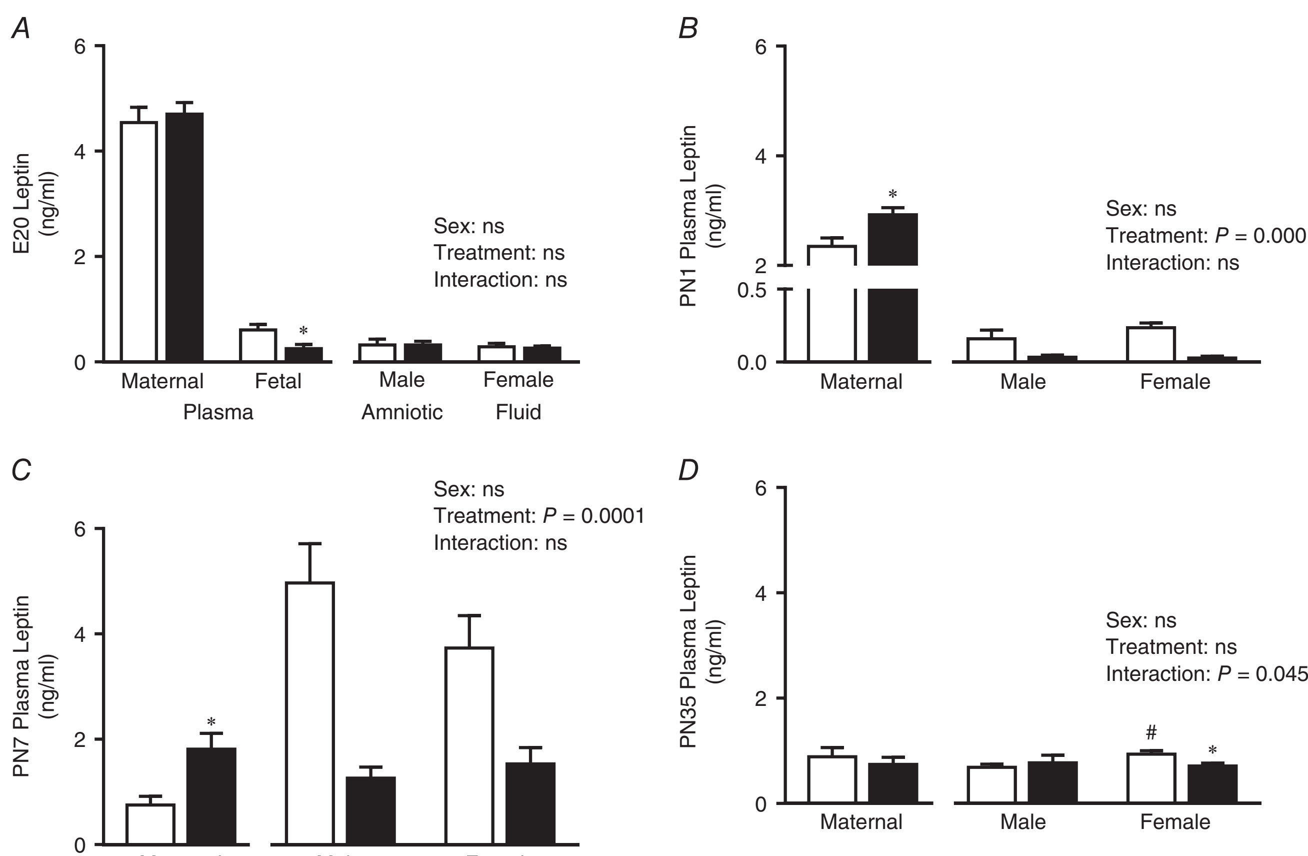


**Figure 1. The effect fetal growth restriction has on plasma and amniotic leptin concentrations**
Maternal and fetal plasma leptin concentrations and amniotic leptin concentrations at embryonic day 20 (E20) (*A*, $n = 5$—11) maternal and pup plasma leptin concentrations on postnatal day (PN) 1 (*B*, $n = 3$–8), PN7 (*C*; $n = 5$–9) and PN35 (*D*; $n = 4$–9). Maternal and fetal (E20) plasma was analysed with a Student's *t* test determine differences between pups (Control *vs*. Restricted) and mothers (sham operated (Control) *vs*. uteroplacental insufficiency surgery (Restricted)) at each time point. Amniotic and offspring plasma was analysed with a two-way ANOVA to determine differences between treatments and sexes, with a Student's *t* test if an interaction was present. Data are expressed as means ± SEM; ns, not significant; *significant differences ($P < 0.05$) between treatment groups; #significant differences between males and females. Control fetuses denoted by open bars; Restricted fetuses denoted by filled bars.

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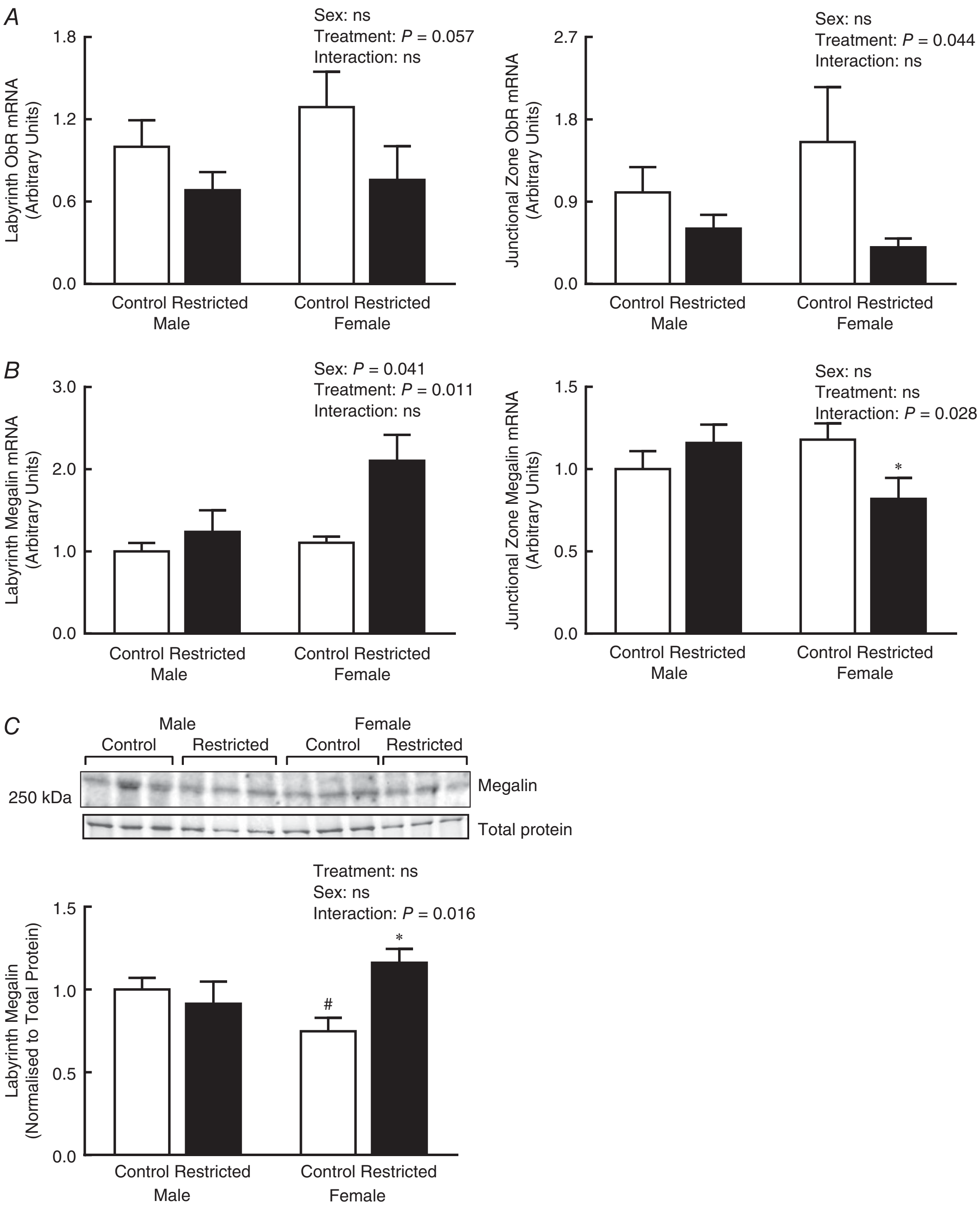


**Figure 2. The effect fetal growth restriction has on placental leptin transporter expression on embryonic day 20**

$n = 7$–8 per group, with $n = 1$ representing 1 pup from 1 litter. Placental gene expression of the leptin receptor (ObR; *A*) and gene (*B*) and protein (*C*) expression of megalin in male (left two bars) and female (right two bars) fetuses in the labyrinth (left graph) and junctional zone (right graph). Protein expression bands of interest were normalised relative to stain-free total protein (Parviainen *et al*. 2013). Gene data were analysed with a two-way ANOVA to determine differences between treatment and sex for each placental region (labyrinth and junctional zone), with a Student's *t* test used to identify where interactions lie. Data are expressed as means ± SEM; ns, not significant; *significant differences ($P < 0.05$) between treatment groups; #significant differences between males and females. Control fetuses denoted by white open bars; Restricted fetuses denoted by filled bars.

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**Table 1. Placental gene expression of downstream pathways activated by the leptin receptors ObR and megalin at embryonic day 20**

| | | Male | | Female | | Two-way ANOVA | | |
|---|---|---|---|---|---|---|---|---|
| | | Control | Restricted | Control | Restricted | Sex | Treatment | Interaction |
| Leptin | Labyrinth | 1.00 ± 0.38 | 0.62 ± 0.14 | 1.27 ± 0.55 | 0.84 ± 0.15 | ns | ns | ns |
| | Junctional zone | 1.00 ± 0.19 | 1.58 ± 0.38 | 1.05 ± 0.21 | 3.23 ± 0.91 | ns | $P = 0.008$ | ns |
| ObR signalling | | | | | | | | |
| JAK2 | Labyrinth | 1.00 ± 0.19 | 1.41 ± 0.10 | 1.13 ± 0.09 | 1.29 ± 0.10 | ns | $P = 0.007$ | ns |
| | Junctional zone | 1.00 ± 0.07 | 0.97 ± 0.03 | 0.99 ± 0.06 | 0.79 ± 0.07 | ns | ns | ns |
| STAT3 | Labyrinth | 1.00 ± 0.12 | 1.54 ± 0.11 | 1.12 ± 0.08 | 1.36 ± 0.13 | ns | $P = 0.002$ | ns |
| | Junctional zone | 1.00 ± 0.08 | 0.76 ± 0.05 | 0.96 ± 0.09 | 0.97 ± 0.09 | ns | ns | ns |
| STAT5a | Labyrinth | 1.00 ± 0.15 | 1.19 ± 0.07 | 0.76 ± 0.10 | 1.39 ± 0.19 | ns | $P = 0.007$ | ns |
| | Junctional zone | 1.00 ± 0.12 | 1.05 ± 0.18 | 1.30 ± 0.11 | 1.80 ± 0.34 | $P = 0.027$ | ns | ns |
| SOCS3 | Labyrinth | 1.00 ± 0.12 | 1.05 ± 0.12 | 1.26 ± 0.12 | 1.06 ± 0.11 | ns | ns | ns |
| | Junctional zone | 1.00 ± 0.20 | 0.60 ± 0.03 | 1.13 ± 0.09 | 0.61 ± 0.09 | ns | $P = 0.001$ | ns |
| Megalin signalling | | | | | | | | |
| PI3K | Labyrinth | 1.00 ± 0.17 | 0.91 ± 0.12 | 1.37 ± 0.12 | 0.66 ± 0.04* | ns | $P = 0.003$ | $P = 0.018$ |
| | Junctional zone | 1.00 ± 0.19 | 0.95 ± 0.15 | 1.13 ± 0.23 | 1.81 ± 0.19 | $P = 0.014$ | ns | ns |
| Akt3 | Labyrinth | 1.00 ± 0.10 | 1.34 ± 0.20 | 1.07 ± 0.13 | 1.24 ± 0.11 | ns | ns | ns |
| | Junctional zone | 1.00 ± 0.08 | 1.05 ± 0.09 | 1.24 ± 0.08 | 1.21 ± 0.13 | $P = 0.047$ | ns | ns |
| mTOR | Labyrinth | 1.00 ± 0.07 | 0.91 ± 0.09 | 1.03 ± 0.07 | 0.86 ± 0.08 | ns | ns | ns |
| | Junctional zone | 1.00 ± 0.10 | 0.92 ± 0.07 | 0.96 ± 0.07 | 1.05 ± 0.10 | ns | ns | ns |
| AMPK$\alpha$ | Labyrinth | 1.00 ± 0.13 | 1.24 ± 0.06 | 1.02 ± 0.06 | 1.27 ± 0.13 | ns | $P = 0.039$ | ns |
| | Junctional zone | 1.00 ± 0.10 | 1.34 ± 0.05 | 1.22 ± 0.08 | 1.33 ± 0.06 | ns | $P = 0.006$ | ns |
| AMPK$\beta$ | Labyrinth | 1.00 ± 0.13 | 1.27 ± 0.10 | 1.29 ± 0.13 | 1.48 ± 0.21 | ns | ns | ns |
| | Junctional zone | 1.00 ± 0.12 | 0.94 ± 0.04 | 1.43 ± 0.12 | 1.06 ± 0.14 | $P = 0.023$ | ns | ns |

$n = 7$–8 per group, with $n = 1$ representing 1 pup from 1 litter. Data were analysed with a two-way ANOVA to determine the main differences between treatments and sexes, with a Student's $t$ test used to identify where interactions lie. Data presented as arbitrary units with the mean ± SEM; ns, not significant; *significant differences ($P < 0.05$) between Control and Restricted pups.

observed in milk $Na^+/K^+$ ratio, total protein and lactose across pups or mothers (Table 3). Pup plasma PTHrP concentrations were reduced in uteroplacental insufficiency surgery (Restricted) mothers (Table 3), whereas pup body total calcium (Table 3) and ionic calcium (Fig. 3*D*) were reduced in Restricted pups. Milk intake in both males and females was reduced in Restricted pups suckling uteroplacental insufficiency surgery (Restricted) mothers (Restricted-on-Restricted) compared to both Restricted pups suckling sham-operated (Control) mothers (Restricted-on-Control) and Control pups suckling uteroplacental insufficiency surgery (Restricted) mothers (Control-on-Restricted) (Fig. 3*E* and *F*; $P < 0.013$, Student's $t$ test).

Mothers suckled by Restricted pups had increased plasma leptin concentrations at PN7 (Fig. 4*A*). However, this increase in maternal plasma leptin concentrations did not alter milk leptin concentrations (Fig. 4*B*). Pup plasma leptin concentrations were reduced in Restricted females suckling uteroplacental insufficiency surgery (Restricted) mothers (Restricted-on-Restricted) compared to Control females suckling uteroplacental insufficiency surgery mothers (Control-on-Restricted) and Restricted females suckling sham-operated mothers (Restricted-on-Control; Fig. 4*D*; $P < 0.011$, Student's $t$ test). A similar trend was also observed in male plasma leptin concentrations when we performed subsequent analysis (Fig. 4*C*), but this interaction did not reach statistical significance ($P = 0.077$, two-way ANOVA).

**Milk fatty acid composition.** n-6 Omega fatty acids were increased in the stomach contents of pups suckling uteroplacental insufficiency surgery (Restricted) mothers and in Restricted pups (Table 4). Stomach contents of n-3 Omega fatty acids were increased in Restricted pups (Table 4). The n-3:n-6 ratio tended to show an interaction (Table 4; $P = 0.057$, two-way ANOVA), but it did not reach statistical significance. Total long chain polyunsaturated fatty acids (LC-PUFA) and total long chain monounsaturated fatty acids (LC-MUFA) were increased in the stomach contents of Restricted pups, with total LC-PUFA also increased in pups suckling uteroplacental insufficiency surgery (Restricted) mothers (Table 4). No changes were observed in medium chain saturated fatty acids (Table 4). Long chain saturated fatty acids were reduced in Restricted pups and in pups suckling

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**Table 2. Cross-fostered litter size at postnatal day (PN) 6 and body weight from birth (PN1) to weaning (PN35)**

| Pup-on-mother | | Control-on-Control | Control-on-Restricted | Restricted-on-Control | Restricted-on-Restricted | Two-way ANOVA Pup | Mother | Interaction |
|---|---|---|---|---|---|---|---|---|
| Litter size at PN6 | | 8.9 ± 0.6 | 8.6 ± 0.8 | 4.3 ± 0.6 | 3.1 ± 0.2 | $P = 0.0001$ | ns | ns |
| Body weight (g) | | | | | | | | |
| PN1 | Male | 4.17 ± 0.12 | 4.13 ± 0.10 | 3.68 ± 0.13 | 3.56 ± 0.09 | $P = 0.0001$ | ns | ns |
| | Female | 3.87 ± 0.12 | 3.98 ± 0.06 | 3.46 ± 0.10 | 3.39 ± 0.08 | $P = 0.0001$ | ns | ns |
| PN3 | Male | 5.62 ± 0.11 | 5.42 ± 0.15 | 4.73 ± 0.15 | 4.23 ± 0.23 | $P = 0.0001$ | $P = 0.040$ | ns |
| | Female | 5.38 ± 0.12 | 5.16 ± 0.20 | 4.59 ± 0.14 | 4.33 ± 0.18 | $P = 0.0001$ | ns | ns |
| PN6 | Male | 8.54 ± 0.21 | 8.30 ± 0.23 | 7.13 ± 0.22 | 6.39 ± 0.32 | $P = 0.0001$ | $P = 0.055$ | ns |
| | Female | 8.17 ± 0.21 | 7.91 ± 0.37 | 7.06 ± 0.31 | 6.76 ± 0.24 | $P = 0.0001$ | ns | ns |
| PN10 | Male | 14.6 ± 0.3 | 14.1 ± 0.4 | 12.4 ± 0.4 | 10.8 ± 0.7 | $P = 0.0001$ | $P = 0.045$ | ns |
| | Female | 14.2 ± 0.3 | 13.6 ± 0.6 | 12.2 ± 0.5 | 11.2 ± 0.6 | $P = 0.0001$ | ns | ns |
| PN14 | Male | 22.1 ± 0.5 | 21.2 ± 0.6 | 19.3 ± 0.6 | 16.2 ± 1.0 | $P = 0.0001$ | $P = 0.006$ | ns |
| | Female | 21.7 ± 0.4 | 20.6 ± 0.8 | 19.7 ± 0.8 | 17.1 ± 0.8 | $P = 0.0001$ | $P = 0.008$ | ns |
| PN17 | Male | 27.4 ± 0.6 | 26.3 ± 0.6 | 24.4 ± 0.6 | 20.5 ± 1.3 | $P = 0.0001$ | $P = 0.004$ | ns |
| | Female | 26.9 ± 0.5 | 25.6 ± 0.7 | 24.3 ± 0.7 | 21.2 ± 1.0 | $P = 0.0001$ | $P = 0.007$ | ns |
| PN21 | Male | 34.3 ± 0.6 | 33.6 ± 1.0 | 31.6 ± 0.8 | 26.8 ± 1.6 | $P = 0.0001$ | $P = 0.012$ | ns |
| | Female | 33.8 ± 0.6 | 32.8 ± 1.0 | 31.0 ± 0.8 | 27.1 ± 1.2 | $P = 0.0001$ | $P = 0.014$ | ns |
| PN24 | Male | 43.7 ± 1.1 | 42.3 ± 0.9 | 39.7 ± 1.1 | 33.8 ± 2.1 | $P = 0.0001$ | $P = 0.013$ | ns |
| | Female | 42.1 ± 0.8 | 40.4 ± 1.1 | 38.0 ± 1.0 | 34.3 ± 1.5 | $P = 0.0001$ | $P = 0.028$ | ns |
| PN28 | Male | 58.7 ± 1.2 | 57.4 ± 1.1 | 54.5 ± 2.4 | 45.8 ± 2.7 | $P = 0.0001$ | $P = 0.015$ | ns |
| | Female | 54.9 ± 1.0 | 54.1 ± 1.4 | 51.6 ± 1.4 | 45.6 ± 1.8 | $P = 0.0001$ | $P = 0.024$ | ns |
| PN35 | Male | 87.7 ± 1.7 | 86.6 ± 1.7 | 81.7 ± 1.3 | 73.3 ± 2.8 | $P = 0.0001$ | $P = 0.020$ | ns |
| | Female | 79.1 ± 1.2 | 77.8 ± 1.8 | 74.6 ± 1.4 | 69.1 ± 2.0 | $P = 0.0001$ | $P = 0.039$ | ns |

$n = 12$–18 litter averages per group. Data are analysed with a two-way ANOVA reporting differences between pup and mother groups with a Student's $t$ test used to identify where interactions lie. Data presented as the means ± SEM; ns, not significant.

uteroplacental insufficiency surgery (Restricted) mothers (Table 4). Table 4 presents the cumulative total percentage of fatty acids in the stomach contents. The entire list of fatty acids analysed from the stomach contents are reported in Table S1 in the Supporting information section.

## Discussion

This study has demonstrated that uteroplacental insufficiency in rats reduces pup plasma leptin concentrations, similar to what occurs following human uteroplacental insufficiency (Koklu *et al.* 2007; Nezar *et al.* 2009). Furthermore, this study has also demonstrated sex-specific alterations in placental leptin transporter expression and its downstream signalling pathways following uteroplacental insufficiency, which may impact on leptin transport. This suggests that sex-specific programmed disease risks in pups born small may in part be attributed to changes in placental leptin transportation and signalling. We have previously reported that uteroplacental insufficiency reduces birth weight and the growth trajectory in both male and female pups (O'Dowd *et al.* 2008). This study demonstrates that improving the lactational environment of these Restricted pups, by cross-fostering them onto a sham-operated (Control) mother, restores plasma leptin concentrations and results in 'catch-up' growth, presumably via increased milk intake and nutrient delivery. An important finding of this study is that the pup plays an important role in modulating mammary development and milk fatty acid composition, which may prevent the organ deficits and disease outcomes in Restricted offspring suckling a sham-operated (Control) mother (Restricted-on-Control) (Wlodek *et al.* 2007; Moritz *et al.* 2009).

### Fetal plasma leptin concentrations and placental leptin transporter expression

Leptin transfer from the maternal to fetal circulatory systems is mediated by the placenta (Smith & Waddell, 2003). Currently it remains unknown what proportion of maternally derived leptin is transported to the fetus and which leptin transporter is responsible. Despite no changes in maternal plasma and amniotic leptin concentrations at E20, as well as the increased junctional zone *leptin* mRNA expression, growth-restricted fetuses have reduced plasma leptin concentrations. This increase in *leptin* in the junctional zone (the hormone producing region of the placenta) suggests the placenta may be increasing

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**Table 3. Maternal and pup plasma, mammary and milk composition in the four cross-foster groups on postnatal day 6**

| | | | | | Two-way ANOVA | | |
|---|---|---|---|---|---|---|---|
| Pup-on-mother | Control-on-Control | Control-on-Restricted | Restricted-On-Control | Restricted-on-Restricted | Pup | Mother | Interaction |
| Maternal | | | | | | | |
| Body weight (g) ($n = 8$–9) | 250.9 ± 6.7 | 246.3 ± 5.1 | 239.3 ± 6.7 | 237.7 ± 3.5 | ns | ns | ns |
| Plasma PTHrP (pmol $l^{-1}$) ($n = 7$–9) | 8.2 ± 0.4 | 9.1 ± 1.2 | 9.7 ± 0.7 | 9.5 ± 0.4 | ns | ns | ns |
| Plasma corticosterone (ng $ml^{-1}$) ($n = 4$–8) | 593 ± 59 | 726 ± 106 | 682 ± 71 | 737 ± 37 | ns | ns | $P = 0.034$ |
| Mammary | | | | | | | |
| PTHrP mRNA ($n = 3$–6) | 1.0 ± 0.1 | 0.8 ± 0.3 | 1.4 ± 0.4 | 0.8 ± 0.2 | ns | ns | ns |
| $\alpha$-Lactalbumin mRNA ($n = 5$–7) | 1.2 ± 0.25 | 0.5 ± 0.13 | 0.6 ± 0.04 | 0.7 ± 0.22 | ns | ns | ns |
| $\beta$-Casein mRNA ($n = 5$–7) | 1.2 ± 0.19 | 0.4 ± 0.11 | 0.6 ± 0.10 | 0.3 ± 0.04 | $P = 0.001$ | $P = 0.024$ | ns |
| Milk | | | | | | | |
| PTHrP (pmol $l^{-1}$) ($n = 6$–9) | 1818 ± 166 | 2958 ± 592 | 1912 ± 273 | 2341 ± 434 | ns | ns | ns |
| $Na^+/K^+$ ($n = 7$–8) | 7.6 ± 1.2 | 6.5 ± 1.6 | 5.8 ± 1.0 | 7.4 ± 1.5 | ns | ns | ns |
| Ionic $Ca^{2+}$ (mmol $l^{-1}$) ($n = 6$–8) | 8.9 ± 0.6 | 8.7 ± 1.0 | 11.2 ± 0.8* | 6.9 ± 0.9‡ | ns | $P = 0.013$ | $P = 0.020$ |
| Total calcium (mmol $l^{-1}$) ($n = 5$–8) | 64.7 ± 4.0 | 65.8 ± 3.7 | 52.0 ± 10.9 | 53.7 ± 4.9 | $P = 0.041$ | ns | ns |
| Total protein (mg $l^{-1}$) ($n = 3$–5) | 28.2 ± 4.4 | 22.0 ± 3.7 | 27.9 ± 7.5 | 23.8 ± 2.9 | ns | ns | ns |
| Lactose (mM) ($n = 3$–6) | 30.2 ± 7.9 | 38.4 ± 3.8 | 25.4 ± 6.8 | 28.8 ± 9.0 | ns | ns | ns |
| Pup | | | | | | | |
| Plasma PTHrP (pmol $l^{-1}$) ($n = 6$–9) | 86.0 ± 4.6 | 51.7 ± 12.0 | 88.8 ± 12.7 | 66.3 ± 7.7 | ns | $P = 0.014$ | ns |
| Body total calcium (mmol $g^{-1}$) ($n = 7$–9) | 0.081 ± 0.001 | 0.076 ± 0.002 | 0.073 ± 0.002 | 0.073 ± 0.001 | $P = 0.001$ | ns | ns |

Data were analysed with a two-way ANOVA reporting differences between pup and mother groups with a Student's *t* test used to identify where interactions lie. Data presented as the means ± SEM with sex pooled per litter; ns, not significant; *significant differences ($P < 0.05$) between Control and Restricted pups on the same mother group; ‡significant differences between sham-operated (Control) and uteroplacental insufficiency surgery (Restricted) mothers with the same pup group.

leptin production, but previous rodent studies have demonstrated that placentally derived leptin is not a major source of leptin in the rat (Kawai *et al.* 1997), implying that this upregulation may not be as effective in increasing fetal leptin. An important limitation of this study is that plasma from male and female fetuses was pooled at E20, which may obscure any sex-specific differences at this age. Interestingly, megalin protein expression was only increased in the labyrinth of Restricted females, despite both males and females having increased *megalin* mRNA, which probably suggests alterations in mRNA translation and/or protein degradation in Restricted males. This suggests that the mechanisms regulating leptin concentrations in the fetus are sex-specific, whereby Restricted females may attenuate the reduced transfer of leptin from the maternal interface by increasing leptin transport across the placenta via megalin. As Restricted males have no changes in placental cell surface availability of megalin, it is possible that their reduced fetal plasma leptin concentrations may be due to reduced fetal leptin production and/or reduced fetal adipocyte mass. However, further research is required to elucidate the plasma leptin concentrations in male and female growth restricted fetuses, and to identify the exact mechanism that may modulate any sex-specific alterations in fetal leptin concentrations.

A shortcoming of this study is that we were unable to quantify labyrinth ObR expression with Western blotting. ObR has a large number of glycosylation sites that affects its size (Kamikubo *et al.* 2008), its molecular weight appears to be tissue specific (Guerra *et al.* 2007; Aquila *et al.* 2008), and different physiological states (including pregnancy) can affect its size (Lammert *et al.* 2002), all of which limit the identification of the correct long (ObRb) and short (ObRa) isoforms. As there are several different isoforms of ObR (a–f) it is important for future studies to characterise their expression separately as they all have

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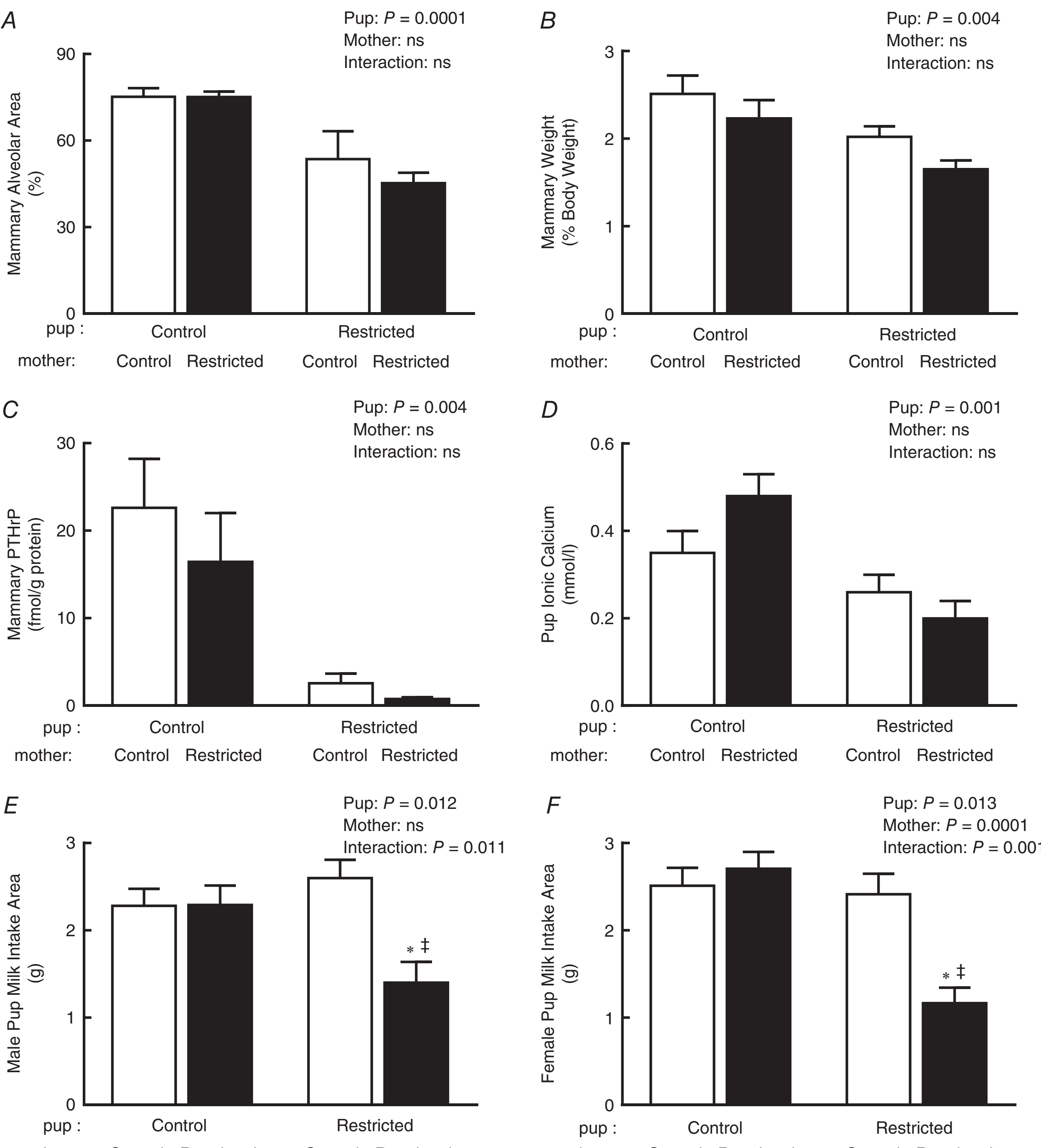


**Figure 3. Cross-fostering effects on mammary structure, pup ionic calcium concentrations and milk consumption at postnatal day 6**

*n* = 6–10 dams or offspring litter averages per group. Mammary alveolar area (*A*) and relative mammary weight (*B*) was reduced in mothers suckled by Restricted pups. Mammary PTHrP content was reduced in mothers suckled by Restricted pups (*C*), which coincided with the reduced pup plasma ionic calcium at postnatal day 6 (*D*). *E* and *F*, milk intake area (*E*, male pups; *F*, female pups) between days 3 and 17 of lactation was reduced in Restricted pups suckling uteroplacental insufficiency surgery (Restricted) mothers compared to both Restricted pups suckled by sham-operated (Control) mothers and Control pups suckled by uteroplacental insufficiency surgery (Restricted) mothers. Data are analysed with a two-way ANOVA reporting differences between Pup and Mother groups and are expressed as means ± SEM; ns, not significant. *significant differences ($P < 0.05$) between Control and Restricted pups on the same mother group; ‡significant differences between sham-operated (Control) and uteroplacental insufficiency surgery (Restricted) mothers with the same pup group. Control pups denoted by open bars; Restricted pups denoted by filled bars.

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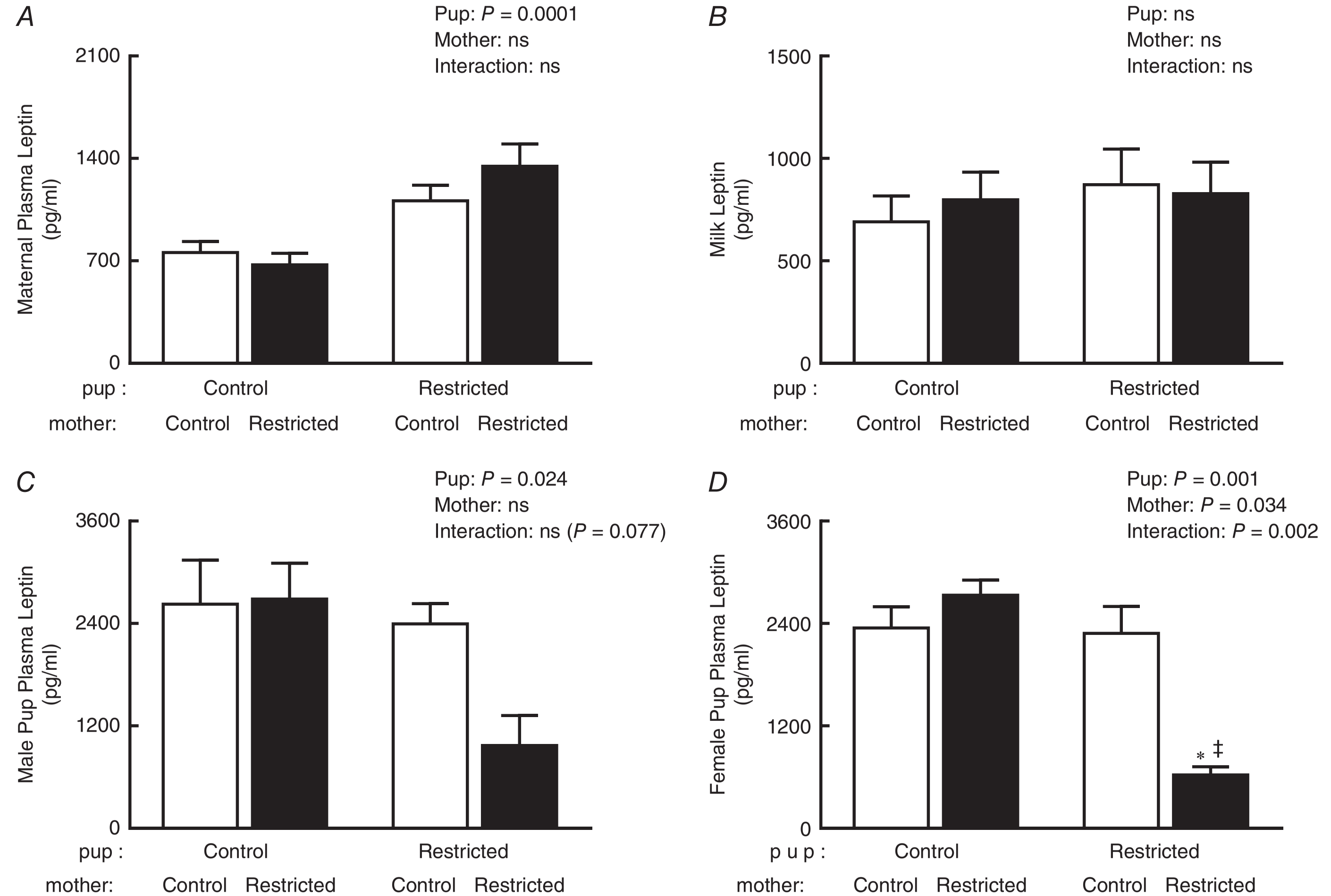


**Figure 4. Cross-fostering effects on maternal, milk and pup leptin concentrations at postnatal day 7**
$n = 4$–8 dams or offspring litter averages per group. *A*, maternal plasma leptin concentrations were increased *in utero*placental insufficiency surgery (Restricted) mothers. Despite no changes in milk leptin concentrations (*B*), plasma leptin concentrations tended to be reduced in Restricted males (*C*), and were significantly reduced in Restricted females suckling uteroplacental insufficiency surgery (Restricted) mothers (*D*). Data were analysed using two-way ANOVA to report differences between Pup and Mother groups and Student's *t* test to identify where interactions lie. Data are expressed as means ± SEM; ns, not significant. *significant differences ($P < 0.05$) between Control and Restricted pups on the same mother group; ‡significant differences between sham-operated (Control) and uteroplacental insufficiency surgery (Restricted) mothers with the same pup group. Control pups denoted by open bars; Restricted pups denoted by filled bars.

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**Table 4. Fatty acid composition in stomach contents of consumed milk presented as a cumulative total percentage of fatty acids on postnatal day 6 in the four cross-foster groups**

| | | | | | Two-way ANOVA | | |
|---|---|---|---|---|---|---|---|
| Pup-on-mother | Control-on-Control | Control-on-Restricted | Restricted-on-Control | Restricted-on-Restricted | Pup | Mother | Interaction |
| Fatty acids | | | | | | | |
| n-6 | 14.4 ± 0.4 | 17.4 ± 0.7 | 18.0 ± 0.7 | 19.6 ± 0.7 | $P = 0.0001$ | $P = 0.001$ | ns |
| n-3 | 2.4 ± 0.09 | 2.4 ± 0.18 | 2.8 ± 0.16 | 3.2 ± 0.09 | $P = 0.0001$ | ns | ns |
| n-3:n-6 | 0.17 ± 0.004 | 0.14 ± 0.006 | 0.16 ± 0.010 | 0.16 ± 0.006 | ns | ns | $P = 0.057$ |
| Total LC-PUFA | 16.8 ± 0.4 | 19.8 ± 0.8 | 20.8 ± 0.7 | 22.8 ± 0.8 | $P = 0.0001$ | $P = 0.002$ | ns |
| Total LC-MUFA | 22.4 ± 0.9 | 22.4 ± 1.4 | 24.4 ± 0.8 | 27.1 ± 1.8 | $P = 0.016$ | ns | ns |
| Saturated fatty acids | | | | | | | |
| Medium (6–12) | 19.8 ± 1.0 | 21.1 ± 0.7 | 21.1 ± 0.6 | 18.9 ± 2.6 | ns | ns | ns |
| Long (14–20) | 40.4 ± 1.1 | 35.2 ± 2.1 | 32.5 ± 1.2 | 29.8 ± 0.5 | $P = 0.0001$ | $P = 0.017$ | ns |

$n = 5$–7 per group, with $n = 1$ representing data from 1 pooled litter. Data were analysed with a two-way ANOVA reporting differences between pup (Control *vs.* Restricted) and mother (sham-operated (Control) *vs.* uteroplacental insufficiency surgery (Restricted)) groups with a Student's $t$ test used to identify where interactions lie. Data presented as the means ± SEM with sex pooled per litter; ns, not significant. Also see Table S1 in Supporting information section for individual fatty acids.

different physiological functions and roles. Specifically, both the long (ObRb) and one of the short (ObRa) isoforms are responsible for transcellular leptin transportation (Frühbeck, 2006), whereas the soluble isoform (ObRe) is responsible for maintaining elevated maternal leptin concentrations during gestation by reducing leptin clearance (Attig *et al.* 2011; Briffa *et al.* 2015*b*). However, only the long isoform (ObRb) has a transmembrane domain that is capable of activating signal transduction pathways (Bjørbaek *et al.* 1997). Importantly, previous studies have demonstrated that these three isoform (ObRa, b and e) are dysregulated in compromised pregnancies (such as maternal glucocoticoid administration) (Smith & Waddell, 2002, 2003).

Only one study to date has investigated changes in amniotic leptin concentrations in growth restricted humans, where it is reduced in small-for-gestational-age babies (Aydın *et al.* 2014). This is in contrast to our finding of no changes in amniotic leptin concentrations. These differences may be due to the study including babies that were born below the third percentile, identifying that these babies were severely growth restricted, whereas our rats were 15–20% lighter. Leptin clearly activates a number of signalling pathways in the placenta, which have previously been linked to trophoblast function. Specifically, STAT3 activity has been correlated with trophoblast invasiveness (Maymó *et al.* 2011) and PI3K has been associated with trophoblast invasive differentiation in early pregnancy (Pérez-Pérez *et al.* 2008). In this study, placentae associated with Restricted fetuses have increases in labyrinth *JAK2*, *STAT3* and *STAT5a* mRNA, which may suggest that the placenta is activating this signalling cascade to maintain normal placental growth in an adverse *in utero* environment. However, the effect these signalling pathways have on placental function in late gestation remains unknown and further research is required to identify whether these gene changes translate to alterations in total and phosphorylated protein expression. Changes in *JAK–STAT* mRNA occurred independently of changes in *ObR* mRNA and the *PI3K* pathway appears to be largely unaffected in response to uteroplacental insufficiency, suggesting that other signalling cascades, which also regulate the expression of these targets, may be dysregulated such as the Forkhead box O that also regulates cell growth, proliferation and differentiation. Despite increases in plasma leptin concentrations at PN1 and PN7 in uteroplacental insufficiency surgery (Restricted) mothers, plasma leptin concentrations remained lower in Restricted pups until weaning (females). This suggests that there may be alterations in pup leptin production and/or milk leptin absorption, which may further adversely impact on pup growth and development postnatally. However, we cannot at this time tell whether the Restricted pups just have reduced plasma leptin concentrations or a delay in the postnatal plasma leptin surge, similar to what is observed following maternal protein restriction (Delahaye *et al.* 2008), which requires future investigation.

## Cross-fostering effects on mammary morphology, milk content and leptin concentrations

A novel finding of this study is that pups can influence maternal physiology. It is important to note that by cross-fostering Restricted offspring onto a sham-operated (Control) mother we are reducing the number of pups the mother has to feed (by approximately 50%). It is possible that this reduction in litter size may confound the interpretation of the findings; however, the reduction

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in litter size is a direct consequence of the uteroplacental insufficiency surgery, and thus we believe we have adequately accounted for this in our statistical approach. Nevertheless, in both sham-operated (Control) and uteroplacental insufficiency surgery (Restricted) mothers suckled by Restricted pups, we characterised a reduction in mammary alveolar area, relative mammary weight and PTHrP protein. Reductions in maternal PTHrP protein would likely reduce pup plasma PTHrP; however, PTHrP concentrations in the pups were modulated by the mother, such that pups suckling uteroplacental insufficiency surgery (Restricted) mothers had reduced PTHrP concentrations. Despite pup PTHrP being modulated by the mother, Restricted pups had reduced plasma calcium concentrations and total body calcium, which are likely to be due to PTHrP's influence on calcium transport from the maternal blood into milk. This finding strongly suggests that the phenotype and impairment in pup growth is not driven solely by the mother or the pup, but by both. One possible mechanism by which the pup can influence maternal milk production is through the feedback inhibitor of lactation (FIL) (Wilde *et al.* 1998). Milk removal from the alveoli drives milk production with an increased suckling volume reducing FIL expression, ultimately increasing milk production (Wilde *et al.* 1998). Interestingly, despite the Restricted pups reducing maternal alveolar area, pup milk intake was only reduced in Restricted pups suckling uteroplacental insufficiency surgery (Restricted) mothers (Restricted-on-Restricted). The lower amount of milk removed by Restricted pups suckling uteroplacental insufficiency surgery (Restricted) mothers (Restricted-on-Restricted) would leave some milk in the alveoli, increasing FIL expression and slowing milk production. Thus, uteroplacental insufficiency surgery (Restricted) mothers may have altered milk production. However, Control pups suckling a uteroplacental insufficiency surgery (Restricted) mother (Control-on-Restricted) are able to remove a greater amount of milk from the mammary gland due to their large size, normalising their milk intake and FIL expression and/or activity. An interesting finding is that the pup also influences maternal plasma leptin concentrations, with mothers suckled by Restricted pups having increased plasma leptin concentrations. These data suggest that, despite no changes in maternal body weight with cross-fostering, the increased maternal plasma leptin concentrations may be due to the pup's influence on maternal adipose tissue deposition, maternal adipocyte hypertrophy, and/or maternal adipose leptin production. However, no studies to date have investigated maternal adipocyte morphology and adipose leptin secretion in uteroplacental insufficiency surgery (Restricted) mothers. Critically, pup plasma leptin concentrations were restored in Restricted pups suckling sham-operated (Control) mothers (Restricted-on-Control), which may partially be attributable to their increased milk consumption compared to Restricted pups suckling uteroplacental insufficiency surgery (Restricted) mothers (Restricted-on-Restricted).

### Cross-fostering effects of milk fatty acids

Fatty acid analysis highlighted that the pups regulate fatty acid composition in the maternal milk. Specifically, suckling by Restricted pups resulted in increased LC-PUFA and LC-MUFA content, and a reduction in long chain saturated fatty acids in the maternal milk. Additionally, LC-PUFA was increased and long chain saturated fatty acids were reduced in pups suckling uteroplacental insufficiency surgery (Restricted) mothers. These increases in LC-MUFA and LC-PUFA composition would increase the theromogenic capacity in the pup (preventing obesity) and improve pup physiological and metabolic function during critical periods of development, which is protective against the development of metabolic disease (Mennitti *et al.* 2015). Additionally, the reduction in saturated fatty acids during development would be protective against the development of obesity, diabetes and cardiovascular disease (Mennitti *et al.* 2015). These improvements in fatty acid composition are modulated by the pups, suggesting that the pups are somehow regulating maternal milk consumption to improve their growth and development. Despite the benefits of increased LC-MUFAs and LC-PUFAs, as well as reduced saturated fatty acids, on cardiometabolic health, the Restricted pups suckling uteroplacental insufficiency surgery (Restricted) mothers (Restricted-on-Restricted) go on to develop metabolic dysfunction, whereas the Restricted pups suckling sham-operated (Control) mothers (Restricted-on-Control) are protected (Siebel *et al.* 2008). These differences in disease outcomes may be due to the increased milk consumption by the Restricted pups suckling sham-operated (Control) mothers (Restricted-on-Control) which would further enhance the delivery of these beneficial fatty acids, improving pup growth and development (Wlodek *et al.* 2007; Moritz *et al.* 2009), and protecting them against adulthood diseases. Despite increases in n-3 and n-6 Omega fatty acids in Restricted pups and in pups suckling uteroplacental insufficiency surgery (Restricted) mothers (Restricted-on-Restricted; n-3 only), the n-3:n-6 ratio was unaffected, suggesting that Omega fatty acids do not adversely impact on pup growth and development (Morris, 2007). Previous research has demonstrated that feeding mothers a high n-3 Omega fatty acid diet results in reduced pup plasma leptin concentrations, despite no changes in maternal milk leptin concentrations (Korotkova *et al.* 2002). Studies in dexamethasone-treated WKY rats during pregnancy have also demonstrated that a maternal diet high in n-3 Omega

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fatty acids during lactation reduces offspring blood pressure (Wyrwoll *et al.* 2006). Therefore, a maternal diet high in n-3 Omega fatty acids and/or altered n-3 Omega fatty acid composition in the maternal milk may impact on pup plasma leptin concentrations and prevent cardiovascular disease. The mothers in the current study were fed the same chow during gestation and lactation, indicating that the diet is not affecting fetal and pup leptin concentrations. This finding suggests that the increased n-3 Omega fatty acid content in the milk of dams suckled by Restricted pups (both sham-operated (Restricted-on-Control) and uteroplacental insufficiency surgery (Restricted-on-Restricted) mothers) may impact on pup plasma leptin concentrations. However, the increased milk consumption in Restricted pups suckling sham-operated (Control) mothers (Restricted-on-Control), despite increased milk n-3 Omega fatty acids, is likely to be responsible for the restoration of plasma leptin concentrations and improved cardiovascular function in these pups (Moritz *et al.* 2009; Tare *et al.* 2012; Wadley *et al.* 2013). It is also important to consider that although these changes in fatty acid intake during lactation may be beneficial for pup health and development during the early postnatal period, an increased consumption of many of these fatty acids in adulthood may increase their disease risk.

## Conclusion

In summary, this study demonstrates that perinatal growth restriction reduces pup plasma leptin concentrations, which are restored by cross-fostering Restricted pups onto a mother with normal lactation (sham-operated), and this may be attributable to increased milk consumption and nutritional intake. We identified that growth restriction alters placental leptin transporter expression and signalling in a region and sex-specific manner. This study demonstrates that Restricted pups enhance milk fatty acid composition, which is probably an attempt to enhance pup growth and organ development. However, despite the enhanced milk fatty acid composition, the limited ability of the uteroplacental insufficiency surgery (Restricted) mothers to supply adequate milk is detrimental to the Restricted pups (Restricted-on-Restricted) development and health in adulthood. In contrast, the increased nutrient delivery, milk intake and plasma leptin concentrations in Restricted pups suckling sham-operated (Control) mothers (Restricted-on-Control) prevents the deleterious changes reported in the Restricted pups suckling uteroplacental insufficiency surgery mothers (Restricted-on-Restricted). Thus, further investigation is required into the role leptin plays in placental function, organogenesis and the organ-specific signalling targets that leptin activates. This study highlights an important window of intervention in rodents to enhance offspring growth and development by enhancing leptin delivery to offspring during lactation, which may prevent the deleterious adult health conditions in offspring born growth restricted.

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## Additional information

### Competing interests

The authors declare no conflicts of interest.

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## Author contributions

J.F.B., R.O., K.M.M., T.R., M.E.W. and D.H.H. designed the study. J.F.B. and R.O., with assistance from L.R.J. and T.R, performed all experiments. All authors participated in the interpretation of the results and contributed to writing the manuscript. All authors approved the submission of this version to *The Journal of Physiology*. All persons designated as authors qualify for authorship, and all those who qualify for authorship are listed.

## Funding

This research was supported by the National Health and Medical Research Council (NHMRC) of Australia (M.E.W. 208948; M.E.W. and K.M. 400003), an Early Career Researcher (ECR) grant from the University of Melbourne (D.H.H.), and a La Trobe University Faculty of Health Sciences Research Grant Award (T.R.). J.F.B. holds an Elizabeth and Vernon Puzey Postdoctoral Fellowship at the University of Melbourne.

## Acknowledgements

We thank Ursula Lorenc for her technical assistance and Professor Jane Moseley for her assistance in this project. We also thank Samantha van der Linde for her assistance with leptin ELISAs, Professor Andrew Sinclair for his assistance with the fatty acid analysis, Professor Kevin Nicholas for his assistance with mammary gland morphology. We also thank Dr Andrew Siebel, Mr Andrew Jefferies and Ms Kerryn Westcott for their assistance with the animal work involved in this project.

## Translational perspective

Uteroplacental insufficiency reduces birth weight and adversely affects fetal organ development, increasing adult disease risk. Plasma leptin concentration peaks during the completion of organ development. Leptin is transferred to the fetus (via the placenta) and to the pup (via the lactating mammary gland). Mothers with growth-restricted pups have compromised milk production and composition. Cross-fostering improves postnatal nutrition and restores the organ deficits associated with growth restriction. This study investigated the effect of uteroplacental insufficiency on pup plasma leptin concentrations and placental leptin transporters. We additionally examined if cross-fostering improves mammary development, milk composition and pup plasma leptin concentrations. Growth-restricted fetuses had reduced plasma leptin concentrations that persisted throughout lactation, reduced placental leptin and sex-specific alterations in placental leptin transporters. Mothers suckled by Restricted pups have impaired mammary development, altered milk fatty acid composition and increased plasma leptin concentrations, which did not correlate to altered milk leptin. Despite these changes, cross-fostering Restricted pups onto sham-operated mothers improved postnatal growth, milk intake and plasma leptin concentrations compared to Restricted pups suckling uteroplacental insufficiency surgery mothers. Human studies are now necessary to ascertain whether breastfeeding of growth-restricted babies by their mothers further compromises neonatal growth due to compromised mammary development and milk composition, and whether their baby consumes less milk. These data may provide evidence for supplementing the milk of mothers who have growth restricted or premature babies. However, further studies are required to determine if donated breast milk or infant formula (normal or specially designed) would be beneficial for infant growth and development.

## Supporting information

The following supporting information is available in the online version of this article.

**Table S1 - related to Table 4.** Fatty acid composition in stomach contents of consumed milk as a percentage of total fatty acids on postnatal day 6 in the four cross-foster groups ($n$ = 5–7 per group, with $n$ = 1 representing data from 1 pooled litter). Data are analysed with a two-way ANOVA reporting differences between pup and mother groups with a Student's $t$ test used to identify where interactions lie. Data presented as the means ± SEM with sex pooled per litter; ns, not significant; *significant differences ($P < 0.05$; Student's $t$ test) between Control and Restricted pups on the same mother group; ‡significant differences ($P < 0.05$; Student's $t$ test) between sham-operated (Control) and uteroplacental insufficiency surgery (Restricted) mothers with the same pup group.

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