The effect of systemic and ovarian infusion of glucose, galactose and fructose on ovarian function in sheep

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Abstract
Glucose is a critical metabolic fuel in most mammals although many foodstuffs also contain high levels of the monosaccharides, galactose and fructose. The aims of this work were to determine the insulin response to challenges of these sugars (experiment 1) and to examine the effect of systemic (experiment 2) and direct ovarian (experiment 3) infusion of these monosaccharides on ovarian function in ewes with autotransplanted ovaries. In experiment 1, both fructose (fourfold increase peaking in 2 h) and galactose (twofold increase; 30 min) elicited markedly different \( P<0.001 \) insulin responses than glucose (sevenfold increase; 20 min) although the total amount released following fructose and glucose challenge was similar. In experiment 2, low-dose systemic fructose infusion had no acute effect on insulin but did depress FSH \( P<0.05 \), and following the end of fructose infusion, a transient increase in FSH and insulin was observed \( P<0.05 \), which was associated with an increase \( P<0.05 \) in ovarian oestradiol and androstenedione secretion. Systemic infusion of neither glucose nor galactose had a significant effect on ovarian steroidogenesis although glucose acutely suppressed insulin levels. In contrast, ovarian arterial infusion of fructose and glucose had no effect on ovarian function whereas galactose suppressed ovarian follicle number and steroid secretion \( P<0.05 \). In conclusion, this work indicates that fructose and galactose can influence ovarian function in vivo in sheep and that different mechanisms are involved. Thus, fructose exerts stimulatory effects through indirect modulation of peripheral insulin and/or gonadotrophin levels whereas galactose exerts primarily suppressive effects by direct actions on the ovary.


Introduction
Nutrition is one of the key environmental factors that lead to infertility or sub-fertility in both clinical medicine and animal production. Severe under-nutrition is associated with anovulatory infertility (e.g. anorexia nervosa; Katz & Vollenhoven 2000) and negative energy balance is associated with poor fertility (e.g. the high genetic merit dairy cow in early lactation; Butler 2000, de Vries & Veerkamp 2000). Conversely, over-nutrition and obesity are also associated with poor reproductive performance (Norman & Clark 1998, Crosignani et al. 2002). Further, the age of puberty is largely dependent on the nutritional status and size of an individual (Foster & Olster 1985) and the duration and severity of postpartum anovulation is directly associated with the nutritional status of the mother and the amount of nutritional supplementation provided to the suckling young (McNeilly et al. 1994). However, while the effects of nutrition are generally well described, the physiological mechanisms that underlie these effects are generally poorly understood.

In sheep, it has been known for hundreds of years that a short-term increase in nutrient supply (flushing) can lead to a marked increase in prolificacy but it is only recently that we have begun to understand the physiological mechanisms underlying these effects. It is clear from a large body of published research that nutritional effects on reproduction are complex and that nutrition modulates ovarian activity through effects at multiple levels through changes in circulating metabolic hormones (e.g. GH, insulin, insulin-like growth factor 1 (IGF1) and leptin) and local paracrine and autocrine factors (e.g. IGF2, IGF-binding proteins, activins and cytokines) acting to modulate the action of key reproductive hormones (e.g. gonadotrophins, steroids and inhibins) at the target organ (Webb et al. 1999). The identification of the insulin/IGF system as a major mediator of nutritional influences on reproduction was
a major advance in this area as this system exerts both endocrine and locally mediated effects on ovarian folliculogenesis, oogenesis and embryogenesis (Giudice 2001). However, it is still unclear the extent to which the ovarian effects of nutrition are controlled by monosaccharides such as glucose modulating either by pancreatic insulin and/or hepatic IGF1 release or by direct ovarian effects.

Glucose is a critical metabolic fuel in mammals and while most mammals derive their glucose from their diet, ruminants derive glucose from dietary precursors such as propionic acid and gluconeogenic amino acids. Evidence produced in our laboratories, however, suggests that glucose is an important modulator of ovarian function in the ewe. We have shown that both SLC2A1 (GLUT1; the facultative glucose transporter) and SLC2A4 (GLUT4; the inducible insulin-dependent glucose transporter) are present in granulosa and theca cells (Williams et al. 2001) and that glucose downregulates both SLC2A1 and SLC2A4 expression in cultured granulosa cells (Campbell et al. 2010). Further, we have shown that there is a significant uptake of glucose by the ovary in both the follicular and luteal phases of the oestrous cycle (Scaramuzzi et al. 2010) and that the administration of gluconeogenic amino acids to ewes will increase insulin concentrations and stimulate folliculogenesis (Downing et al. 1999). Conversely, more recent data have shown that systemic infusion of high doses of glucose (10 mM/h) results in an acute increase in peripheral insulin concentrations, a decline in ovarian oestradiol secretion, a marked stimulation in the number of small antral follicles and a depression in aromatase and phosphorylated AKT and AMPK expression in granulosa cells (C Gallet, J Dupont, BK Campbell, D Monniaux & R Scaramuzzi 2010, unpublished observations). Collectively, these data suggest that glucose primarily affects ovarian function indirectly by modulation of the insulin/IGF system but it is also possible that glucose has direct effects on ovarian function.

Apart from glucose, other monosaccharides such as fructose and galactose are important constituents of ruminant and non-ruminant diets. Fructose is a physiologically significant monosaccharide, particularly in human nutrition where it occurs in high levels in fruit and in corn syrup (Elliott et al. 2002). Further, it is also the predominant blood sugar in foetal ruminants (Daniels et al. 1974) and an important energy substrate within the testis (Burant et al. 1992). Conversely, there is currently little evidence for a direct effect of fructose on the ovary although we have been able to show that fructose can be used as an energy source during the gonadotrophin-induced differentiation of granulosa cells in vitro (Campbell et al. 2010) despite the absence of SLC2A5 (GLUT5) mRNA expression (Kol et al. 1997, Campbell et al. 2010). In ruminants, fructose is thought to be rapidly metabolised to glucose in the liver (Luck et al. 1957), which may then trigger insulin release, but in primates and humans, fructose has been reported to have little effect on insulin and leptin release or on the concentrations of blood glucose (Curry 1989, Havel 1997, Tefi et al. 2004). In contrast, enzymes involved in the metabolism of galactose are abundantly and actively expressed in the ovary (Heidenreich et al. 1993) and in humans ovarian dysfunction is observed in individuals with deficiencies or mutations in these enzymes (Liu et al. 2000). In ruminants, however, the supplementation of diets high in galactose, such as those containing lupin grain (van Barneveld 1999), stimulates folliculogenesis and increases fecundity in ewes (Knight et al. 1975) although evidence of the effects of this intervention on ovarian concentrations is equivocal (Munoz-Gutierrez et al. 2002, Somchit et al. 2007).

In summary, there is evidence to suggest that other monosaccharides, in addition to glucose, can modulate ovarian function but in all cases, it is unclear whether these effects are mediated directly or indirectly through the insulin/IGF system. In the present experiment, we have attempted to address this question by utilising the ovarian autotransplant model. This experimental model allows the infusion of test substances, such as locally active growth factors (Campbell et al. 1994, 1995) or hormones (Campbell et al. 1996, Nicklin et al. 2007), directly into the ovary to expose follicular cells to high local concentrations. Further, the preparation allows repeated collection of ovarian venous blood from conscious animals and transdermal ultrasound scanning of the ovary, thus facilitating the assessment of the effect of treatment on ovulatory follicle development. The objective of this study was therefore to determine the ovarian and endocrine response to both systemic and direct ovarian infusion of low doses of the monosaccharides, glucose, galactose and fructose, at doses unlikely to stimulate the insulin/IGF system.

Results

Experiment 1: insulin response to a bolus injection of monosaccharide

Bolus injections of each monosaccharide resulted in acute increases (P<0.001) in circulating insulin concentrations but there were marked differences in the magnitude and duration of the responses obtained (Fig. 1a) with highly significant (P<0.01) effects of monosaccharide and time by monosaccharide interaction (P<0.001). Glucose injection resulted in the most rapid (20 min) and largest (sevenfold) increase in insulin concentrations but these had fallen to basal concentrations within 2 h of injection. Galactose injection induced the lowest response (twofold), which peaked within 30 min of injection and had returned to basal concentrations by 2 h so that the area under the response curve for galactose was 50% of that observed for glucose (11.6 cm²). In contrast, fructose injection induced a
Experiment 1: monosaccharide response to a bolus injection of monosaccharide

Analysis of the effect of the injection of a glucose bolus on circulating glucose concentrations showed the expected rapid increase (eightfold; \( P<0.001 \)) followed by rapid clearance of glucose to basal concentrations within 2 h of treatment (Fig. 1b). Glucose injection had no effect on circulating galactose concentrations but there was a small increase in fructose concentrations (1 mM) observed in the sample taken 5 min after treatment (data not shown). In contrast, the injection of the other two sugars resulted in very different profiles. Galactose injection resulted in an acute sixfold increase (\( P<0.001 \)) in circulating galactose concentrations but the clearance rate was slow and concentrations had not returned to basal concentrations by 180 min after injection (Fig. 1b). Galactose injection had no effect on circulating glucose or fructose concentrations (data not shown). Fructose injection, however, resulted in only a small onefold increase in circulating fructose concentrations in the 10 min following infusion but also induced an acute increase in circulating glucose concentration, which was similar to that induced by glucose injection in terms of both timing and magnitude (Fig. 1b). Fructose injection, however, had no effect on circulating galactose concentrations (data not shown).

Experiment 2: systemic monosaccharide infusion

Infusion of the low doses of glucose, fructose and galactose chosen for this experiment did not result in any statistically significant changes in circulating monosaccharide concentrations, although glucose and fructose concentrations tended to be higher during the infusion period of these monosaccharides (Table 1). Overall, fructose concentrations prior to infusion were around fivefold lower than for glucose whereas galactose concentrations were 250-fold lower (Table 1). Surprisingly, a significant (\( P<0.01 \)) increase in glucose concentrations was observed during the post-infusion period, which encompassed the follicular phase, in vehicle-treated controls and in ewes infused with glucose and galactose, but not fructose. In fructose-treated ewes, glucose concentrations were significantly lower during the infusion period relative to the post-infusion period (\( P<0.01 \)) and the same comparison with the pre-infusion period also approached statistical significance (\( P<0.1 \)).

In contrast, infusion of glucose and galactose, but not fructose, resulted in a significant depression (\( P<0.05 \)) in peripheral insulin concentrations with time over the initial 24 h of infusion although, as concentrations in saline-infused controls also tended to decline over this period, only glucose differed significantly (\( P<0.05 \)) from controls from 24 to 42 h from the start of infusion (Fig. 2a). However, over the follicular phase, following the end of the infusion, insulin concentrations tended to increase in all experimental groups, including the controls, but in sheep treated with fructose and galactose, this increase was sustained with the result that insulin concentrations were significantly elevated over controls (\( P<0.05 \)) during the period 36–72 h after the end of the infusion (Fig. 2a). Insulin concentrations in glucose-treated sheep were identical to controls over this period.

Monosaccharide infusion had no effect on circulating LH concentrations (data not shown) but there were effects on FSH. During the infusion period, FSH concentrations remained stable in saline-, glucose- and galactose-treated ewes (Fig. 2b). In contrast, fructose infusion resulted in an acute decline in FSH concentrations, which was significantly different (\( P<0.05 \)) from controls.
Table 1 Experiment 2: peripheral concentrations of monosaccharides in ewes infused with saline (control), glucose, galactose or fructose at a rate of 9 mg/h for 72 h during the late luteal phase before induction of luteal regression. Pre-infusion concentrations are those recorded for each animal prior to starting infusion; during infusion are mean values determined from 6 hourly samples collected over the entire 72 h of infusion and post infusion are mean values determined from 6 hourly samples collected over the entire 72 h period post infusion, which encompassed the entire follicular phase.

<table>
<thead>
<tr>
<th></th>
<th>Pre-infusion</th>
<th>During infusion</th>
<th>Post infusion</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Glucose</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>52.3±2.3*</td>
<td>51.2±1.6*</td>
<td>56.7±1.8*I</td>
</tr>
<tr>
<td>Glucose</td>
<td>51.7±2.9*</td>
<td>52.8±1.2*</td>
<td>59.5±1.5*I</td>
</tr>
<tr>
<td>Galactose</td>
<td>52.3±1.4*</td>
<td>54.0±1.5*</td>
<td>60.0±1.5*I</td>
</tr>
<tr>
<td>Fructose</td>
<td>55.3±2.8*†</td>
<td>51.7±1.4*</td>
<td>57.3±2.0*I</td>
</tr>
<tr>
<td><strong>Galactose</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>0.33±0.09*</td>
<td>0.35±0.07*</td>
<td>0.36±0.06*</td>
</tr>
<tr>
<td>Glucose</td>
<td>0.42±0.09*</td>
<td>0.38±0.10*</td>
<td>0.35±0.07*</td>
</tr>
<tr>
<td>Galactose</td>
<td>0.41±0.14*</td>
<td>0.39±0.10*</td>
<td>0.37±0.08*</td>
</tr>
<tr>
<td>Fructose</td>
<td>0.38±0.07*</td>
<td>0.36±0.07*</td>
<td>0.35±0.06*</td>
</tr>
<tr>
<td><strong>Fructose</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>10.3±0.6*</td>
<td>11.0±0.6*</td>
<td>11.0±0.6*</td>
</tr>
<tr>
<td>Glucose</td>
<td>11.7±0.7*</td>
<td>13.5±1.8*</td>
<td>12.8±1.2*</td>
</tr>
<tr>
<td>Galactose</td>
<td>11.1±0.9*</td>
<td>11.3±1.0*</td>
<td>13.2±1.0*</td>
</tr>
<tr>
<td>Fructose</td>
<td>10.9±0.5*</td>
<td>11.3±0.7*</td>
<td>12.1±0.9*</td>
</tr>
</tbody>
</table>

Values are mean±S.E.M. Statistically significant differences are indicated by different superscripts across each row; *P<0.05.

From 30 to 42 h of the infusion. Induction of luteal regression resulted in an acute increase in FSH concentrations in all experimental groups, including the controls, but the magnitude (20%) and longevity (6 h) of this increase differed from controls in ewes treated with fructose (40% over 18 h) and galactose (30% over 18 h) with the result that follicular phase concentrations of FSH were significantly (*P<0.05) elevated over controls in fructose- and galactose-treated ewes, 12 h after initiation of luteal regression (Fig. 2b). Thereafter, all ewes exhibited the expected 50% suppression in FSH over the remaining 48–60 h of the follicular phase (Campbell et al. 1990) followed by variable profiles because of the asynchronous induction of the pre-ovulatory gonadotrophin surge during the final 24 h of the experiment (Fig. 2b). The time of the LH surge after luteal regression did not differ between vehicle-treated controls (56±4 h) and those animals that received glucose (53±2 h), fructose (57±4 h) or galactose (53±3 h).

Examination of the effect of monosaccharide infusion on ovarian steroidogenesis showed no effect of treatment on either basal concentrations of secretion determined from 6 hourly samples taken during the infusion or more frequent samples taken around the time of an LH challenge at the end of infusion (Figs 3 and 4). However, during the subsequent follicular phase, fructose infusion resulted in a significant increase in both mean oestradiol secretion (*P<0.05) and oestradiol pulse amplitude (*P<0.01) during the final period of intensive blood sampling (Fig. 3) and an increase in both ovarian oestradiol and androstenedione secretion in the 24 h prior to the LH surge (*P<0.05; Fig. 4). Over this period, ewes treated with galactose exhibited identical steroid secretory profiles as controls and although glucose resulted in slightly higher levels of secretion immediately prior to the LH surge, overall glucose had no significant effect on ovarian steroid secretion across the follicular phase. Examination of subsequent luteal function, however, revealed that progesterone concentrations in ewes treated with glucose (2.6±0.5) and fructose (2.4±0.4) were significantly (*P<0.05) higher on day 7 of the cycle than those observed in control (1.3±0.4)- and galactose (1.6±0.3)-treated ewes.

Ultrasound scanning of the ovaries revealed that there were no statistically significant differences between treatment groups in the ovarian antral follicle population following systemic monosaccharide infusion. However, there was a tendency (P<0.1) for both glucose and fructose infusion to increase the number of antral follicles and for galactose to depress the number of both large and small antral follicles (Table 2). A similar

Figure 2 Experiment 2: effect of systemic infusion (9 mg/h) of glucose (open triangle), fructose (closed square) or galactose (open circle) on peripheral insulin (a) or FSH (b) concentrations over the entire experimental period. Shaded area indicates mean±S.E.M. values for vehicle-treated controls. Both insulin and FSH values have been expressed as a percentage of pre-infusion values for each experimental animal. Data have been expressed relative to the time of induction of luteal regression. Values are mean±S.E.M.
A trend was observed in ovulation rate, assessed by ultrasound on day 7, but again these differences did not reach statistical significance (Table 2).

**Experiment 3: ovarian monosaccharide infusion**

Direct ovarian arterial infusion of monosaccharide at a dose rate of 0.18 mg/h had no effect on peripheral insulin or monosaccharide concentrations (data not shown). Interestingly, however, across all animals the concentration of galactose in ovarian venous blood (0.52 ± 0.05 mg/dl) was significantly (P<0.01) higher than in jugular venous blood (0.40 ± 0.04). Monosaccharide infusion had no effect on the level or pattern of FSH (data not shown) or LH secretion during the follicular phase following infusion. The time of the LH surge after luteal regression did not differ between vehicle-treated controls (61 ± 4 h) and those animals that received glucose (56 ± 4 h), fructose (67 ± 4 h) or galactose (61 ± 4 h).

The pulsatile pattern of ovarian oestradiol secretion observed in response to LH pulses, determined from periods of frequent blood sampling at the end of arterial infusion and 12–15 h after the end of infusion, showed no significant effect of fructose infusion at either time point on any of the pulsatile parameters measured (Table 3). Glucose, however, tended to increase both overall mean secretion and maximum peak height at both time points (P<0.10) and this effect was statistically significant (P<0.05) for maximum peak height over the period 12 h after infusion (Table 3). However, across the subsequent follicular phase, relative to controls infusion of neither glucose nor fructose had a statistically significant effect on either oestradiol (Fig. 5a) or androstenedione (Fig. 5b) secretion although androstenedione concentrations tended (P<0.1) to be lower in glucose-treated animal during the early follicular phase from −48 to −24 h prior to the LH surge. In contrast, galactose infusion significantly suppressed oestradiol pulse amplitude (P<0.05) over the period 12 h after infusion (Table 3) and over the remainder of the follicular phase.
phase resulted in an overall suppression in both ovarian oestradiol and androstenedione secretion that was statistically significant for androstenedione from −48 to −18 h prior to the LH surge and −18 to −6 h prior to the LH surge for oestradiol (Fig. 5). Examination of subsequent luteal function, however, revealed no effect of treatment on either the pattern or level of progesterone attained by day 7 of the cycle (data not shown).

Direct ovarian arterial infusion of low-dose glucose or fructose had no significant effect on the number of small or large antral follicles or ovulation rate. Galactose infusion had no effect on follicle number at the end of infusion but resulted in a depression in the number of both small (P<0.1) and large (P<0.05) antral follicles towards the end of the subsequent follicular phase (Table 2). Similarly, ovulation rate was lower, relative to controls, following galactose treatment and this difference approached statistical significance (Table 2; P<0.1).

**Discussion**

The results of this study provide strong in vivo evidence that low doses of the monosaccharides, glucose, galactose and fructose, are all capable of modulating ovarian function but suggest that they do so by distinct mechanisms that are differentially mediated either directly or indirectly through the insulin system. Thus, it is clear that both the type and dose of monosaccharide exposure has a major effect on the characteristics of the insulin response to treatment observed and overall these data support the hypothesis that the effects of fructose and glucose are mediated by insulin whereas galactose appears to have direct suppressive effects on ovarian follicle development. Further, this paper reports a novel effect of fructose on peripheral FSH concentrations, suggesting that this sugar, at least, may also exert effects on ovarian function through the modulation of gonadotrophic drive for the gonadotrophin-responsive and gonadotrophin-dependent stages of follicle development.

One of the most surprising and significant results obtained in these studies was the differential effect of dose and type of monosaccharide on insulin release in sheep. The results of experiment 1, which involved a standard ‘glucose tolerance test’, in which a large bolus of glucose was injected directly into the peripheral circulation demonstrated the classic acute insulin response and rapid clearance of glucose from the blood observed in most species (Bloomfield et al. 2007). A similar challenge with galactose, however, resulted in a smaller but more prolonged increase in insulin and slow clearance from the peripheral circulation whereas a fructose bolus, despite being apparently rapidly converted to glucose (Luick et al. 1957), resulted in a much more prolonged period of insulin release. Thus, it appears that the control of insulin release is far more complex than indicated by the responses obtained to a standard glucose challenge and this suggestion is supported by the results obtained in experiment 2 following the systemic infusion of much lower doses of monosaccharide; doses that were designed not to perturb peripheral insulin concentrations.

Surprisingly, infusion of these low doses of glucose resulted in a significant decrease in insulin concentrations whereas fructose infusion had no effect and the galactose response was intermediate. Further, cessation of infusion resulted in a noticeable rebound in insulin concentrations in animals treated with all three sugars and this seemed to be related to an increase in peripheral glucose concentrations across the follicular phase in all experimental groups, apart from those treated with fructose (Fig. 2). In addition, changes in insulin concentrations in all ewes were observed that appeared to be related to stage of the oestrous cycle and this is consistent with reports in the literature that insulin concentrations in sheep are affected by changes in the circulating concentrations of progesterone and oestradiol (McCann et al. 1989). We have previously shown that infusion of glucose at higher doses of 10 mM/h for 3 days at a similar stage of the cycle results in an initial insulin

**Table 2** Experiments 2 and 3: follicle number and ovulation rate in ewes subjected to systemic (experiment 2) or ovarian arterial (experiment 3) infusion of saline (control) or glucose, galactose or fructose. Follicle number was determined by ultrasound at either the end of monosaccharide infusion or during the latter stages of the subsequent follicular phase, 48 h after induction of luteal regression (PG+48 h) and follicles have been classified as either small (2–3.5 mm diameter) or large (≥3.5 mm). Ovulation rate indicates number of corpora lutea visualised by transdermal ultrasound on day 7 of the subsequent luteal phase.

<table>
<thead>
<tr>
<th>Experiments</th>
<th>Stage determined</th>
<th>Follicle size</th>
<th>Control</th>
<th>Glucose</th>
<th>Fructose</th>
<th>Galactose</th>
</tr>
</thead>
<tbody>
<tr>
<td>2: Systemic infusion</td>
<td>End of infusion</td>
<td>Small (2–3.5 mm)</td>
<td>16.6±1.5</td>
<td>18.4±1.0</td>
<td>13.9±2.1</td>
<td>12.0±2.1†</td>
</tr>
<tr>
<td></td>
<td>Large (≥3.5 mm)</td>
<td>1.9±0.3</td>
<td>2.8±0.3†</td>
<td>2.1±0.3</td>
<td>1.9±0.4</td>
<td>1.4±0.2†</td>
</tr>
<tr>
<td>PG+48 h</td>
<td>Large (≥3.5 mm)</td>
<td>14.1±1.3</td>
<td>13.8±1.5</td>
<td>17.9±1.7†</td>
<td>9.8±1.9†</td>
<td>1.4±0.2†</td>
</tr>
<tr>
<td>Day 7</td>
<td>Ovulation rate</td>
<td>2.3±0.4</td>
<td>3.0±0.3†</td>
<td>3.1±0.3†</td>
<td>1.4±0.2†</td>
<td></td>
</tr>
<tr>
<td>3: Arterial infusion</td>
<td>End of infusion</td>
<td>Small (2–3.5 mm)</td>
<td>11.3±0.8</td>
<td>13.0±0.7</td>
<td>13.1±0.9</td>
<td>9.6±1.2</td>
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<tr>
<td></td>
<td>Large (≥3.5 mm)</td>
<td>2.5±0.3</td>
<td>2.8±0.3</td>
<td>2.6±0.4</td>
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<tr>
<td>PG+48 h</td>
<td>Large (≥3.5 mm)</td>
<td>9.9±0.9</td>
<td>11.0±1.0</td>
<td>9.4±0.8</td>
<td>6.6±0.9†</td>
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<tr>
<td>Day 7</td>
<td>Ovulation rate</td>
<td>2.3±0.3</td>
<td>2.5±0.2</td>
<td>2.3±0.4</td>
<td>1.5±0.2†</td>
<td></td>
</tr>
</tbody>
</table>

Values are mean±s.e.m. *Indicates P<0.05 whereas † indicates P<0.1 for comparison with vehicle-treated control at the same time point.
response followed by a decline in insulin as glucose concentrations fall (C Gallet, J Dupont, BK Campbell, D Monniaux & R Scaramuzzi 2010, unpublished observations). Similarly, recent experiments involving larger numbers of animals exposed to the same low doses, utilised in the current experiment, have resulted in essentially identical insulin profiles as those observed in this study (V Onions, RJ Scaramuzzi and BK Campbell, unpublished observations). It would therefore appear that the control of insulin release in response to exposure to much larger doses of glucose (C Gallet, J Dupont, BK Campbell, D Monniaux & R Scaramuzzi 2010, unpublished observations).

In contrast, fructose, although an important energy source in early development (Daniels et al. 1974), has not been considered as a major candidate for the mechanisms by which nutrition modulates ovarian function in the adult. This is primarily because it has long been believed that fructose is rapidly metabolised to glucose in ruminants and that this glucose may then modulate insulin release (Luick et al. 1957). The results of experiment 1, while supporting these earlier data in

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**Table 3** Experiment 3: parameters of pulsatile oestradiol secretion in ewes infused with saline (control), glucose, galactose or fructose through the ovarian artery at a rate of 0.18 mg/h for 12 h during the late luteal phase before induction of luteal regression. Values estimated from intensive periods of blood sampling conducted at the end of the infusion (9.5–12 h at 15 min intervals relative to start of the infusion) and 12–15 h (at 10 min intervals) after the end of the infusion and sponge withdrawal.

<table>
<thead>
<tr>
<th></th>
<th>During infusion</th>
<th>After infusion</th>
</tr>
</thead>
<tbody>
<tr>
<td>Overall mean (ng/ml)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>2.44 ± 0.09</td>
<td>2.47 ± 0.12</td>
</tr>
<tr>
<td>Fructose</td>
<td>2.54 ± 0.10</td>
<td>2.58 ± 0.13</td>
</tr>
<tr>
<td>Galactose</td>
<td>2.40 ± 0.08</td>
<td>2.54 ± 0.11</td>
</tr>
<tr>
<td>Glucose</td>
<td>2.67 ± 0.09</td>
<td>2.76 ± 0.11</td>
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<tr>
<td>Pulse maximum (ng/ml)</td>
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</tr>
<tr>
<td>Control</td>
<td>2.73 ± 0.10</td>
<td>2.68 ± 0.12</td>
</tr>
<tr>
<td>Fructose</td>
<td>2.83 ± 0.09</td>
<td>2.75 ± 0.10</td>
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<tr>
<td>Galactose</td>
<td>2.63 ± 0.10</td>
<td>2.66 ± 0.12</td>
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<tr>
<td>Glucose</td>
<td>2.96 ± 0.08*</td>
<td>2.95 ± 0.09*</td>
</tr>
<tr>
<td>Pulse nadir (ng/ml)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>2.13 ± 0.11</td>
<td>2.18 ± 0.11</td>
</tr>
<tr>
<td>Fructose</td>
<td>2.14 ± 0.13</td>
<td>2.33 ± 0.16</td>
</tr>
<tr>
<td>Galactose</td>
<td>2.10 ± 0.09</td>
<td>2.31 ± 0.12</td>
</tr>
<tr>
<td>Glucose</td>
<td>2.32 ± 0.11</td>
<td>2.52 ± 0.12</td>
</tr>
<tr>
<td>Pulse amplitude (ng/ml)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>0.60 ± 0.11</td>
<td>0.49 ± 0.06</td>
</tr>
<tr>
<td>Fructose</td>
<td>0.69 ± 0.08</td>
<td>0.42 ± 0.07</td>
</tr>
<tr>
<td>Galactose</td>
<td>0.53 ± 0.10</td>
<td>0.35 ± 0.06*</td>
</tr>
<tr>
<td>Glucose</td>
<td>0.64 ± 0.08</td>
<td>0.42 ± 0.06</td>
</tr>
</tbody>
</table>

Values are mean±s.e.m. Statistically significant differences are indicated by different superscripts across each row, P<0.05.

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*Figure 5* Experiment 3: effect of arterial infusion (0.18 mg/h) of glucose (open triangle), fructose (closed square) or galactose (open circle) on ovarian secretion of oestradiol (a) and androstenedione (b) over the entire experimental period. Shaded area indicates mean±s.e.m. values for vehicle-treated controls. Data have been expressed relative to the time of the pre-ovulatory LH surge. Values are mean±s.e.m.
terms of the conversion of fructose to glucose, show that the insulin response to fructose challenge is much more prolonged than that induced by glucose and suggest a more complicated relationship. Why this should be so is unknown but it is worth noting that the standard assay for both these sugars utilises a common enzyme and fructose values are calculated by difference. Therefore, the specificity of the assays and hence the sugar profiles obtained may be open to question. In experiment 2, the systemic infusion of a low dose of fructose, in contrast to glucose, had no acute effect on insulin release but was followed by a marked increase in insulin when fructose infusion was stopped, at a time that coincided with the subsequent follicular phase. Further, alone among the sugars we tested, fructose infusion also resulted in a significant decline in circulating FSH concentrations. The mechanism for this decrease could either be a direct effect on the hypo-pituitary axis or an indirect effect through the ovary to stimulate follicular development and hence increase the level of negative inhibitory feedback controlling FSH release (Baird et al. 1991). As no change in follicle number or in oestradiol secretion was observed over this period, it appears likely that the effect of fructose infusion on FSH may be direct, and it is significant that expression of SLC2A5 (GLUT5), the specific fructose transporter, has been observed in the brain in many species (Maher et al. 1994, Douard & Ferraris 2008). This interpretation is supported by the fact that after ending fructose infusion, FSH actually increased by around 50% over the initial 12 h of the follicular phase before exhibiting a normal follicular phase decline. This increase in FSH, at a time when follicle selection is thought to occur (Campbell et al. 2003) in combination with the elevation in insulin that occurred at around the same time, is the most likely explanation for the stimulatory effects of systemic fructose on ovarian oestradiol and androstenedione secretion and follicle development over the subsequent follicular phase. This interpretation is supported by the fact that direct arterial infusion of fructose in experiment 3 had little effect on ovarian function, although clearly the period of exposure was much less in this instance. Thus, overall it would appear that low doses of fructose can exert quite marked effects on ovarian function and that it is likely that these effects are mediated indirectly through effects on either pituitary FSH release and/or circulating insulin concentrations. Given that fructose intake, in the form of corn syrup, has increased markedly in humans from developed countries consuming ‘western diets’ and has been associated with obesity and diabetes, this finding requires further investigation to elucidate exact mechanisms in terms of nutritional effects on ovarian function in both monogastric and ruminant species.

In contrast to glucose and fructose, the findings of this study indicate that galactose exerts its effects on ovarian function by direct actions. Experiment 1 showed that even pharmacological doses of galactose did not elicit a marked insulin response and the results of experiment 2 showed that systemic infusion of low doses of galactose had little effect on insulin concentrations during the infusion or on ovarian steroid secretion over the subsequent follicular phase whereas direct ovarian arterial infusion (experiment 3) had a marked suppressive effect on ovarian steroidogenesis and follicle development. However, it cannot be concluded that galactose has no indirect actions as the magnitude of the insulin rebound at the end of galactose infusion was actually similar to that observed for fructose. However, in the absence of any change in FSH or perhaps because of direct suppressive effects on the ovary, unlike fructose, galactose-treated animals tended (P<0.1) to have fewer antral follicles and lower concentrations of progesterone over the subsequent follicular phase. The hypothesis that galactose has direct inhibitory effects on ovarian function is not consistent with findings in humans that galactosaemia is associated with ovarian dysfunction (Liu et al. 2000). The underlying mechanisms that result in these suppressive effects are unknown and future research is required to both elucidate these mechanisms and to determine whether the level of dietary intake of foods rich in galactose can be related to changes in ovarian function. Studies utilising cultured ovarian somatic cells in vitro have shown that while galactose can be metabolised readily by theca cells (Onions et al. 2009), granulosa cell proliferation and oestradiol production are depressed when galactose is provided as an energy source when compared to fructose or glucose (Campbell et al. 2010). These in vitro findings therefore suggest that galactose is exerting its inhibitory effects on ovarian function through granulosa cell function although we have also been able to show that both granulosa and theca cells express the galactose transporter SLC2A8 (GLUT8; Onions et al. 2009, Campbell et al. 2010). Further, galactose can also be utilised as a substrate for the synthesis of essential factors such as nucleotide sugars (Heidenreich et al. 1993, Liu et al. 2000) and therefore may have local actions at multiple levels.

In conclusion, this work has provided compelling new evidence that the monosaccharides, fructose and galactose, can influence ovarian function in vivo in sheep and that different mechanisms are involved. Thus, fructose appears to exert stimulatory effects through indirect modulation of peripheral insulin and/or gonadotrophin concentrations whereas galactose appears to exert primarily suppressive effects by direct actions on the ovary. Further work is therefore required to investigate the possibility that dietary fructose and galactose intake may also modulate some of the well-known but poorly understood effects of nutritional intake on ovarian function in both ruminant and monogastric species.
Materials and Methods

Experimental animals

These experimental procedures were carried out under the terms of a licence for Animal Scientific Procedures from the Home Office following local ethical committee approval. Mature Border Leicester Merino cross ewes with ovarian autotransplants (Campbell et al. 1994), in which the surgery had been performed at least 5 years earlier, were used for this experiment. The experiments was carried out during the breeding season (October–February) and oestrous cycles were synchronised using intravaginal progesterone (medroxyprogestrone acetate) impregnated sponges (Dunlop, Dumfries, UK) that were inserted for 10 days, with an injection of Cloprostenol (Estrumate; Intervet, Cambridge, UK) at the time of sponge withdrawal (0 h). In both the experiments, the absence or presence of oestrous behaviour prior to sponge insertion was confirmed by the inclusion of a harnessed ram within the flock. The animals are routinely fed a maintenance diet of hay and straw without any high protein or energy supplements.

On the day prior to the start of blood sampling, all animals had cannulae inserted into both jugular veins and the carotid artery (experiment 3) as described previously (Campbell et al. 1994). Following cannulation, all sheep were placed in metabolism crates in ventilated rooms and treated prophylactically with antibiotics (3 ml i.m. every 3 days; Clamoxil, SmithKline Beecham, Surrey, UK) and heparin (5000 IU i.v. every 12 h; Leo Laboratories, Aylesbury, Bucks, UK). The sheep were well habituated to these housing conditions and to frequent handling.

Monosaccharide infusion

Monosaccharide (Sigma–Aldrich) was prepared as a solution in 0.9% (w/v) saline with 0.1% (v/v) normal sheep plasma and sterilised by filtration through a 0.22 µm filter. This solution was infused into either the jugular vein (experiments 1 and 2) or the carotid artery (experiment 3) using syringe driven pumps fitted with a plastic 50 ml syringe. For arterial infusions, blood flow was directed to the ovary by occlusion of the carotid artery cranial to ovarian carotid arterial anastomosis using a paediatric sphygmomanometer (Downs Surgical, Mitcham, Surrey, UK) inflated to a pressure of 200 mmHg as previously described (Campbell et al. 1994).

Experimental design

Experiment 1: insulin response to a bolus injection of monosaccharide

A bolus of 21 g monosaccharide in 50 ml saline was infused directly into the jugular vein over a period of 1–2 min (n=4–5/group) and samples of jugular venous blood were collected from the contra-lateral vein 5, 10, 20, 45, 65, 120 and 180 min after the end of injection. Samples were collected into tubes containing fluoride to inhibit glycolytic enzymes and EDTA as an anti-coagulant (Sarstedt, Leics, UK) in order to facilitate the assay of monosaccharide concentrations in the plasma. These samples were also assayed for insulin.

A schematic outline of the experimental design is presented in Fig. 6a. The treatment groups were i) saline only controls (n=9); ii) glucose at 9 mg/h (n=9); iii) galactose at 9 mg/h (n=9); and iv) fructose at 9 mg/h (n=9) infused over a 72 h period prior to sponge withdrawal. Owing to the limited number of ovarian autotransplants available, the experimental design was a crossover with a 1-month washout period between each of three replicate experiments. Samples of ovarian venous blood (8 ml) were collected at 6 hourly intervals from the beginning of the infusion period until 72 h after sponge withdrawal in order to determine the effect of treatment on the timing and characteristics of the pre-ovulatory

Experiment 2: systemic monosaccharide infusion

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Figure 6 Schematic diagram of the experimental design for experiments 2 (a) and 3 (b). For both experiments, luteal regression was induced by injection of Cloprostenol (PG) at the time of sponge withdrawal on day 10 of the cycle (0 h). In experiment 2, systemic infusion of monosaccharide (9 mg/h) or vehicle (n=9) was performed for 72 h prior to induction of luteal regression whereas in experiment 3, ovarian arterial infusion of monosaccharide (0.18 mg/h) or vehicle (n=10) was performed for 12 h prior to luteal regression (hatched horizontal bar). During the entire experimental period, samples of ovarian venous blood (5 ml) were collected at 6 hourly intervals (lightly shaded horizontal bar). In addition, in each experiment there were three periods of more frequent blood sampling (solid horizontal bars) immediately prior to infusion, during the infusion and at the end of the infusion. Idealised profiles show pattern of progesterone (dotted line), oestradiol (dashed line) and LH (solid line) expected in untreated animals.
steroid and gonadotrophin surges. These samples were split so that 5 ml were placed in tubes containing heparin (50 IU/tube) and 3 ml were placed into tubes containing fluoride and EDTA. In addition, there were three periods of more frequent blood sampling (15 min intervals for 2.5 h), designed to examine the effect of infusion on the pulsatile pattern of ovarian oestradiol secretion. These were carried out immediately prior to infusion (−3 to −0.5 h relative to start infusion), at the end of the infusion (70–72.5 h relative to start infusion) and 33.5–36 h after the end of the infusion and sponge withdrawal. Finally, jugular venous samples (5 ml) were taken every day for the 7 days after the end of the intensive experimental period in order to assess post-ovulatory luteal function.

Experiment 3: arterial monosaccharide infusion

A schematic outline of the experimental design is presented in Fig. 6b. Experiment 3 was performed 12 months after experiment 2, during the following breeding season, and utilised a number of different ovarian autotransplants. The treatment groups (n = 10) were the same as for experiment 2 but the rate of arterial infusion for all sugars was 0.18 mg/h and this was administered for 12 h prior to the induction of luteal regression. The period of arterial infusion is limited to 12 h due to oedema that occurs in the cranial end of the vascular skin loop as a result of the sustained pressure from the paediatric sphygmomanometer needed to occlude the carotid artery. The experimental design was a crossover with an 8-week washout period between each of the two replicate experiments. Samples of ovarian venous blood (5 ml) were collected at 6 hourly intervals from the beginning of the infusion period until 72 h after sponge withdrawal in order to determine the effect of treatment on the timing and characteristics of the pre-ovulatory steroid and gonadotrophin surges. Additional 5 ml samples of jugular venous blood were collected at 6 hourly intervals over the infusion period and were placed into tubes containing fluoride and EDTA (Sarstedt) for determination of peripheral sugar concentrations. In addition, there were three periods of more frequent blood sampling, designed to examine the effect of infusion on the pulsatile pattern of ovarian oestradiol secretion. These were carried out immediately prior to infusion (−3 to −0.5 h at 15 min intervals relative to start of the infusion), at the end of the infusion (9.5–12 h at 15 min intervals relative to start of the infusion) and 12–15 h (at 10 min intervals) after the end of the infusion and sponge withdrawal. Finally, jugular venous samples (5 ml) were taken every day for the 7 days after the end of the intensive experimental period in order to assess post-ovulatory luteal function.

Hormone and sugar assays

Blood samples were centrifuged at 4 °C and 3000 g for 20 min to obtain plasma that was stored at −20 °C. Oestradiol (Campbell et al. 1994), LH (Kendall et al. 2004), FSH (Campbell et al. 1994), androstenedione (Campbell et al. 1994), progesterone (Souza et al. 1997b) and insulin (Somchit et al. 2007) concentrations in ovarian and/or jugular venous plasma were determined by RIA as previously described. All hormone assays had intra-assay and inter-assay coefficients of variation of <10 and 15% respectively.

Monosaccharide assays were carried out by colourimetric assay using a clinical chemistry type auto analyser (Rx IMOLA, Randox Laboratories, Belfast Co., Antrim, UK). Glucose was determined using a commercially available reagent kit (GL3816; Randox Laboratories, Belfast Co.) according to the manufacturer’s instructions using the Hexokinase method. Fructose was calculated by the difference between running the glucose assay, but with the addition of phosphoglucose isomerase 50 μl (276U) to 20 ml glucose kit reagent 1 (P5381, Sigma–Aldrich) to convert all the fructose into glucose, and the glucose reading. Galactose was based on the methods of Kurz & Wallenfels (1974) adapted for a clinical chemistry type auto-analyser (Rx IMOLA, Randox Laboratories, Belfast Co.). Briefly, a 1 M solution of Tris–HCl buffer (pH 8.6) was prepared. Galactose dehydrogenase (5 U/ml, cat 104981) was purchased from Roche and NAD from Sigma–Aldrich. Deproteinisation was found not to be required. Daily a 1 mg/ml solution of NAD in Tris-HCl buffer (pH 8.6) was prepared and galactose dehydrogenase was added at 0.5 ml (2.5 U) to 43.4 ml of the Tris–NAD buffer solution. The small batches were used to maintain peak enzyme activity. The analyser pipetted 0.145 ml reagent solution and then added 0.035 ml sample, the rate of reaction between 5 and 50 s post sample addition was assessed and compared with a standard curve derived from dilutions prepared from a galactose standard (Sigma–Aldrich). The curve proved to be linear up to 0.28 mM. There was no cross reactivity in the glucose assay for galactose or in the galactose assay for glucose. There was a 0.035% cross reactivity with fructose in the galactose assay. The inter-assay and intra-assay coefficients of variation were <2% for each of the monosaccharide assays.

Ovarian scanning procedure

In order to estimate effects of infusion on the ovarian follicle population and corpora lutea, all animals were scanned prior to and after infusion, and at 12 h intervals post infusion until the end of the intensive blood sampling period at the expected time of the LH surge (60 h after sponge withdrawal). In addition, the ovaries of all animals were scanned on day 7 of the subsequent luteal days to determine the presence, size and number of corpora lutea. Transdermal scanning was performed as previously described (Souza et al. 1997a) using a real-time Aloka 500 ultrasound scanner with a linear 7.5 MHz transducer probe (Dynamic Imaging, Livingston, UK) and recorded onto video tape. The position, size and area of all visible follicles and corpora lutea at each scan in each animal were subsequently determined using National Institutes of Health (NIH, Bethesda, MD, USA) image analysis software.

Statistical analysis

Hormone profile data were analysed using repeated measures ANOVA. Hormonal data in which there was significance between animal variations prior to the start of experimentation were converted to percentages of pretreatment values prior to analysis. Other hormonal data were log transformed prior to statistical analysis. Data from different replicate experiments were presumed to be independent. The characteristic of
pulsatile oestradiol secretion was determined using the Munro pulse analysis programme (Zaristow Software, East Lothian, UK). Analysis of pulse characteristic and follicle and corpus luteum size was performed by ANOVA following appropriate transformation. Analysis of monosaccharide concentrations in ovarian and jugular venous blood was performed utilising paired t-test.

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

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