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Effects of systemic progesterone during the early luteal phase on the availabilities of amino acids and glucose in the bovine uterine lumen

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Abstract. The uterine histotroph provides essential nutrition to the developing conceptus during the preimplantation period of pregnancy. The objective of the present study was to examine the effects of cycle stage and progesterone (P4) concentrations in the blood on the recoverable quantities of amino acids and glucose in the histotroph during the preimplantaion period of conceptus development. Following oestrus, dairy heifers were assigned to low, control or high P4 groups ($n = 6$ heifers per treatment and time point). The uterine horn ipsilateral to the corpus luteum was flushed on either Day 7 or Day 13. The present study quantified 24 amino acids and glucose in the uterine flushings using HPLC and fluorometry, respectively. Heifers in the low P4 group had lower plasma concentrations of P4 throughout the cycle, whereas heifers in the high group had higher plasma concentrations of P4 between Days 3 and 7 compared with the control group ($P < 0.05$). Total recoverable neutral (Ser, Gln, Gly, Thr, Cit, β-Ala, Tau, Ala, Tyr, Trp, Met, Val, Phe, Ile, Leu, Pro and Cys), acidic (Glu) and basic (His, Arg, Orn and Lys) amino acids were greater ($P < 0.05$) on Day 13 than on Day 7. There was no significant difference in the amount of Asp or Asn between Day 7 and Day 13. The amount of amino acids recovered on Day 7 was similar across treatment groups. On Day 13, the amount of Asn, His and Thr was lower ($P < 0.05$) in the low P4 heifers compared with the controls and/or high P4 heifers. Quantities of glucose were not altered by cycle stage or P4 treatment. In conclusion, the stage of oestrous cycle and P4 play important roles in modulating amino acids in the histotroph, a potentially critical factor for early embryonic and/or conceptus survival.

Additional keywords: embryo loss, fertility, histotroph.

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Introduction

The ability of the developing embryo to produce sufficient quantities of interferon-τ (IFNT) during the preimplantation phase is essential for maternal recognition of pregnancy, maintenance of a functional corpus luteum (CL), and subsequent maintenance of pregnancy in domestic ruminants, and must occur by Day 16 in cattle (Betteridge *et al.* 1980; Northey and French 1980; Spencer *et al.* 2007). Embryonic development, and therefore production of IFNT, during this critical preimplantation period is reliant on maternal provision (via secretion from endometrial glands) of a complex milieu of growth factors, enzymes, cytokines, transport proteins, vitamins, minerals, amino acids, glucose and other nutrients, collectively termed histotroph (Bazer *et al.* 1987; Gray *et al.* 2001; Gray *et al.* 2002; Gray *et al.* 2006; Spencer *et al.* 2007).

The central role that progesterone (P4) plays in the establishment and maintenance of pregnancy is well established (Bazer *et al.* 2008). Several studies have reported an association between the P4 concentrations and embryonic survival (Starbuck *et al.* 2001; Diskin and Sreenan 2005; Stronge *et al.* 2005; McNeill *et al.* 2006; Parr *et al.* 2012). Indeed, changes in systemic concentrations of P4 during the early luteal phase modulates embryonic development (Carter *et al.* 2008; Clemente *et al.* 2009; Forde *et al.* 2011*a*). The mechanisms by

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which systemic P4 affects embryonic development are proposed to be indirect via effects on the uterine endometrium (Clemente *et al.* 2009) that alter the timing and expression of endometrial genes (McNeill *et al.* 2006; Simmons *et al.* 2009; Satterfield *et al.* 2010; Forde *et al.* 2011*a*; Mullen *et al.* 2012*b*) and ultimately the composition of histotroph (Spencer *et al.* 2007; Forde *et al.* 2009; Forde *et al.* 2011*a*; Mullen *et al.* 2012*b*). The importance of the histotroph to survival of the conceptus (i.e. the embryo and its associated extraembryonic membranes) was clearly demonstrated in sheep when embryos transferred into uteri lacking endometrial glands for the secretion of histotroph failed to elongate (Gray *et al.* 2001, 2002).

During early embryonic development, amino acids and glucose serve important biological functions as major energy sources (Tiffin *et al.* 1991; Rieger *et al.* 1992; Partridge and Leese 1996; Lane and Gardner 1997; Kim *et al.* 2011*a*), as cell signalling molecules (Kimball and Jefferson 2004; Wu 2009) and substrates for protein synthesis (Alexiou and Leese 1992). In addition, they can act as intracellular osmolytes (Dawson *et al.* 1998; Tartia *et al.* 2009), regulators of pH (Baltz 1993; Edwards *et al.* 1998), antioxidants (Nasr-Esfahani *et al.* 1992), chelators (Van Winkle *et al.* 1990), substrates for synthesis of biologically important nitrogenous compounds (Kim *et al.* 2011*a*) and regulators of cell proliferation and differentiation (Gao *et al.* 2009*b*; Kim *et al.* 2011*b*).

Despite the requirement of histotroph by the early developing embryo and the significantly increased early embryonic mortality in dairy cattle over recent decades (Diskin *et al.* 2006; Diskin and Morris 2008), studies examining its composition and the effects of systemic P4 in the bovine are limited. Recent studies with cattle examined the effects of cycle stage on amino acid and energy substrates in oviducal fluids on Days 0, 2, 3, 4 and 6 and uterine fluids on Days 6, 8 and 14 of the oestrous cycle (Hugentobler *et al.* 2007, 2008). In addition, the same group examined the effects of P4 infused on Day 1–4 on ions, amino acids and energy substrates in oviducal and uterine fluids collected on Days 3 and 6, respectively (Hugentobler *et al.* 2010). These studies revealed novel information on the temporal and P4-induced effects on concentrations of amino acids and glucose in the bovine uterine lumen. However, there are no reports of the effects of systemic P4 during the early luteal phase on amino acids or glucose in the uterine lumen at later stages of the preimplantation period, coincident with critical stages of conceptus development in cattle. The hypothesis was that the availability of amino acids and glucose in the bovine uterus would change in accordance with the concentration of systemic P4 and stage of cycle. Therefore, the objective of the present study was to examine the effects of altered systemic concentrations of P4 during the early luteal phase on amino acid and glucose availability on Days 7 and 13 of the oestrous cycle in the bovine uterine lumen.

Materials and methods

Experimental design and collection of uterine flushings

All experimental procedures involving animals were licensed by the Department of Health and Children, Ireland, in compliance with the Cruelty to Animals Act (Ireland 1876) and the European Community Directive 86/609/EC and were sanctioned by the University College Dublin Animal Research Ethics Committee.

The heifers used in the present study were part of a larger cohort from which uterine flushings were collected, as described previously (Mullen *et al.* 2012*b*). Briefly, 40 Holstein–Friesian (HF) heifers were subjected to repeated prostaglandin (PG) $F_{2\alpha}$-induced synchronisation of oestrus and assigned to one of three groups representing high P4, control and low P4 ($n = 10$, 18 and 12 respectively). To generate groups divergent for systemic P4, intramuscular injections of $PGF_{2\alpha}$ on Days 3, 3.5 and 4 and insertion of progesterone releasing intravaginal device (PRID) on Days 3–6 were performed to induce low and high systemic concentrations of P4, respectively. Heifers that were unresponsive to treatment or underwent complete CL regression in the low P4 group were excluded, as described previously (Beltman *et al.* 2009). Heifers that received a PRID to increase plasma concentrations of P4 were chosen as those with the greatest deviation from the mean to avoid an overlap in P4 profiles. The uterine horns of heifers were flushed on each of two separate cycles to obtain a uterine flush sample on both Day 7 and Day 13. There were six heifers per treatment group that yielded flushings on both days, with the exception of one heifer in the high P4 group that failed to yield a suitable uterine flushing on Day 7. Thus, the total numbers of samples on Days 7 and 13 were 17 and 18, respectively (Table 1).

Uterine flushings were recovered from the uterine horn ipsilateral to the CL by an experienced commercial operative (BoviGenetics, Cavan, Ireland), and involved the careful introduction and removal of 50 mL of 100 mM Tris buffer (pH 7.2; Sigma Aldrich, Dublin, Ireland). All uterine flushings were

Table 1. Experimental design and flushing volume recovered from the ipsilateral uterine horn on Day 7 and Day 13 of the oestrous cycle of cyclic dairy heifers

X: flushing sample not available

Status	ID	Uterine horn	Flush volume recovered (mL)	
			Day 7	Day 13
High P4	8143	Ipsilateral	48	48
	8167	Ipsilateral	47	48
	8231	Ipsilateral	X	49
	8232	Ipsilateral	46	47
	8238	Ipsilateral	46	48
	8177	Ipsilateral	47	45
Control	8152	Ipsilateral	48	44
	8155	Ipsilateral	48	48
	8185	Ipsilateral	45	49
	8190	Ipsilateral	38	43
	8235	Ipsilateral	46	48
	8146	Ipsilateral	49	48
Low P4	8145	Ipsilateral	39	49
	8158	Ipsilateral	41	49
	8169	Ipsilateral	49	48
	8201	Ipsilateral	43	48
	8212	Ipsilateral	46	49
	8193	Ipsilateral	46	49

immediately centrifuged on-site at 4000*g* for 30 min at 4°C. The supernatants were aliquoted and snap frozen in liquid nitrogen before being transported on dry ice for storage at −80°C.

Analysis of amino acids and glucose

The analysis of amino acids and glucose in uterine flushes was performed as described previously (Gao *et al.* 2009*a*). Briefly, uterine flushings (0.5 mL) were deproteinised with an equal volume of 1.5 M $HClO_4$, followed by the addition of 0.25 mL of 2 M K_2CO_3. The extract was then analysed for glucose using a flourometric method involving hexokinase and glucose-6-phosphate dehydrogenase, as described previously (Wu 1995). Amino acids in the extract were determined by flourometric HPLC involving precolumn derivatisation with *o*-phthaldialdehyde, as described previously (Wu *et al.* 1997). Integration of chromatographic peaks was performed using Millenium-32 software (Waters, Milford, MA, USA).

Circulating P4 concentrations

Plasma concentrations of P4 were determined by radioimmunoassay (RIA) in a direct assay (Coat-A-Count; Siemens Medical Solutions Diagnostics, Los Angeles, CA, USA) without prior extraction, as described previously (Forde *et al.* 2011*a*). The intra- and inter-assay CV for samples containing mean concentrations of 0.43, 2.59 and 7.02 ng mL^{-1} P4 were 16.2% and 8.8%, 8.0% and 3.5%, and 6.7% and 2.9%, respectively. The minimum detectable concentration of P4 in the assay was 0.04 ng mL^{-1}.

Statistical analysis

To account for variations in the volume of uterine flushing recovered (Table 1), total recovered quantities of each substrate were calculated (by multiplying the concentration by the volume of flushing obtained) and used for subsequent comparisons herein.

The effect of treatment on circulating P4 concentrations and on recovered quantities of amino acids and glucose were calculated using PROC MIXED in SAS version 9.1 (SAS Institute, Cary, NC, USA). Terms for day and treatment × day were included in the model. Heifer was fitted as a repeated-measures term and the covariance structure chosen was unstructured. The DIFF option along with LS means in PROC MIXED was used for multiple comparisons. $P \leq 0.05$ was considered significant.

The relationship between circulating P4 concentrations averaged between Days 3–7, 8–10 and 11–13 and amino acid and glucose recovery were evaluated using regression analysis. Where a significant linear relationship was recorded, circulating P4 concentrations were fitted as a quadratic term and retained in the final model if statistically significant ($P \leq 0.05$).

Results

Progesterone model

Plasma concentrations of P4 from Day 3 to 13 ranged between 0.76 and 6.87 ng mL^{-1}, 0.59 and 7.49 ng mL^{-1} and 0.45 and 5.92 ng mL^{-1} for the high, control and low P4 groups, respectively (Fig. 1). Plasma concentrations of P4 were higher for heifers in the high P4 group from Day 3 to Day 7 and lower in the low P4 group from Day 3 to the day of collection of uterine flushings compared with levels in the control group (area under the curve (AUC) $P < 0.05$).

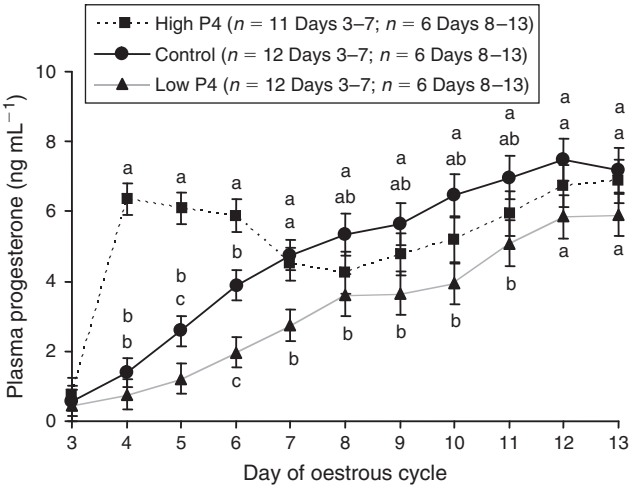

Fig. 1. Plasma concentrations of progesterone (P4) in cyclic heifers in the high, control and low P4 treatment groups. Heifers were treated with a PRID device from Day 3 until Day 6 (■), whereas other heifers (▲) were treated with prostaglandin $F_{2\alpha}$ on Days 3, 3.5 and 4 or served as control untreated heifers (●). Data are the mean ± s.e.m. Values for different treatment groups on a given day with different letters differ significantly ($P < 0.05$).

Volume of uterine flushing recovered

The mean (± s.d.) volume of uterine flushing recovered on Day 7 and 13 was 45.4 ± 3.3 and 47.6 ± 1.8 mL, respectively (Table 1).

Effects of P4 and cycle stage on recoverable amounts of glucose and amino acids in uterine flushings

Total recoverable glucose in uterine flushings was similar across both Day 7 and Day 13, as well as across P4 treatment groups ($P > 0.05$; Table 2).

Total recoverable amounts of Arg, His, Lys, Orn, Glu, Ser, Gly, Thr, Ala, β-Ala, Tau, Cys, Cit, Tyr, Trp, Met, Val, Phe, Ile, Pro, Gln and Leu were higher on Day 13 than on Day 7 (day effect $P < 0.01$; Fig. 2; Table 2). Total recoverable amounts of Asp and Asn were not altered by cycle stage (day effect $P > 0.05$; Table 2).

The effects of P4 treatment on total recoverable amounts of any of the amino acids on either Day 7 or Day 13 were not significant ($P > 0.05$), with the exceptions of His, Asn and Thr, for which heifers with lower plasma concentrations of P4 yielded lower amounts of His, Asn, and Thr on Day 13 than did heifers in the control or high P4 groups ($P < 0.02$; Figs 3–5).

To illustrate the effect of day, and in the absence of significant treatment by day interactions, the mean quantities of amino acids recovered on each day across P4 groups are shown in Fig. 2.

Table 2. Effects of day of the oestrous cycle, progesterone status and the interaction between day and progesterone status on total recoverable amino acids and glucose in bovine uterine flushings

Data are the mean ± s.e.m. Within treatment groups, values with different superscript letters differ significantly ($P \leq 0.02$). P4, progesterone

		Day 7	Day 13	High P4	Control	Low P4	*P*-value		
							Day effect	Treatment effect	Day × treatment interaction
Basic amino acids	ARG	1007 ± 75	1357 ± 73	1182 ± 94	1141 ± 89	1223 ± 89	0.002	0.813	0.121
	HIS	16 ± 2	31 ± 2	25 ± 3^{ab}	28 ± 3^{a}	17 ± 3^{b}	0.000	0.033	0.110
	LYS	705 ± 67	1032 ± 65	850 ± 83	846 ± 79	909 ± 79	0.002	0.826	0.172
	ORN	182 ± 20	272 ± 19	215 ± 25	241 ± 23	225 ± 23	0.003	0.737	0.105
Acidic amino acids	ASP	61 ± 6	60 ± 5	68 ± 7	58 ± 7	54 ± 7	0.892	0.343	0.385
	GLU	201 ± 24	302 ± 23	279 ± 30	243 ± 29	231 ± 29	0.006	0.496	0.547
Small neutral amino acids (≤C4)	ASN	11 ± 1	14 ± 1	15 ± 2^{a}	13 ± 2^{ab}	9 ± 2^{b}	0.114	0.042	0.650
	SER	124 ± 13	194 ± 13	173 ± 16	171 ± 15	133 ± 15	0.001	0.144	0.524
	GLY	3198 ± 187	4202 ± 181	3733 ± 233	3723 ± 222	3644 ± 222	0.001	0.954	0.163
	THR	1075 ± 58	1334 ± 56	1204 ± 72	1285 ± 68	1124 ± 68	0.003	0.266	0.083
	ALA	407 ± 39	681 ± 38	551 ± 49	533 ± 47	549 ± 47	<0.0001	0.957	0.468
	β-ALA	2232 ± 188	3425 ± 182	2916 ± 234	2825 ± 223	2745 ± 223	<0.0001	0.871	0.074
	TAU	828 ± 96	1424 ± 93	1194 ± 119	1056 ± 114	1129 ± 113	0.000	0.704	0.383
	CYS	722 ± 60	1096 ± 58	907 ± 75	935 ± 71	885 ± 71	0.000	0.885	0.558
Large neutral amino acids (>C5)	CIT	506 ± 55	720 ± 53	629 ± 68	597 ± 65	613 ± 65	0.009	0.942	0.085
	TYR	118 ± 13	209 ± 13	165 ± 16	163 ± 16	163 ± 16	<0.0001	0.991	0.407
	TRP	18 ± 3	34 ± 2	26 ± 3	28 ± 3	23 ± 3	<0.0001	0.499	0.652
	MET	21 ± 3	34 ± 3	29 ± 4	28 ± 4	25 ± 4	0.005	0.689	0.369
	VAL	183 ± 20	324 ± 19	245 ± 25	261 ± 24	254 ± 24	<0.0001	0.898	0.666
	PHE	136 ± 20	250 ± 19	183 ± 24	202 ± 23	195 ± 23	0.000	0.854	0.524
	ILE	146 ± 20	282 ± 19	205 ± 24	218 ± 23	220 ± 23	<0.0001	0.892	0.663
	PRO	852 ± 64	1238 ± 62	1025 ± 79	1077 ± 76	1033 ± 76	0.000	0.875	0.503
	GLN	205 ± 18	340 ± 17	276 ± 22	276 ± 21	264 ± 21	<0.0001	0.893	0.431
	LEU	266 ± 28	433 ± 27	337 ± 35	355 ± 34	355 ± 34	0.000	0.910	0.885
Glucose		370 ± 96	501 ± 93	470 ± 119	417 ± 114	420 ± 114	0.337	0.938	0.152

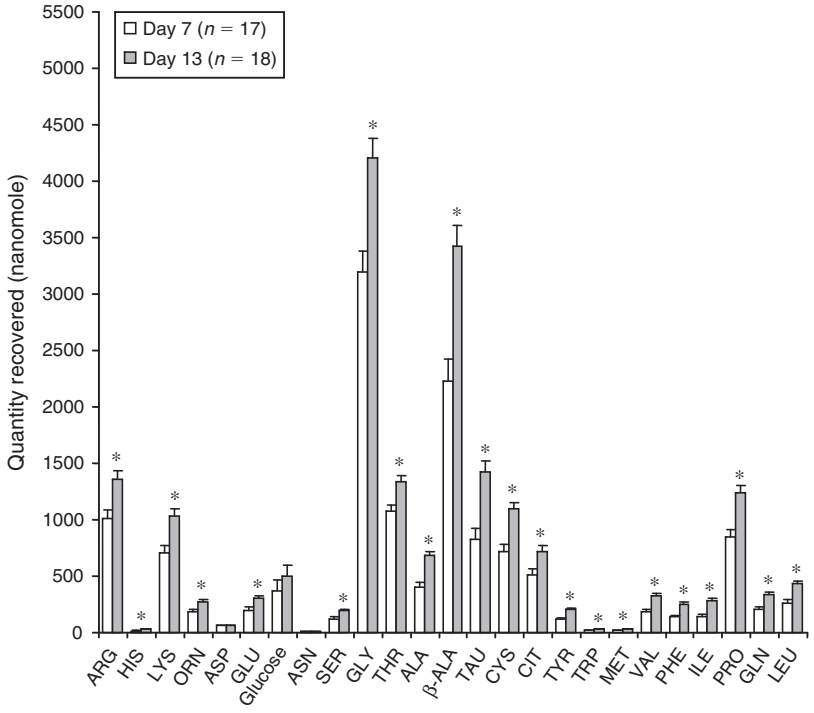

Fig. 2. Quantities of amino acids and glucose in uterine flushings from dairy cattle on Day 7 ($n = 17$) and Day 13 ($n = 18$) of the oestrous cycle, as determined by HPLC and fluorometry, respectively. Data are the mean ± s.e.m. Asterisks indicate a significant effect of day for individual amino acids ($P < 0.01$).

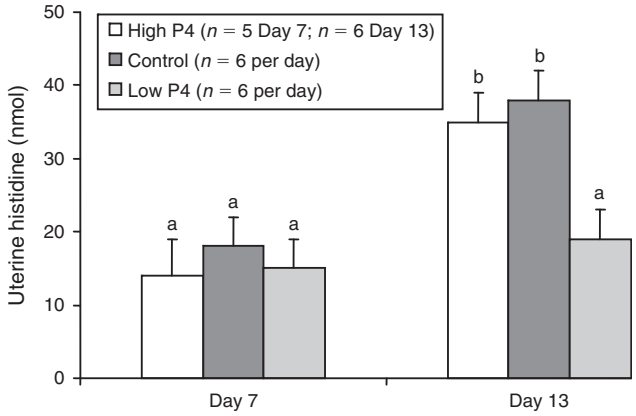

Fig. 3. Quantities of histidine recovered from uterine flushes on Days 7 and 13 of the oestrous cycle in the high, control and low progesterone (P4) cyclic dairy heifers, as determined by HPLC analysis. Data are the mean ± s.e.m. Values with different letters differ significantly ($P \leq 0.01$).

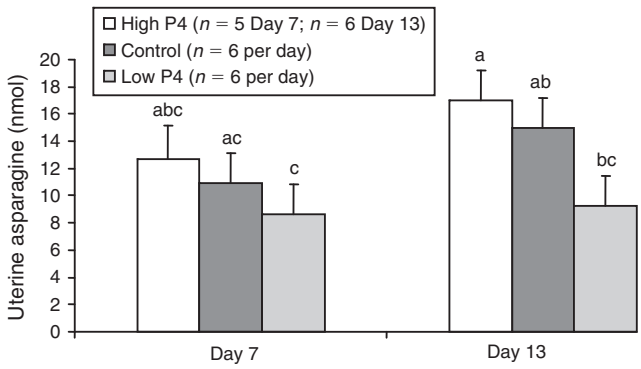

Fig. 4. Quantities of asparagine recovered from uterine flushes on Days 7 and 13 of the oestrous cycle in the high, control and low progesterone (P4) cyclic dairy heifers, as determined by HPLC analysis. Data are the mean ± s.e.m. Values with different letters differ significantly ($P \leq 0.01$).

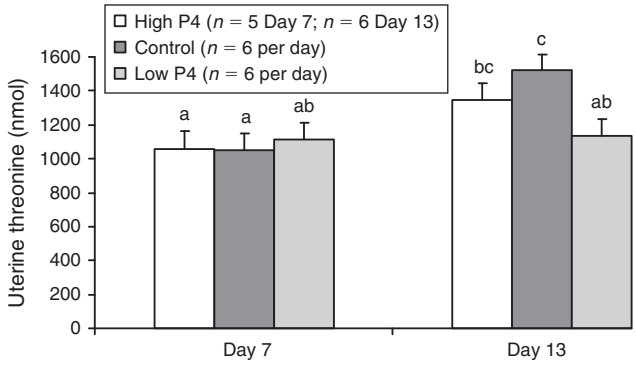

Fig. 5. Quantities of threonine recovered from uterine flushes on Days 7 and 13 of the oestrous cycle in the high, control and low progesterone (P4) cyclic dairy heifers, as determined by HPLC analysis. Data are the mean ± s.e.m. Values with different letters differ significantly ($P \leq 0.01$).

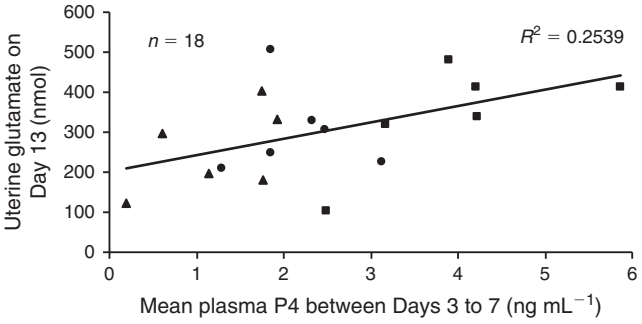

Fig. 6. Total recoverable glutamate in the histotroph on Day 13 of the oestrous cycle and its relationship with plasma progesterone (P4) concentrations averaged from Day 3 to Day 7. Results are from heifers treated with a PRID device from Day 3 until Day 6 (■), heifers treated with prostaglandin $F_{2\alpha}$ on Days 3, 3.5 and 4 (▲) and control untreated heifers (●).

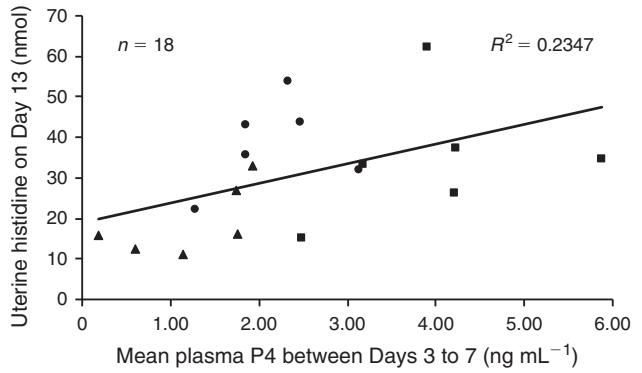

Fig. 7. Total recoverable histidine in the histotroph on Day 13 of the oestrous cycle and its relationship with plasma progesterone (P4) concentrations averaged from Day 3 to Day 7. Results are from heifers treated with a PRID device from Day 3 until Day 6 (■), heifers treated with prostaglandin $F_{2\alpha}$ on Days 3, 3.5 and 4 (▲) and control untreated heifers (●).

Relationship between circulating P4 concentrations during the early luteal phase and amino acids and glucose amounts in uterine flushings

There were no significant relationships ($P > 0.05$) between mean P4 plasma concentrations on Days 3–7, 8–10 or 11–13 and the amount of either amino acids or glucose in uterine flushings collected on Day 7. However, the amount of recoverable Ser on Day 7 tended to have a positive linear relationship with mean plasma P4 concentrations averaged between Days 3 and 7 ($P < 0.1$).

On Day 13, mean plasma P4 concentrations between Days 3 and 7 had a positive linear relationship with Glu and His recovery ($P < 0.05$; Figs 6, 7), as well as a tendency for greater Asn and Tau recovery ($P \leq 0.08$) in uterine flushings. Plasma concentrations of P4 averaged from Day 8 to Day 10 had a positive linear relationship with Ser and a tendency for higher Met recovery in uterine flushings on Day 13 ($P < 0.03$ and $P < 0.1$, respectively; Fig. 8). Although there was no significant relationship between mean P4 concentrations from Day 11 to

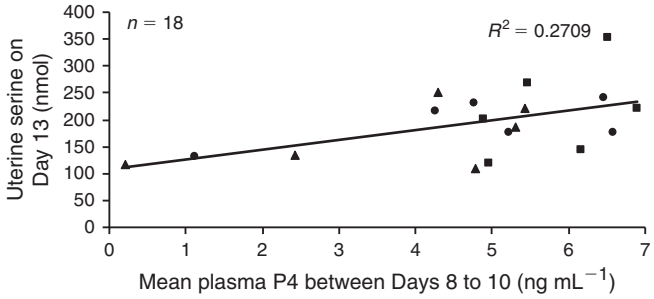

Fig. 8. Total recoverable serine in the histotroph on Day 13 of the oestrous cycle and its relationship with plasma progesterone (P4) concentrations averaged from Day 3 to Day 7. Results are from heifers treated with a PRID device from Day 3 until Day 6 (■), heifers treated with prostaglandin $F_{2\alpha}$ on Days 3, 3.5 and 4 (▲) and control untreated heifers (●).

Day 13 and amino acid recovery, there was a trend for a positive linear relationship with Ser in uterine flushings collected on Day 13 ($P < 0.1$).

Discussion

The main findings of the present study were that: (1) the most abundant amino acids in bovine histotroph were Gly and β-Ala, for which there was an approximate 250-fold difference compared with the least abundant amino acid, Asn; (2) the maternal provision of 23 amino acids increased in uterine histotroph between Days 7 and 13, which coincides with the increasing requirements of rapidly developing conceptuses for nutrients; (3) low circulating P4 concentrations at different stages of the early luteal phase modulated the availability of amino acids His, Thr and Asn on Day 13 in uterine histotroph, an important period in conceptus development during the preimplantation period; and (4) the availability of glucose in the uterine fluid during this developmental period was not significantly altered by P4 or cycle stage.

Within the first 2 weeks after oestrus, available evidence in cattle suggests that the uterine environment is largely independent of the developing embryo and/or conceptus, but then undergoes significant temporal modulation and assumes a pregnant state until there is either recognition of pregnancy by approximately Day 16 or luteolysis occurs in advance of a return to oestrus (Spencer *et al.* 2007; Forde *et al.* 2011*b*, 2012; Mullen *et al.* 2012*a*). During these first 2 weeks of pregnancy the embryo undergoes rapid growth and development, increasing over 160-fold in protein content between Day 7 and Day 13 (Grealy *et al.* 1996). Therefore, given the importance of histotroph to conceptus survival, analysis of the uterine environment in high-fertility dairy heifers and its response to systemic P4 during this critical time period was expected to yield additional information regarding factors required for successful establishment of pregnancy in the bovine.

In the present study, Gly and β-Ala were the most abundant amino acids in the uterine fluid, followed by Thr, Arg, Tau and Pro. The identification of Gly as a highly abundant amino acid in female reproductive tract fluids is consistent with previous observations in cattle (Fahning *et al.* 1967; Tiffin *et al.* 1991; Moore and Bondioli 1993; Steeves and Gardner 1999; Elhassan *et al.* 2001; Hugentobler *et al.* 2007), sheep (Hill *et al.* 1997; Moses *et al.* 1997; Gao *et al.* 2009*b*), horses (Engle *et al.* 1984) and rabbits (Miller and Schultz 1987). The relatively high abundance of Gly in the uterine lumen across species suggests that it has an important role in embryo and/or conceptus development, and this is further supported by its abundance in the fetuses of swine throughout pregnancy (Wu *et al.* 1999). Indeed, the addition of Gly to the culture medium enhances the development of porcine and bovine embryos *in vitro* (Moore and Bondioli 1993; Lee and Fukui 1996; Takahashi and Kanagawa 1998; Mito *et al.* 2012). Although the mechanisms are not well defined, Gly may serve several functions during embryo and/or conceptus development, including acting as a precursor to protein or nucleic acid synthesis, regulation of intracellular pH and osmoregulation (Edwards *et al.* 1998; Steeves *et al.* 2003; Tartia *et al.* 2009; Moravek *et al.* 2012). The developing embryo is particularly susceptible to changes in intracellular pH (FitzHarris and Baltz 2009) and increased osmolarity (Dawson *et al.* 1998; Hammer and Baltz 2003). The abundance (and temporal increase) of effective osmoregulators such as Gly (Dawson *et al.* 1998; Moravek *et al.* 2012), β-Ala (Dawson *et al.* 1998; Hammer and Baltz 2003), Pro (Dawson *et al.* 1998) and Tau (Menezo and Guerin 1997) in uterine flushings in the present study, coupled with the significant production of Gly by the embryo *in vitro* (Lee and Fukui 1996), suggests that these amino acids play an important role in osmotic regulation during the preimplantation period in the bovine. In addition, the organic osmoregulatory effects of Gly and β-Ala are additive during murine embryo development, indicating a common mode of action (Dawson *et al.* 1998).

A significant effect of day of cycle was evident in the present study, with total recoverable quantities of 22 amino acids being higher, and two (Asn and Asp) being similar, on Day 13 compared with Day 7. Day 7 and Day 13 of pregnancy were selected because they represent days for two critical developmental events for rapidly developing bovine embryos as they reach the blastocyst stage and then initiate elongation as a conceptus, respectively (Spencer *et al.* 2007). These results indicate an increasing requirement for a large proportion of the amino acids examined by the developing conceptus during the initiation of elongation. To the best of our knowledge only two other studies (Fahning *et al.* 1967; Hugentobler *et al.* 2007) have examined the effects of cycle stage on amino acid availability in bovine uterine fluid. However, the present study, which includes the analysis of amino acids Orn, β-Ala, Cys, Cit and Pro, represents the most comprehensive information to date with regard to the identification of all protein amino acids and major non-protein amino acids in bovine histotroph.

Hugentobler *et al.* (2007) reported a temporal increase in the concentrations of eight amino acids (Ser, Thr, Tyr, Met, Val, Ile and Leu) in uterine fluid from either Days 6 or 8 and Day 14 in cyclic beef heifers, which is consistent with our findings. However, in contrast with the results of the present study, Hugentobler *et al.* (2007) reported a temporal decrease in the concentrations of Gly, Tau, His and Phe and no significant differences in the concentrations of Asp, Glu, Asn, Gln, Arg, Ala, Trp or Lys between Days 6, 8 and 14. Although we also

found no temporal effect on uterine Asp or Asn, we did observe a temporal increase in the recovery of Gly, Tau, His, Phe, Glu, Gln, Arg, Ala, Trp and Lys as the cycle progressed. The reasons for these differences in results are not clear as both studies used comparable methods for amino acid analysis; however, there were differences in the methods used for sample collection. Hugentobler *et al.* (2007) collected neat uterine fluid *in vivo* using a moderate period (3 h) of anaesthesia in comparison to the collection of uterine flushings. Whether the use of general anaesthesia, as pointed out by Hugentobler *et al.* (2007), affects the uterine environment remains unknown. However, a uterine flushing approach can result in alterations in the concentration of analytes detected, therefore normalisation according to recovery volume is required and results expressed as total quantities recovered. It should also be noted that examination of any secretome introduces the risks of the introduction of intracellular material due to cell damage that, despite one's best efforts, cannot be ruled out as a source of variation among studies.

Of particular interest in the present study was the temporal increase and relative abundance of Arg, Lys, His and Cit in uterine flushings. Arg, Lys and His (all basic amino acids that share the same transport systems) are essential amino acids that cannot be synthesised by blastocysts, but are required during *in vitro* culture for murine blastocyst development and expansion, suggesting an important role during conceptus development and the establishment of pregnancy (for a review, see Bazer *et al.* 2012). Subsequent studies revealed that Arg or Leu are central to murine blastocyst expansion and further development, initiating signalling pathways (including mammalian target of rapamycin), increasing mRNA expression and production of nitric oxide and polyamines essential for conceptus development and successful pregnancy (Bazer *et al.* 2012). Other studies have demonstrated the importance of Arg during pregnancy. For example, intravenous administration of L-Arg or L-Cit (a precursor for the synthesis of Arg) enhanced Arg availability in the ovine fetus, which enhanced fetal growth and survival in multiple birth pregnancies and prevented fetal growth restriction in malnourished ewes (Lassala *et al.* 2009, 2010, 2011). Furthermore, in humans, Arg deficiency in preterm infants results in hyperammonaemia and cardiovascular, pulmonary, neurological and intestinal dysfunction (Becker *et al.* 2000; Wu *et al.* 2004).

Other temporally modulated amino acids of interest include Orn and Cys. Orn is an intermediary in the synthesis of polyamines, derived from the conversion of Arg to Orn by arginase followed by conversion of Orn to polyamines by ornithine decarboxylase (ODC1), which is essential for embryogenesis and placental development (Fozard *et al.* 1980; Bazer *et al.* 2012). Cys, a member of the functional amino acids (defined as regulators of key metabolic pathways benefiting survival, growth, development, reproduction and health; Wu *et al.* 2009), has been shown to improve the development of porcine blastocysts *in vitro* (Choe *et al.* 2010).

The models used herein to modulate systemic concentrations of P4 were described previously by Beltman *et al.* (2009) and Carter *et al.* (2008). They were designed primarily to generate divergent P4 profiles primarily between Days 3 and 7 of the oestrous cycle, as reported in several studies in beef cattle, to examine the effects of P4 on endometrial gene expression and subsequent embryo and/or conceptus development (Carter *et al.* 2008, 2010; Forde *et al.* 2011*a*). This approach addresses the difficulty associated with determining the effects of systemic P4 on the composition of uterine fluid due to the variation in endogenous concentrations of P4 among animals on the same day and among cycles (Hugentobler *et al.* 2010; Parr *et al.* 2012). This model results in significant divergence in concentrations of systemic P4 during Days 3–7 and, to a lesser extent, between Days 8 and 13 after oestrus; in beef heifers, its use has clearly demonstrated the effects of a high and low P4 environment during the early luteal phase on the timing of endometrial gene expression and resultant advancement or retardation of conceptus development (Carter *et al.* 2008; Forde *et al.* 2009, 2011*a*).

Despite the effects of systemic P4 on endometrial gene expression in beef cattle (Carter *et al.* 2008, 2010; Forde *et al.* 2009, 2011*a*) and amino acid content in uterine flushings from sheep and pigs (Satterfield *et al.* 2010; Bazer *et al.* 2012), we found only subtle effects on uterine amino acid composition in Holstein–Friesian heifers. The high P4 environment had no effect on amino acid or glucose recovery on either Day 7 or Day 13, whereas the low P4 environment modulated the availability of three amino acids His, Thr and Asn on Day 13, with reduced recovery in low P4 heifers compared with their high P4 or control counterparts. This would suggest that the curvilinear relationship observed in several studies between systemic concentrations of P4 during the early luteal phase and embryo survival rates in beef and dairy cattle (Diskin and Sreenan 2005; Stronge *et al.* 2005; McNeill *et al.* 2006), most recently demonstrated by Parr *et al.* (2012), is, at least in the case of high P4, not contributed to by modulation of uterine amino acid or glucose availability (before implantation). However, the reduced uterine availability of His, Thr and Asn observed in the low P4 heifers may contribute to the retardation of conceptus elongation on Day 14 (following embryo transfer on Day 7) observed in a similar low P4 environment (generated using the model herein) by Forde *et al.* (2011*a*) and the suboptimal pregnancy rates associated with low P4 in the aforementioned studies (Diskin and Sreenan 2005; Stronge *et al.* 2005; McNeill *et al.* 2006; Parr *et al.* 2012). Recoverable levels of Asn and Thr in uterine flushings are modulated by P4 in ovine and porcine uteri (Bazer *et al.* 2012), but this is the first report of an effect of P4 on the availability of His in uterine fluid. His, a basic amino acid, is a precursor for histamine synthesis via histidine decarboxylase that is essential for preimplantation embryo development in mice (Dey and Johnson 1980; Hudgins *et al.* 1982) and it has an important role in tissues undergoing rapid growth (Russell and Snyder 1968; Dzodzœ and Rosengren 1971). Threonine is a limiting factor for fetal growth in rats (Metcoff *et al.* 1981), whereas evidence supporting an important role for Asn is limited. Asn added to culture medium along with Asp, Glu, Arg and Ile has embryotrophic effects (Lee *et al.* 2004).

However, it should be noted that because the effects in the present study were only significant in the low P4 environment, it cannot be rule out, despite the short half-life of $PGF_{2\alpha}$ of 8 min (Kindahl *et al.* 1976) and endogenous production throughout the

oestrous cycle (Murakami *et al.* 2001), that $PGF_{2\alpha}$ administration did not contribute directly or indirectly to the effects on the uterine environment.

In cattle, only two studies by Hugentobler *et al.* (2007, 2010) have related systemic progesterone concentrations to uterine amino acid composition by comparing: (1) endogenous variation of systemic P4 throughout the oestrous cycle with uterine constituents on Days 6, 8 and 14; and (2) the effects of intravenous administration of P4 on Days 1–4 on oviducal and uterine amino acids on Days 3 and 6, respectively. Hugentobler *et al.* (2007, 2010) found no effect of endogenous systemic concentrations of P4 on uterine amino acid composition (possibly due to a lack of sufficient variation), but did identify a positive association between administered P4 and uterine fluid content of glucose and Val on Day 6. This suggests a temporal delay in the uterine response to systemic P4, in agreement with previous observations by our group on the uterine abundance of retinol-binding protein (RBP; Mullen *et al.* 2012*b*), wherein systemic P4 during Days 3–7, while showing weak relationships with histotroph constituents on Day 7, more effectively modulated the uterine environment later in the cycle, between Day 7 and Day 13. Interestingly, in the present study, linear regression analysis of mean plasma P4 concentrations between Days 3–7, 8–10 and 11–13 identified positive linear relationships between P4 during the early luteal phase (Days 3–7) and Ser (an immediate precursor of glycine) availability on Day 7 and P4 during the mid-luteal phase (Days 8–10 and 11–13) and Ser availability on Day 13. These results indicate that: (1) P4 alters the timing of nutrient availability in a specific manner (supporting a stage- or function-specific role of amino acids on embryonic development; Lee *et al.* 2004); and (2) systemic P4 after Day 7 also modulates the composition of the histotroph during the preimplantation period of pregnancy in cattle.

Glucose is an essential source of energy to the developing embryo (Petters *et al.* 1990) and can be used to form not only glycogen, but also nucleic acids, proteins and lipids. However, oocytes and early embryos up to the 8-cell stage have a preference for pyruvate before switching to glucose at the blastocyst stage (Leese 1991; Boland *et al.* 2001). In fact, high concentrations of glucose during the initial cleavage stage are inhibitory to embryonic development in the bovine (Takahashi and First 1992), porcine (Flood and Wiebold 1988), murine (Chatot *et al.* 1989), hamster (Schini and Bavister 1988) and human (Conaghan *et al.* 1993) due to the Crabtree effect, resulting in accelerated glycolysis (Schini and Bavister 1988). Although the bovine embryo does not require glucose until Day 3 or 4 *in vitro* (Kim *et al.* 1993), it is an essential nutrient from the early morula stage onwards (Rieger *et al.* 1992), which corresponds to approximately Day 6. Our findings that the cycle stage, although numerically higher on Day 13 than on Day 7, did not significantly affect uterine glucose availability are consistent with the results of previous studies with cyclic beef heifers (Hugentobler *et al.* 2008) in which glucose availability was examined on Days 6, 8 and 14. In addition, in the sheep, uterine glucose recovery was not affected by the day of the oestrous cycle, but increased significantly between Days 10 and 16 of pregnancy (Gao *et al.* 2009*b*). In contrast with the present study, systemic P4 affected several components of the histotroph in ewes, including recoverable glucose, Asp, Asn, Ser, Ala, Gln, β-Ala, Cit, Arg and Lys on Day 9 and Lys and Arg on Day 12, which may reflect differences in hormonal regulation of the histotroph between species.

In conclusion, the results of the present study add novel information on the maternal environment during early embryonic development in the bovine and affirm previous observations for a central role of cycle stage in concert with the P4 environment in modulating histotroph composition and, possibly, an important role in the nutrition of developing embryos before maternal recognition of pregnancy. In addition, these results may assist in the optimisation of culture media for *in vitro* studies of embryonic development.

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