

# Steroidogenesis in bovine granulosa cells: the effect of short-term changes in dietary intake

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The nutritional status of a cow is a key factor in the regulation of both follicle growth and oocyte quality. In this study, the effect of diets designed to increase circulating insulin and insulin-like growth factor I (IGF-I) concentrations on steroid production by granulosa cells *in vitro* was examined to analyse the mechanisms through which these changes occur. Hereford × Friesian heifers ( $n = 24$ ) were offered maintenance or twice maintenance diets during the experimental period (17 days). Circulating concentrations of FSH did not differ between the two dietary groups, whereas insulin and IGF-I concentrations showed significant diet × day of oestrous cycle interactions. Ovaries were collected on day 3 of the first follicle wave after synchronization of oestrus. Granulosa cells were isolated from small (1–4 mm) and medium-sized (4–8 mm) follicles and cultured in the presence of long R3-IGF-I or bFSH or both. After 4 days in culture, granulosa cells isolated from small follicles, but not

medium-sized follicles, collected from cattle offered the twice maintenance diet secreted significantly higher ( $P < 0.05$ ) amounts of oestradiol compared with granulosa cells collected from cattle offered the maintenance diet. The effect was apparent in either the presence or absence of FSH and long R3-IGF-I. This nutritional effect on aromatase activity in granulosa cells was not apparent after day 6 of culture. There was no effect of diet on progesterone production by granulosa cells after 4 or 6 days of culture. These results support the hypothesis that dietary-induced changes in circulating insulin and IGF-I concentrations have a direct effect on the steroidogenic potential of bovine granulosa cells from small follicles. The dietary-induced increases in aromatase activity in small follicles combined with the increased concentration of metabolic hormones are possible mechanisms through which short-term changes in nutrition may affect follicle dynamics.

## Introduction

Nutritional status is a major factor controlling fertility in cattle and the direct effects of nutrition on ovarian function have been well documented (Robinson, 1990; O'Callaghan and Boland, 1999; Robinson *et al.*, 1999; Boland *et al.*, 2001). Specific examples of the influence of dietary intake on ovarian function are the correlations between the diameters of preovulatory follicles and weight loss in beef cattle (Rhodes *et al.*, 1995), and between negative energy balance and follicle growth in postpartum dairy cattle (Beam and Butler, 1999), and the effects of diet on developmental competence of oocytes in heifers (Sinclair *et al.*, 2000; Armstrong *et al.*, 2001).

Although the effects of short-term changes in nutrition on bovine ovarian function have been described, the precise mechanisms involved in regulation of follicle growth are not understood. Recent studies have indicated that short-term changes in nutrition regulate follicle recruitment without affecting circulating concentrations of FSH (Gutierrez *et al.*, 1997a). Circulating insulin concentrations

were positively correlated with dietary energy and it was proposed that this hormone is a key factor in mediating the effects of dietary intake on ovarian function. Supporting this contention are the observations that bovine granulosa cells in culture are dependent on the presence of physiological concentrations of insulin (Holtorf *et al.*, 1989; Gutierrez *et al.*, 1997b; Glister *et al.*, 2001) and that the infusion of insulin into beef heifers resulted in an increase in the diameter of the dominant follicle (Simpson *et al.*, 1994).

Subsequent studies have shown that diets designed specifically to increase circulating insulin and insulin-like growth factor I (IGF-I) concentrations affect the ovarian IGF system directly (Armstrong *et al.*, 2001). In particular, mRNAs encoding IGF-binding protein 2 (IGFBP-2) in granulosa cells and IGFBP-4 in theca cells from small follicles were reduced in cattle offered a high energy diet compared with those offered a lower energy diet. Armstrong *et al.* (2001) hypothesized that a decrease in the steady state expression of mRNAs encoding IGFBP-2 and -4 would increase the bioavailability of IGF in small follicles and, thereby, increase their response to FSH, resulting in the increased growth rate of small follicles.

As a consequence of these nutritionally induced changes

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**Table 1.** Chemical analysis of the cattle feed (1663 harvest feed nuts) used for maintenance and twice maintenance diets of heifers

	Hay	Concentrate
Dry matter (g kg <sup>-1</sup> )	913	863
Metabolizable energy (MJ per kg dry matter)	8.3	10.9
Crude protein (g per kg dry matter)	83	190
Organic matter	942	927
Acid detergent fibre	288	148
Neutral detergent fibre	664	220

in the ovarian IGF system, we predicted that granulosa cells isolated from small follicles collected from the ovaries of cattle at a high plane of nutrition would produce more oestradiol *in vitro* than granulosa cells collected from cattle at a lower plane of nutrition. Therefore, this study was designed to analyse the effects of short-term changes in dietary intake on steroidogenesis in bovine granulosa cells *in vitro*.

## Materials and Methods

### Materials

Medium 199, McCoy's 5A modified medium, Hepes, amphotericin, L-glutamine, Dulbecco's phosphate-buffered saline without calcium and magnesium (DPBS-), McCoy's 5a medium with sodium bicarbonate, PenStrep solution containing 10 000 iu penicillin and 10 mg streptomycin per ml, bovine serum albumin (BSA), tissue culture grade transferrin, selenium, bovine insulin, neutral red, trypsin, testosterone and trypan blue were all purchased from Sigma Aldrich Co. Ltd (Poole). Bovine FSH (USDA-bFSH-I-2; bioactivity potency 854 iu mg<sup>-1</sup>) was generously donated by the US Department of Agriculture. Human recombinant IGF-I and the IGF-I analogue long R3-IGF-I (LR3-IGF-I, which has a reduced affinity to IGF-BPs), were acquired from Gropep Pty Ltd (Adelaide).

### Dietary treatments and experimental design

Twenty-four cattle were selected from a herd of 2–3-year-old Hereford × Friesian heifers, with an average body weight of 449.4 ± 31.0 kg (mean ± SD; *n* = 32) and body condition score ranging from 1.75 to 2.50 (on a scale of 1–5). The animals were divided randomly into three experimental groups (*n* = 8) and kept individually in pens with wood shavings as bedding material. Within each experimental group, heifers (*n* = 4) were allocated randomly into two treatment groups and fed either a maintenance or twice maintenance diet, offered as two meals per day at 09:00 h and 16:30 h.

The compositions of the two diets (Table 1) were calculated according to AFRC (1993), and consisted of 2 kg hay per day and either 2.5 kg concentrate per day (maintenance group) or 6.5 kg concentrate per day (twice maintenance group).

For 1 week before the start of the experiment the animals were adapted to their respective diets. Controlled feeding (Fig. 1) began on the day of insertion of a controlled intra-vaginal drug-releasing device (CIDR; InterAg, Hamilton). The oestrous cycles of the heifers were synchronized by a PGF<sub>2α</sub> (Estrumate; Coopers Animal Health Ltd, Crewe) injection 2 days before CIDR removal (day 10) and ovulation was induced by a GnRH agonist (Buserelin; Hoechst, Hounslow) injection on day 12. Heifers were killed on day 17 (day 3 of the first follicle wave) at a local abattoir. Blood sampling (10 ml) and ultrasonography were performed daily after feeding between 09:00 h and 11:00 h.

### Isolation of granulosa cells

After the heifers were killed, the ovaries were placed immediately in collection medium (medium 199, with 20 mmol Hepes l<sup>-1</sup>, 100 iu penicillin ml<sup>-1</sup>, 0.1 mg streptomycin ml<sup>-1</sup> and 0.1% (w/v) BSA) at 37°C, and transported to the laboratory. The ovaries were trimmed, washed briefly in 70% (v/v) ethanol and maintained in collection medium (at 37°C) until dissection. The follicles were blunt-dissected and classified according to external diameter into small (< 4 mm) and medium-sized (4–8 mm) follicles. Granulosa cells were isolated from each group of follicles as described by Gutierrez *et al.* (1997b) and suspended in culture medium consisting of McCoy 5A modified medium supplemented with 1% (v/v) PenStrep solution, 10 ng bovine insulin ml<sup>-1</sup>, either 0 or 10 ng LR3-IGF-I ml<sup>-1</sup>, 3 mmol glutamine l<sup>-1</sup>, 20 mmol Hepes l<sup>-1</sup>, 5 mg apo-transferrin ml<sup>-1</sup>, 4 ng sodium selenite ml<sup>-1</sup>, 0.1% (w/v) BSA and 10<sup>-7</sup> mol testosterone l<sup>-1</sup>. The number of cells was counted using a haemocytometer and viability estimates measured after trypan blue dye exclusion ranged from 10 to 25%.

### Cell culture

Three cultures were performed. Each culture was performed using granulosa cells isolated from eight heifers. Within each culture, ovaries were grouped according to whether they were collected from heifers offered the maintenance or twice maintenance diet and within both of these groups follicles were subdivided further according to follicle size before pooling and isolation of granulosa cells. Cells (5.0–7.5 × 10<sup>4</sup> per 50 µl culture medium) were seeded into individual wells in 96-well culture plates (Nunclon; Life Technologies, Paisley) according to the procedure described by Gutierrez *et al.* (1997b) in a final volume of 250 µl culture medium. On each of days 2 and 4 of culture, 80% (200 µl) of the culture medium was removed (taking care not to disturb the loosely adherent cells) and replaced with fresh medium. After day 6 of culture, the number of granulosa cells was estimated by the uptake of neutral red, as described by Campbell *et al.* (1996). The granulosa cell conditioned culture medium was

stored at  $-20^{\circ}\text{C}$  until it was assayed for oestrogen and progesterone.

### Radioimmunoassays

Oestradiol and progesterone concentrations in unextracted culture medium were measured by radioimmunoassay (Corrie *et al.*, 1981; Webb *et al.*, 1985), validated previously for unextracted culture medium (Gong *et al.*, 1994). Steroid measurements during days 2–4 and 4–6 of culture were adjusted for carryover from days 0–2 and 2–4 of culture, respectively. Plasma IGF-I concentration was measured by radioimmunoassay after acid gel filtration to remove IGF-BPs (Gutierrez *et al.*, 1997c), using a rabbit polyclonal antibody raised against human recombinant IGF-I (Armstrong *et al.*, 1990). The sensitivity of the assay was 22 pg per assay tube and the inter- and intra-assay coefficients of variation were 13 and 8%, respectively. FSH concentrations were measured by radioimmunoassay as described by Gong *et al.* (1995). The sensitivity of the assay was 0.1 ng ml<sup>-1</sup> and the intra-assay coefficient of variation was 1.2%. Insulin concentrations were measured by radioimmunoassay (Gong *et al.*, 1993). The sensitivity of the assay was 0.04 ng ml<sup>-1</sup> and the intra-assay coefficient of variation was 7.4%.

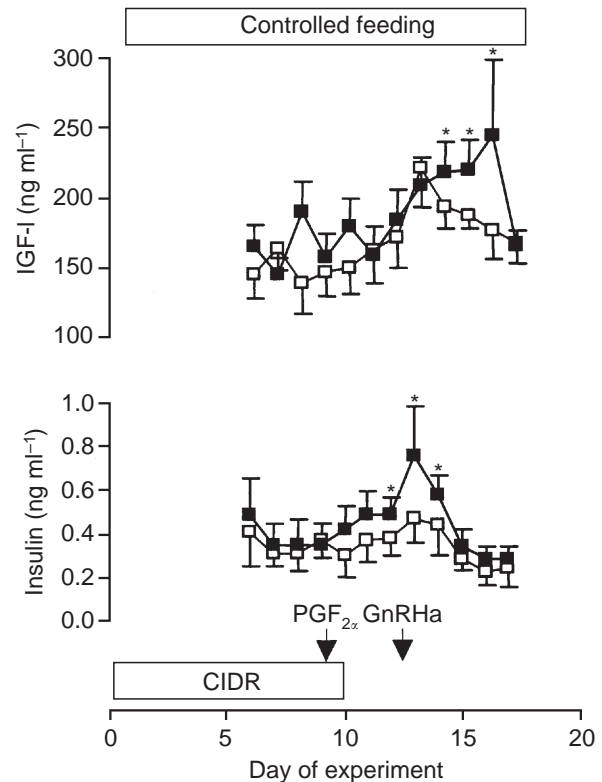
### Follicle dynamics

The pattern of follicular development was examined daily from day 6 to day 16 of the experimental period, using an ultrasound scanner (Aloka Echo Camera SSD-210 DX II) fitted with a 7.5 MHz linear array transrectal probe (Aloka Co. Ltd). Follicles were classified according to their diameter as small (2–4 mm) or medium-sized (4–8 mm).

### Statistical analyses

Data relating to plasma insulin, IGF-I, FSH, follicular growth, animal weight and body condition score were analysed using repeated measures (split-plot) ANOVA (Genstat 5, Version 4.21; Lawes Agricultural Trust, Rothamstead, 1993). The analysis allowed for variation due to treatment (diet), animal within treatment, day of experiment and interaction of day and treatment. The significance of the effect of level of feeding was tested using the animal within treatment as an error term.

Three separate cell culture experiments were performed. Each experiment used eight cows, four of which were fed the twice maintenance diet and four the maintenance diet. Within each experiment, granulosa cells were pooled according to size and the effect of treatments within diets was measured in quadruplicate. Effects of days of culture, diet and hormone treatments (LR3-IGF-I and FSH) on steroid production were analysed by split-plot ANOVA allowing for variation due to culture and different pools of cells (random effects). Oestradiol and progesterone production were analysed after logarithmic transformation to correct for heterogeneity of variance. Data from the cell culture experiments are presented as overall means of the three separate



**Fig. 1.** Serum insulin and insulin-like growth factor I (IGF-I) concentrations during and after synchronization of oestrus in cattle offered maintenance (□) or twice maintenance (■) diets. PGF<sub>2α</sub> and gonadotrophin-releasing hormone agonist (GnRH) treatments are shown. The periods of progesterone treatment (CIDR) and controlled feeding are also shown. Values are mean ± SEM. \*Indicates a significant difference between diets on that day ( $P < 0.05$ ).

cultures (after correction for number of cells) with the corresponding pooled standard error of the difference.

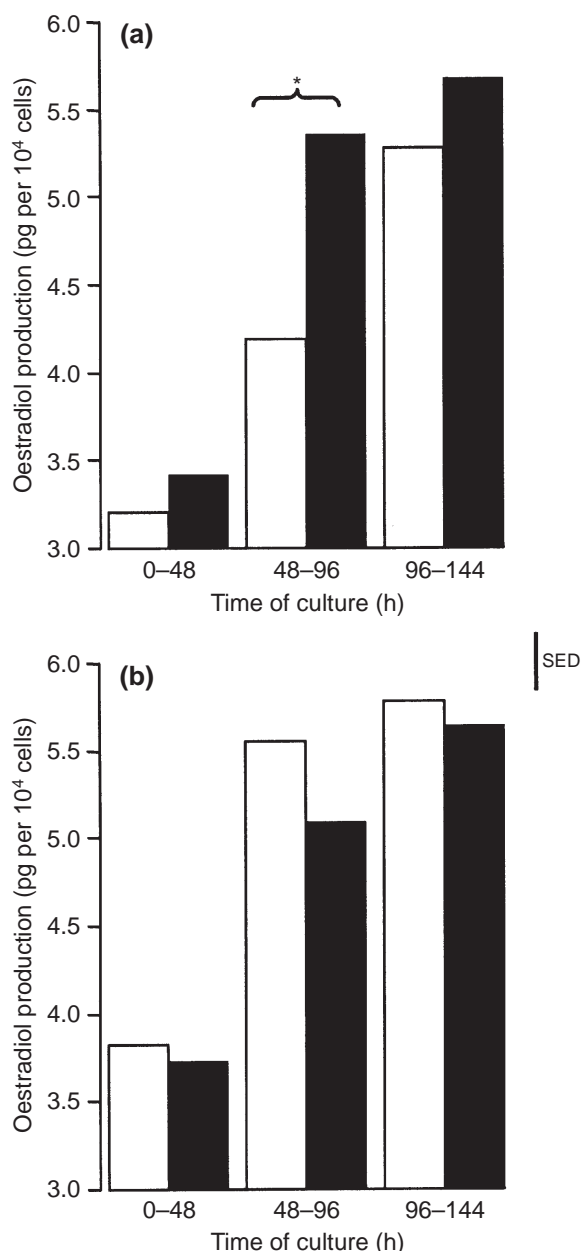
## Results

### Animal weights and body condition score

There was no significant effect of diet on body condition score ( $2.2 \pm 0.07$ ) or body weight ( $440.6 \pm 10.5$  kg) of the heifers throughout the 17 day period of controlled feeding.

### Follicle dynamics

Within 24 h of GnRH treatment, 10 of 12 heifers fed the maintenance diet and nine of 12 heifers fed the twice maintenance diet had ovulated. The other heifers all ovulated within 48 h of the GnRH treatment. The diameter of the largest follicle on the day before death was  $6.9 \pm 0.7$  mm and  $7.1 \pm 0.5$  mm in heifers offered the maintenance and twice maintenance diets, respectively, and was not significantly different between the two groups. The diameter of the largest follicle on the day of GnRH treatment was  $14.8 \pm 0.3$  mm and  $15.1 \pm 0.4$  mm in cattle



**Fig. 2.** Oestradiol production (shown on a natural logarithmic scale) by bovine granulosa cells during 0–48 h, 48–96 h and 96–144 h of culture. Granulosa cells were isolated from (a) small (1–4 mm) and (b) medium-sized (4–8 mm) ovarian follicles collected from heifers offered either maintenance (□) or twice maintenance (■) diets. Granulosa cells were cultured under serum-free conditions in the presence of FSH (0.1 ng ml<sup>-1</sup>), insulin (10 ng ml<sup>-1</sup>) and long R3 insulin-like growth factor I (IGF-I; 10 ng ml<sup>-1</sup>). The results are means of three separate experiments with SED representing the pooled standard error of the difference. \*Indicates a significant difference in oestradiol production between diets within follicle size and culture time ( $P < 0.05$ ).

fed the maintenance and twice maintenance diets, respectively, and was not significantly different between the two groups.

On day 3 after GnRH treatment there was a significant

( $P < 0.05$ ) increase in the number of small (1–4 mm) follicles in heifers offered the twice maintenance diet ( $n = 12.3 \pm 2.3$ ) compared with heifers fed on the maintenance diet ( $n = 7.6 \pm 1.4$ ). In contrast, there was no difference in the number of medium-sized (5–8 mm) follicles in heifers offered the twice maintenance ( $n = 7.1 \pm 0.5$ ) and maintenance ( $n = 6.9 \pm 0.7$ ) diets.

#### Systemic hormones

Changes in the circulating concentrations of insulin and IGF-I are shown (Fig. 1). The higher maintenance diet tended to increase insulin concentrations during the experimental period. There was a significant diet  $\times$  day interaction ( $P < 0.05$ ) and the largest difference in insulin concentrations between the two diets was associated with the GnRH-induced ovulation. There was a significant diet  $\times$  day interaction ( $P < 0.05$ ) on IGF-I concentrations, with significant differences ( $P < 0.05$ ) apparent on the 3 days after GnRH treatment only.

There was no effect of diet or diet  $\times$  day interaction on circulating FSH concentrations. The mean plasma FSH concentration throughout the experimental period was 0.49 ng ml<sup>-1</sup> (data not shown).

#### Number of cells

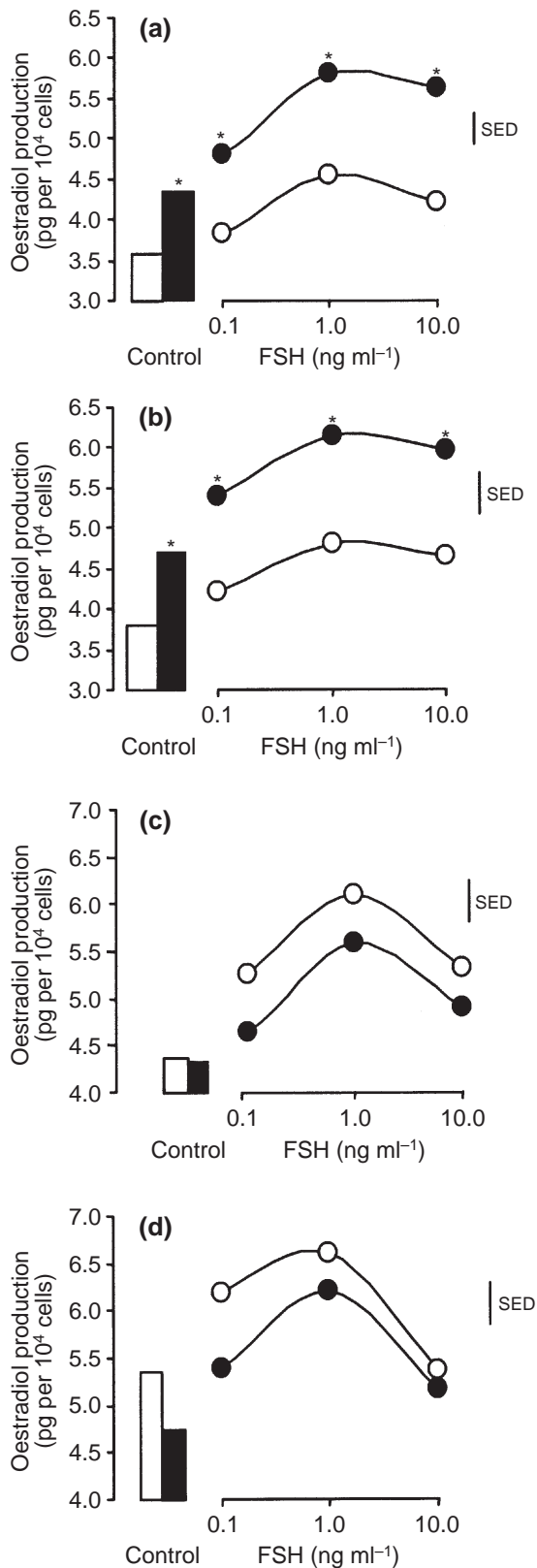
Numbers of cells were estimated at the end (144 h) of culture. There was a tendency for FSH to increase the number of cells ( $P = 0.07$ ); however, diet had no effect on number of cells. In addition, there were no significant interactions between diet, follicle size and FSH, or diet, follicle size and LR3-IGF-I on the number of cells measured at the end of the culture period.

#### Steroid production by granulosa cells

Steroid production is shown as the amount of steroid produced during the 0–48 h, 48–96 h or 96–144 h period after adjusting for carryover of culture medium from the earlier time period. Steroid secretion during the first 0–48 h of culture was adjusted for number of cells using the number of viable cells plated at the start of the culture, and secretion during 48–96 h and 96–144 h of culture was adjusted for number of cells using cell counts assessed at the end of each culture period.

After an initial lag period of 48 h, oestradiol production increased for up to 144 h in culture (Fig. 2). Oestradiol production by granulosa cells from small follicles collected from heifers offered a twice maintenance diet was significantly higher ( $P < 0.05$ ) than in follicles collected from heifers offered a maintenance diet after 96 h, but not 144 h, of culture. This effect of diet on oestradiol production by granulosa cells from small follicles was not observed with granulosa cells isolated from medium-sized follicles.

The effect of FSH on oestradiol production by granulosa cells from small and medium-sized follicles after culture for 4 days is shown (Fig. 3). Diet had no effect on the sensitivity of granulosa cells towards FSH; however, granulosa cells



**Fig. 3.** The effect of FSH on oestradiol production (pg per 10<sup>4</sup> cells; natural logarithmic scale) by bovine granulosa cells during 48–96 h of culture. Granulosa cells were isolated from (a,b) small (1–4 mm) and (c,d) medium-sized (4–8 mm) follicles and were cultured

from small follicles collected from heifers offered the twice maintenance diet produced 3.7-fold more oestradiol than did heifers offered the maintenance diet. This effect was independent of the presence of LR3-IGF-I in the culture medium. In contrast, diet had no effect on FSH-regulated oestradiol production by granulosa cells from medium-sized follicles. Cultures containing LR3-IGF-I (Fig. 3b,d) secreted more oestradiol than did corresponding cultures containing no LR3-IGF-I (Fig. 3a,c)

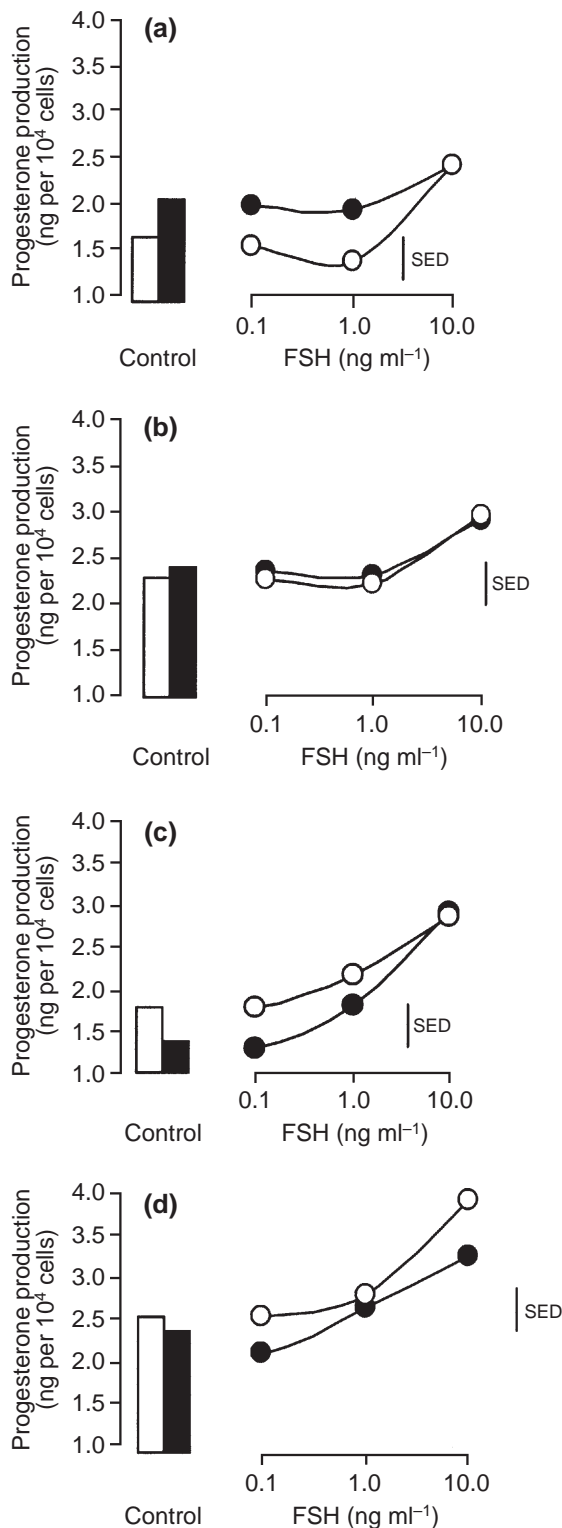
Progesterone production by granulosa cells was increased after 96 h of culture in the presence of FSH (10 ng ml<sup>-1</sup>). Unlike oestradiol production, there was no effect of diet on progesterone production by granulosa cells from either small or medium-sized follicles (Fig. 4). Cells cultured in the presence of LR3-IGF-I (Fig. 4b,d) secreted more progesterone than did corresponding cells cultured in the absence of LR3-IGF-I (Fig. 4a,c).

## Discussion

The results described in the present study indicate that a change in dietary intake from one- to two-times maintenance increased circulating concentrations of insulin and IGF-I. This increase was associated with an increase in both the number of small follicles and the steroidogenic potential of granulosa cells from small but not medium-sized follicles. These data complement previous observations showing that short-term changes in dietary intake can change follicle dynamics in cattle (Gutierrez *et al.*, 1997a; Armstrong *et al.*, 2001; Gong *et al.*, in press) without significant alterations in circulating FSH concentrations. These results, when combined with those of other studies that show a positive correlation between nutritionally (Beam and Butler, 1999) and hormonally (Gong *et al.*, 1991, 1997) induced changes in metabolic hormones and follicular growth, provide further evidence for a role for insulin and IGF-I in regulation of follicle recruitment and granulosa cell differentiation in cattle. Reduced dietary intake also affects follicle development (Murphy *et al.*, 1991), with the continued loss of body reserves resulting in nutritionally induced anoestrus (Richards *et al.*, 1991). The reductions in the circulating concentrations of insulin and IGF-I associated with severe nutrient restriction are also believed to contribute directly to reduced follicular function (Bossis *et al.*, 1999).

A study in which insulin was infused into cattle concomitantly with an FSH superovulatory regimen

under serum-free conditions with insulin (10 ng ml<sup>-1</sup>) in the (a,c) absence and (b,d) presence of long R3 insulin-like growth factor I (IGF-I; 10 ng ml<sup>-1</sup>). Granulosa cells were collected from heifers offered either maintenance (□, ○) or twice maintenance (■, ●) diets. SED represents pooled standard error of the difference from three separate experiments. \*Indicates a significant difference in oestradiol production between diets within follicle sizes and IGF treatment (P < 0.05). The control represents oestradiol production by granulosa cells in the absence of FSH.



**Fig. 4.** The effect of FSH on progesterone production (shown on a natural logarithmic scale) by bovine granulosa cells during the 48–96 h of culture. Granulosa cells were isolated from (a,b) small (1–4 mm) and (c,d) medium-sized (4–8 mm) follicles and were cultured under serum-free conditions with insulin (10 ng ml<sup>-1</sup>) in the (a,c) absence and (b,d) presence of long R3 insulin-like growth factor I (IGF-I; 10 ng ml<sup>-1</sup>). Granulosa cells were collected from

(Simpson *et al.*, 1994) resulted in an increase in the diameter of the largest follicle; however, there was no effect of treatment on the number of small, medium or large follicles. This is in contrast to results presented in the present study and in our recent studies which showed that nutritionally induced changes in insulin concentrations increased both the number of small follicles (Gutierrez *et al.*, 1997a; Gong *et al.*, in press) and the superovulatory response (Gong *et al.*, in press). The reasons for these differences are unclear; however, these results do indicate that, in addition to insulin, nutritionally induced changes in other metabolic hormones such as leptin and IGF-I, as well as metabolic fuels, are important in regulation of follicle development.

Significant increases in circulating concentrations of insulin and IGF-I were associated with the GnRH-induced ovulation. The effect was most pronounced in heifers offered the twice maintenance diet. Similar results were observed by Armstrong *et al.* (2001) and are probably associated with increasing oestradiol concentrations during the follicular phase of the cycle stimulating hepatic IGF-I production (Richards *et al.*, 1991). Other studies have demonstrated a significant interaction between body condition score of heifers and stage of oestrous cycle on circulating insulin concentration, with maximum differences occurring during oestrus (McCann and Hansel, 1986).

The mechanisms by which these nutritionally induced changes in IGF-I and insulin alter follicle dynamics are still unclear. The follicular IGF system has been implicated in this process, with nutritionally- (Hamilton *et al.*, 1999) and insulin- (Simpson *et al.*, 1994) induced changes in the concentration of components of the IGF system in follicular fluid being recorded. More recently, a direct effect of diet on the expression of follicular IGF-BP-2 and -4 was described (Armstrong *et al.*, 2001). These changes in the IGF system are believed to alter the sensitivity or response of follicles to FSH, thereby directly contributing to the observed changes in number of follicles and follicle size.

The results of the present study demonstrate that nutritionally induced changes in metabolic hormones directly affect oestradiol production in cultured granulosa cells from small follicles. In intact follicles, the production of oestradiol by granulosa cells is controlled by the supply of androgen precursors from thecal cells and regulation of aromatase activity by FSH. As the granulosa cell cultures described in the present study contained testosterone, the changes in the amount of oestradiol produced by granulosa cells between cattle offered the maintenance and twice maintenance diets must be a result of changes in the amount or activity of aromatase activity. As discussed above, this is most likely to be the result of metabolic hormones directly affecting the follicular IGF system,

heifers offered either maintenance (□, ○) or twice maintenance (■, ●) diets. SED represents pooled standard error of the difference from three separate experiments. The control represents oestradiol production by granulosa cells in the absence of FSH.

which, in turn, increases the response of granulosa cells from small follicles to FSH.

An alternative explanation for the differences in oestradiol production by granulosa cells isolated from cattle offered the maintenance and twice maintenance diets is that they were due to dietary-induced differences in number of cells between the cultures during 48–96 h of culture. The design of the experiment was such that the number of cells at 96 h had to be estimated from the number of cells obtained after 144 h of culture. However, as there was no effect of diet on the number of cells counted after 144 h, this alternative explanation is unlikely.

Increased nutrition has also been shown to decrease circulating concentrations of progesterone in ruminants, presumably by increasing its clearance rate (Boland *et al.*, 2001; Rabiee *et al.*, 2001). Therefore, it is possible that increased dietary intake can enhance steroidogenesis in granulosa cells through this mechanism. Decreased circulating progesterone concentration would be expected to increase the secretion of LH, which, in turn, would stimulate androgen production by thecal tissue, which is then aromatized to oestrogens by granulosa cells. However, although increased feed intake consistently reduces peripheral concentrations of progesterone in sheep (see Boland *et al.*, 2001), the results in cattle are more variable. Enhanced dietary intake has been shown to increase (McCann and Hansel, 1986; Dunn and Moss, 1992), decrease (Villa-Godoy *et al.*, 1990) or have no effect (Spitzer *et al.*, 1978) on circulating progesterone concentrations in heifers. Although plasma progesterone concentrations were not measured in the present study, the same dietary regimen (feeding with 200% maintenance diet) increased rather than decreased circulating concentrations of progesterone in the study of Gutierrez *et al.* (1997a).

Oestrogens are important regulators of ovarian function (Palter *et al.*, 2001). Their effects on follicle growth are mediated primarily through oestrogen receptor beta and expression of this receptor has been detected in granulosa cells of bovine antral follicles at various stages of development (Rosenfeld *et al.*, 1999). Although follicular concentrations of oestrogen were not measured in the present study, Simpson *et al.* (1994) demonstrated that the direct administration of insulin to heifers increased intrafollicular concentrations of oestrogens and it is likely that the dietary-induced increase in insulin described in the present study will have similar effects. Oestradiol is recognized as a follicle survival factor (Kaipia and Hsueh, 1997) and, therefore, the dietary-induced changes in steroidogenic potential of small follicles could be one mechanism through which short-term changes in dietary intake regulate follicle dynamics in cattle.

Increased oestradiol production by cultured granulosa cells from small follicles collected from cattle offered the twice maintenance diet was observed at day 4 of culture but was not observed at day 6 of culture. The increased oestradiol production is probably a result of dietary-induced changes in the follicular IGF system increasing the

sensitivity of follicles to FSH. However, it is not clear why this effect disappears after day 6 of culture. When granulosa cells are removed from the follicle, it would be expected that any dietary-induced differences in intrafollicular IGF bioavailability in small follicles would be lost. The results presented in the present study indicate that it takes about 6 days of culture for this effect to be manifested. This conclusion is supported by a previous study (Gutierrez, 1997) in which it was shown that bovine granulosa cells maintained their *in vivo* characteristics early in culture and that this was progressively lost the longer the cells were maintained in culture.

In conclusion, the results of the present study support the hypothesis that dietary-induced changes in circulating insulin and IGF-I have a direct effect on the steroidogenic potential of bovine granulosa cells from small follicles. It is hypothesized that these effects are mediated by the intrafollicular IGF system and that increased oestradiol production, combined with the changes in the follicular IGF system in small follicles, is one mechanism through which short-term changes in dietary intake regulate follicle dynamics in bovine ovaries.

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