Evidence that high variation in antral follicle count during follicular waves is linked to alterations in ovarian androgen production in cattle

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Abstract

Androgens have an important role in ovarian follicular growth and function, but circulating androgen concentrations are also associated with ovarian dysfunction, cardiovascular disease, and metabolic disorders in women. The extent and causes of the variation in androgen production in individuals, however, are unknown. Because thecal cells of follicles synthesize androstenedione and testosterone, variation in production of these androgens is hypothesized to be directly related to the inherently high variation in number of healthy growing follicles in ovaries of individuals. To test this hypothesis, we determined whether thecal *CYP17A1* mRNA (codes for a cytochrome P450 enzyme involved in androgen synthesis), LH-induced thecal androstenedione production, androstenedione concentrations in follicular fluid, and circulating testosterone concentrations were lower in cattle with relatively low versus high number of follicles growing during follicular waves and whether ovariectomy reduced serum testosterone concentrations. Results demonstrated that cattle with a low follicle number had lower ($P < 0.05$) abundance of *CYP17A1* mRNA in thecal cells, reduced ($P < 0.01$) capacity of thecal cells to produce androstenedione in response to LH, lower ($P < 0.01$) androstenedione concentrations in ovulatory follicles, and lower ($P < 0.02$) circulating testosterone concentrations during estrous cycles compared with animals with high follicle number. Also, serum testosterone in cattle with low or high follicle number was reduced by 63 and 70%, respectively, following ovariectomy. In conclusion, circulating androgen concentrations are lower in cattle with low versus high number of follicles growing during follicular waves, possibly because of a reduced responsiveness of thecal cells to LH.

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Introduction

Androgens provide substrate for estradiol (E_2) production, which is crucial for the development and function of ovulatory follicles and thus reproductive success (King *et al.* 2007, Walters *et al.* 2008). In addition, circulating androgen concentrations are associated with ovarian disorders such as polycystic ovarian syndrome (PCOS; Dumesic *et al.* 2007), cardiovascular health (Wu & von Eckardstein 2003), and metabolic disease in women (Padmanabhan *et al.* 2006, Chen *et al.* 2008). While the primary tissue sources of androgens in healthy non-pregnant females are the ovaries and adrenal glands, the extent and causes of the variation in androgen production among individuals are unknown. Because thecal cells of growing follicles synthesize two potent

androgens, androstenedione and testosterone, variation in production of these androgens (by individual follicles), and correspondingly in circulating androgen concentrations (from the total pool of follicles), is hypothesized to be directly related to the inherently high variation in total number of healthy growing follicles in ovaries of individuals.

Previous studies from our laboratories show that serial ovarian ultrasonography can be used to reliably identify cattle that consistently have low or high number of follicles during follicular waves (Burns *et al.* 2005, Ireland *et al.* 2007), and that the variation in the peak antral follicle count (AFC) is positively associated with size of the ovarian reserve (total number of morphologically healthy follicles and oocytes in ovaries; Ireland *et al.* 2008), but inversely associated with

secretion of gonadotropins during the estrous cycle (Burns *et al.* 2005, Ireland *et al.* 2008, 2009, Mossa *et al.* 2010). Specifically, young adult cattle with low AFC have chronically high circulating LH concentrations (Jimenez-Krassel *et al.* 2009), and this could lead to an enhanced production of testosterone by thecal cells. However, the capacity to produce progesterone in response to LH is lower for luteinized granulosa cells isolated from dominant follicles and for luteal cells of animals with low versus high AFC, despite similar amounts of LH receptor binding sites, indicating a possible disruption of the LH signaling system in cattle with a relatively low number of follicles growing during follicular waves (Jimenez-Krassel *et al.* 2009).

Consequently, to test the aforementioned hypothesis, this study used the bovine AFC model to determine whether 1) abundance of thecal *CYP17A1* mRNA, which codes for a member of the cytochrome P450 superfamily of enzymes involved in androgen synthesis, 2) androstenedione production by thecal cells *in vitro*, 3) concentrations of androstenedione in follicular fluid (FF) of ovulatory follicles, 4) circulating testosterone concentrations during estrous cycles were greater in cattle with high versus low AFC, and 5) whether serum testosterone concentrations decreased following ovariectomy and removal of the ovarian reserve in cattle.

Results

Alterations in *CYP17A1* mRNA in thecal cells from cattle with low versus high AFC

As reported previously (Ireland *et al.* 2009), diameters of the three largest follicles per pair of ovaries combined, collected during the first 2 to 3 days of the follicular wave, were similar ($P>0.05$; 6.1 ± 0.3 vs 5.4 ± 0.3 mm) for animals in the low versus high AFC groups. However, analysis of mRNA in thecal cells of these follicles showed that the abundance of *CYP17A1* mRNA was 60% lower ($P<0.05$) in cells isolated from cattle with low versus high AFC (0.74 ± 0.22 vs 1.83 ± 0.61). FF samples were exhausted after completion of other assays, thus androgen concentrations in FF could not be determined in this study.

Alterations in basal and LH-induced androstenedione production by thecal cells of antral follicles of cattle with low versus high follicle number

Although there was no difference in basal androstenedione production, LH-stimulated androstenedione production was 2.5-fold higher ($P<0.05$) for thecal cells isolated from cattle in the high versus the low follicle number groups (Fig. 1).

Figure 1 Alterations in basal and LH-induced thecal production of androstenedione in cattle with low versus high AFC. Androstenedione production by theca interna isolated from first-wave dominant follicles of ovaries from animals with low ($n=3$) or high ($n=3$) AFC. Theca interna (2 pieces per well) from each follicle were cultured in duplicate for 24 h with or without 4 ng/ml LH as described previously (Lv *et al.* 2009). Androstenedione production was normalized to total protein contained in thecal tissue after culture. Each bar represents the mean $\pm$ S.E.M. for each follicle number group. Asterisk indicates significant ($P<0.05$) difference between means.

Alterations in intrafollicular concentrations of androstenedione in ovulatory follicles of cattle with low versus high AFC

Within 12 h after the prostaglandin (PG) injection to induce luteolysis and dominant follicle development, diameter (15.74 ± 0.36 vs 14.74 ± 0.42 mm) and progesterone concentrations in FF (123 ± 14 vs 101 ± 7 ng/ml) of ovulatory follicles were similar ($P>0.05$) for animals in the low versus high AFC groups. In contrast, intrafollicular concentrations of androstenedione were 77% ($P<0.03$, 32.67 ± 6.45 vs 109.55 ± 25.87 ng/ml) and of E_2 were 26% ($P<0.01$; 435 ± 59 vs 588 ± 76 ng/ml) lower in FF aspirated from ovulatory follicles of animals with low versus high AFC.

Alterations in serum testosterone concentrations during estrous cycles of beef heifers and dairy cows with low versus high AFC

Serum testosterone concentrations varied ($P<0.01$) during the days of the estrous cycles of both beef heifers and non-lactating dairy cows. Although testosterone concentrations were lower in dairy cows (0.04 ± 0 vs 0.10 ± 0.01), patterns of testosterone secretion were similar for animals with low or high AFC (data not shown). Nevertheless, animals with low AFC had an overall lower ($P<0.02$) serum concentration of testosterone between days 0 and 14 of the estrous cycle for beef heifers and between days 6 and 21 for non-lactating dairy cows compared with animals with high AFC (Fig. 2). In addition, serum testosterone concentrations were lower ($P<0.05$) on days 2 (0.03 ± 0.01 vs 0.09 ± 0.03 ng/ml) and 3 (0.03 ± 0.01 vs 0.08 ± 0.03 ng/ml) of the estrous cycle in beef heifers and on days 8 and 12–16 of the cycle in non-lactating dairy cows (Fig. 2) for animals in the low versus high AFC groups.

Figure 2 Alterations in serum testosterone concentrations during estrous cycles of dairy cows with low versus high AFC. Concentration of testosterone in serum in Holstein cows with low (open symbols, $n=5$ animals) versus high (solid symbols, $n=4$) AFC. Animals were synchronized with two injections of PG administered 11 days apart, and blood samples were collected daily from day 6 to 21 of the estrous cycle. Ovulation occurred on day 1 of the estrous cycle, and ovulation was defined as the day of disappearance of largest dominant follicle as determined by ovarian ultrasonography. ANOVA indicated that there were highly statistically significant ($P<0.02$) differences in testosterone concentrations during the estrous cycle (day) and between AFC groups (group). Asterisks indicate significant ($P<0.05$) difference between means. Each symbol represents the daily mean $\pm$ S.E.M. for animals in each AFC group.

Alterations in serum testosterone concentrations after ovariectomy of cattle with low versus high AFC

The average testosterone concentrations decreased ($P<0.0001$) by 70 and 63% by 9 days after ovariectomy in the high and low AFC groups, respectively, compared with overall average testosterone concentrations before ovariectomy collected on day 1, 5, and 10 of the estrous cycle (day 0). Testosterone concentrations were not different before or after surgery between the high and low AFC groups (Fig. 3).

Discussion

The most significant finding of this study is that ovarian androgen production is positively associated with the number of antral follicles ≥ 3 mm in diameter. Several diverse lines of evidence strongly support this conclusion. First, in cattle with low AFC, the abundance of thecal *CYP17A1* mRNA, which codes for a cytochrome P450 enzyme involved in androgen synthesis, responsiveness of thecal cells to LH treatment and FF concentrations of androstenedione in ovulatory follicles were diminished compared with that in cattle with a high AFC. Secondly, serum testosterone concentrations were lower overall during estrous cycles of young adult beef cattle and older dairy cows with low versus high AFC. Thirdly, surgical removal of ovaries, and thereby the ovarian reserve, markedly reduced serum testosterone concentrations in cattle. Taken together, these findings imply that follicular androgen production by thecal cells

is linked positively to number of follicles growing during follicular waves and correspondingly to the size of the ovarian reserve in cattle, which explains why ovarian androgen production is relatively low during estrous cycles for cattle with low versus high AFC.

Several key questions arise from these novel findings. First, why is androgen production inversely associated with LH secretion? Secondly, what is the impact, if any, of the differences in ovarian androgen production between cattle with low or high AFC on fertility and on other physiological systems?

We previously demonstrated that cattle with low versus high AFC have a twofold greater basal LH concentration (Jimenez-Krassel *et al.* 2009), whereas the present work shows that both the circulating testosterone concentrations during the estrous cycle and LH-induced androstenedione production by theca cells are lower in cattle with a low versus high AFC. Considering that steroidogenic activity of thecal cells is mainly regulated by LH (Li *et al.* 2010), and that the inherent capacity of the pituitary gland to produce gonadotropins is not influenced by AFC (Mossa *et al.* 2010), this discrepancy could be due to a reduced responsiveness of thecal cells to LH for cattle with a low AFC. This hypothesis is confirmed by the recent finding that the capacity of luteinized granulosa cells isolated from dominant follicles and of luteal cells isolated from CL on day 12 of the estrous cycle to produce progesterone basally and in response to LH are lower for cattle with low versus high AFC (Jimenez-Krassel *et al.* 2009). Moreover, two recent studies showed that LH significantly increased androstenedione production in bovine thecal cells (in accordance with our results), and that addition of two inhibitors of the phosphatidylinositol-3-kinase/Akt signaling pathway significantly decreased LH-induced androstenedione production (Fukuda *et al.* 2009), inhibition of the Akt and Erk pathway inhibited both basal and LH-induced androstenedione secretion by

Figure 3 Alterations in serum testosterone concentrations after ovariectomy of cattle with low versus high AFC. Circulating concentrations of testosterone were measured in Holstein cows with low (open symbols, $n=5$ animals) versus high (solid symbols, $n=4$) AFC. Animals were synchronized with PG and blood samples collected at various times before (day 5 and 10 of estrous cycle and 1 h before ovariectomy) and 1 and 9 days after ovariectomy as explained in Materials and Methods. The zero time point represents the pooled mean $\pm$ S.E.M. for day 5 and 10 of the estrous cycle and 1 h before ovariectomy for cows in the low or high AFC groups. Asterisks indicate significant ($P<0.01$) difference from day 0 values.

bovine theca cells *in vitro* (Ryan *et al.* 2008). Recent evidence shows that LH significantly increases *CYP17A1* mRNA expression in thecal cells (Fukuda *et al.* 2009), and in this study, it was shown that *CYP17A1* mRNA expression was higher in thecal cells from cattle with a high AFC, despite lower basal and episodic LH secretion (Jimenez-Krassel *et al.* 2009). Therefore, the reduced androgen production in animals with a low AFC could be due to an alteration in LH signaling which, in turn, may alter *CYP17A1* mRNA expression.

We have recently shown that intrafollicular E_2 concentration in the three largest follicles (5–7 mm) at emergence of the follicular wave is greater in cattle with low versus high AFC (Ireland *et al.* 2009). In contrast, it was found that intrafollicular E_2 concentrations were lower in ovulatory follicles (14–15 mm) in cattle with low versus high AFC in this study. While several explanations are possible, this difference in E_2 production by the two different follicle types could be due to the reduced responsiveness of dominant compared with smaller antral follicles to LH in cattle with low AFC. In support of this possibility, it is well established that granulosa and thecal cells of dominant follicles develop LH receptors in sheep and cattle (Webb & Campbell 2007). Therefore, the lower sensitivity of granulosa and thecal cells to LH in cattle with low versus high AFC could also explain the lower intrafollicular E_2 and androstenedione concentrations at the ovulatory, LH-dependent stage of follicular development, as observed in this study.

Ovarian AFC is inversely associated with circulating FSH concentrations during the estrous cycle (Burns *et al.* 2005, Ireland *et al.* 2008, 2009, Mossa *et al.* 2010), and there is evidence suggesting a reciprocal control between androgens and ovarian sensitivity to FSH. For example, *in vivo* treatment with testosterone increases androgen receptor mRNA in granulosa and thecal cells in small antral follicles of primates, and the expression of androgen receptor mRNA is positively correlated with FSH receptor (*FSHR*) mRNA expression (Weil *et al.* 1999). In addition, dihydrotestosterone (DHT) increases *FSHR* expression in preovulatory follicles in gilts (Cardenas *et al.* 2002), and administration of testosterone or DHT to female rhesus monkeys and pregnant ewes (prenatally treated fetuses) stimulates primordial follicle initiation and increases growing follicle numbers and overall follicle survival (Vendola *et al.* 1999, Steckler *et al.* 2005, Forsdike *et al.* 2007). During oocyte maturation and ovulation, androgens increase abundance of *FSHR* mRNA and number of preovulatory follicles and corpora lutea (Walters *et al.* 2008). Testosterone increases murine follicle responsiveness to FSH (Wang *et al.* 2001), and androstenedione stimulates FSH-mediated differentiation of bovine granulosa cells, as indicated by an increase in aromatase activity and E_2 production (Hamel *et al.* 2005). In women, serum androgen levels are correlated with the ovarian response to FSH, as assessed by the peak E_2 levels

and the number of follicles and number of oocytes and embryos retrieved (Barbieri *et al.* 2005), and significant positive correlations exist between the AFC and both baseline and stimulated steroid serum concentrations (Hugues *et al.* 2010). Therefore, cattle with a high AFC and higher serum androgen concentrations could have an enhanced ovarian sensitivity to FSH, and this could in turn explain why a greater number of follicles are recruited per wave, despite the lower serum concentrations of FSH compared with cattle with a lower AFC. Moreover, we have recently demonstrated that the inherent capacity of the pituitary gland to produce gonadotropins is not different between cattle with high versus low number of follicles during follicular waves (Mossa *et al.* 2010), implicating ovarian factors as the more plausible causes of FSH variation.

The possible effects of elevated gonadotropins and relatively low androgen concentrations on fertility are unknown. In women, ovarian aging is characterized by a reduced ovarian sensitivity to FSH, which is accompanied by a progressive decline in serum androgen levels (Davison *et al.* 2005, Sowers *et al.* 2009). Several studies show that women with an elevated FSH concentration, independent of age, have a poor response to ovarian stimulation, leading to a lower pregnancy rate using assisted reproduction techniques (Scott *et al.* 1989, Martin *et al.* 1996, Sharif *et al.* 1998, El-Toukhy *et al.* 2002). However, Abdalla & Thum (2004) report that elevated basal FSH concentrations are not indicative of deterioration of oocyte and embryo quality and explained the reduction in pregnancy rate as a result of a reduced number of oocytes collected and the subsequently limited choice of embryos available for transfer in women undergoing IVF treatment. Cattle with a low AFC show similarities with aging women, such as increased secretion of FSH (Burns *et al.* 2005, Ireland *et al.* 2008), reduced serum progesterone (Jimenez-Krassel *et al.* 2009) and anti-Müllerian hormone during the estrous cycle (Ireland *et al.* 2008), reduced ovarian reserve (Ireland *et al.* 2008), poor response to superovulation (Ireland *et al.* 2007), and, as our present results show, reduced androgen concentrations during estrous cycles. These findings may suggest an impaired fertility in cattle with low AFC. However, to date, no studies have investigated the association between AFC and reproductive success in cattle. Specifically, the possible effects of the variation in androgen concentrations on reproduction are unknown. For example, the addition of exogenous testosterone (100 nM) to oocyte maturation medium, selected on the basis of physiological concentrations reported in preovulatory bovine follicles (~165 nM; Dieleman *et al.* 1983), improved the developmental competence of bovine oocytes after IVF (Younis *et al.* 1989, Silva & Knight 2000). On the other hand, hyperandrogenism is among the main features of PCOS, one of the most common causes of ovulatory infertility in women (Pasquali & Gambineri 2006).

The present results also indicate ovaries as the primary source of androgens in female cattle, regardless of number of follicles. Serum testosterone concentrations did not differ significantly between the two groups of cattle with low or high follicle number before the removal of the ovaries probably because blood was sampled on day 1, 5, and 9 of the estrous cycle when the differences in testosterone concentrations are not statistically significant, as shown in this study. Nevertheless, results of ovariectomy and removal of the ovarian reserve confirm that the ovary is the main source of androgens in cattle.

In conclusion, this study has demonstrated for the first time that responsiveness of thecal cells to LH is lower in cattle with low versus high number of antral follicles growing during follicular waves as assessed by the reduced capacity of thecal cells to produce androstenedione in response to LH, lower abundance of *CYP17A1* mRNA in thecal cells, lower androstenedione concentrations in ovulatory follicles, and diminished circulating testosterone concentrations during estrous cycles. Further studies will be necessary to investigate the possible effects of the variation in the ovarian reserve, and associated androgen production, on fertility as well as the causes of the high variation in numbers of follicles and oocytes in ovaries of mammals.

Materials and Methods

Animals

Cattle used in experiments were located at the Michigan State University Beef or Dairy Cattle Teaching and Research Centers or Lyons Research Farm, University College Dublin, Ireland. All experiments were performed in compliance with protocols approved by the All University Committee on Animal Use and Care at Michigan State University or the Animal Research Ethics Subcommittee, University College Dublin, the Cruelty to Animal Act (Ireland, 1876), and the European Union Directive 86/609/EC.

Identification of cattle with consistently low versus high AFC during follicular waves

Ovaries of each animal were monitored with an Aloka SSD-900 ultrasound machine using a linear array trans-rectal probe (7.5 MHz transducer), and follicles were counted as described previously (Burns *et al.* 2005, Ireland *et al.* 2007, 2008, 2009). Each antral follicle ≥ 3 mm in diameter was drawn on an ovarian map, and diameter and total number of follicles ≥ 3 mm in diameter per pair of ovaries, hereafter referred to as AFC, were recorded for each animal. Cattle that consistently had a relatively low versus high peak AFC during follicular waves were identified as explained previously (Burns *et al.* 2005). Briefly, a group of adult animals of similar ages and weights were injected two or three times with PGF_{2 α} spaced 11 days apart to initiate luteolysis and to synchronize occurrence of ovulation. Ovaries were then subjected to

once or twice daily ovarian ultrasonography to determine AFC beginning on days 1 to 2 after the last PG injection and continuing until completion of the study. Ovulation day was defined as the first day that the preovulatory follicle was not observed by ultrasonography. Peak AFC was determined for three to five follicular waves per animal, and the average peak value for AFC per wave was used to classify cattle into the low (≤ 15 follicles) or high (≥ 25 follicles) AFC groups (Burns *et al.* 2005, Ireland *et al.* 2007, 2008, 2009, Mossa *et al.* 2010). Animals in the intermediate category (16–24 follicles) were not studied further and were returned to the herd.

In previously published studies (Ireland *et al.* 2007, 2009, Mossa *et al.* 2010), 177 cross-bred beef heifers and 116 dairy cows were examined, and 13% ($n=39$) were ranked in the low, 76% ($n=223$) in the intermediate, and 11% ($n=31$) in high AFC groups. The number of animals used in each experiment is specified below.

Alterations in *CYP17A1* mRNA in thecal cells from cattle with low versus high AFC

Thirty-two cross-bred beef heifers (Hereford $\times$ Angus $\times$ Charolais, 11–13 months of age) were initially used for this experiment, and heifers were assigned to one of two groups as described above: low (≤ 15 follicles, $n=5$ animals) or high (≥ 25 follicles, $n=5$ animals). Remaining animals in the intermediate category ($n=22$) were excluded. Thecal cells were obtained from each of the three largest growing follicles per pair of ovaries during the early stages of the first follicular wave on days 2–3 of the estrous cycle for adult beef heifers ($n=3$ largest follicles per pair of ovaries for five animals per group) with low versus high AFC as explained previously (Ireland *et al.* 2009). Abundance of *CYP17A1* mRNA was measured in thecal cells as explained below.

Alterations in basal and LH-induced androstenedione production by thecal cells of dominant follicles from cattle with low versus high number of follicles

Thecal cells were obtained from first-wave dominant follicles (Ireland *et al.* 1980) isolated from ovaries obtained at an abattoir from cattle with high (≥ 25 follicles ≥ 3 mm in diameter, $n=3$ animals) or low (≤ 15 follicles ≥ 3 mm in diameter, $n=3$ animals) follicle number. The theca interna was dissected from the follicle, scraped clean of granulosa cells, and cut into eight pieces. Pieces of theca (two per well) from each animal were then separately cultured for 24 h with 0 or 4 ng/ml of bLH (NIH AFP-11743B), as described previously (Lv *et al.* 2009). After culture, the media were aspirated and stored at -20°C until determination of androstenedione concentration, and the theca pieces were snap frozen in liquid nitrogen and stored at -80°C . The theca pieces were then lysed by homogenization and sonication in ice-cold RIPA buffer (Sigma) plus Complete Protease Inhibitor Cocktail Tablet (Roche). Lysates were centrifuged (10 000 *g* for 10 min at 4°C), and protein in the supernatant was determined with the use of the DC Protein Assay (Bio-Rad). Data were expressed as nanogram androstenedione per mg protein.

Alterations in intrafollicular concentrations of androstenedione in ovulatory follicles of cattle with low versus high AFC

Fifty-five cross-bred beef heifers (12–14 months of age) were examined, and animals with low ($n=7$), intermediate ($n=38$), and high ($n=10$) AFC were identified as described previously; heifers in the intermediate group were excluded from the experiment and returned to the herd. On day 7 of the estrous cycle during the first follicular wave, PG was injected, and 12 h later, the diameter of the ovulatory follicle was measured using ultrasonography, and FF was collected from the dominant ovulatory follicle (largest) via ultrasound-guided follicular aspiration (Jimenez-Krassel *et al.* 2003). Granulosa cells were separated by centrifugation, and FF was frozen within 15 min of collection. After thawing, concentration of androstenedione, progesterone, and E_2 were determined for each aspirated FF supernatant.

Alterations in serum testosterone concentrations during estrous cycles of beef heifers and dairy cows with low versus high AFC

As reported previously (Ireland *et al.* 2007), 90 cross-bred beef heifers (11–12 months of age) were segregated into the low ($n=14$), intermediate ($n=65$), and high ($n=11$) AFC groups. A subset of heifers with low ($n=7$ animals) versus high ($n=7$) AFC were synchronized with two injections of PG administered 11 days apart. Daily blood samples were collected from the day of injecting second PG to day 14 of the estrous cycle. In a second previously published study (Mossa *et al.* 2010), 116 non-lactating Holstein cows (4–8 years of age) were examined, and cattle with low ($n=5$ animals) versus high ($n=4$) AFC were selected and synchronized with two injections of PG administered 11 days apart. After the second PG injection, daily blood samples were collected from day 6 to 21 of the estrous cycle. Blood samples from cows of the two previously published studies (Ireland *et al.* 2009, Mossa *et al.* 2010) were used as the source of serum samples for testosterone analysis in this study.

Alterations in serum testosterone concentrations after ovariectomy of cattle with low versus high AFC

To investigate the contribution of the ovaries in animals with high or low AFC to circulating androgen concentrations, this study determined whether ovariectomy altered serum testosterone concentrations in cattle with low or high AFC. The same non-lactating Holstein cows (4–8 years of age) with low ($n=5$ animals) or high ($n=4$) AFC used in a previous study (Mossa *et al.* 2010) and for serum testosterone determinations as explained above were also used in this study. Testosterone concentrations were determined in blood samples collected on days 5 and 10 of the estrous cycle. In addition, estrous cycles were synchronized with two injections of PG spaced 11 days apart. Ten days later, cows received two additional injections of PG 16 h apart to ensure rapid luteolysis; and 22–28 h after the fourth PG injection, cows were bilaterally ovariectomized via colpotomy

(Drost *et al.* 1992). Blood samples were collected 1 h before and 1 and 9 days after ovariectomy to monitor alterations in serum testosterone concentrations.

Hormone analyses

Total testosterone in serum was measured following ether extraction of serum samples (0.5 ml) using commercially available RIA kits (Siemens Medical Solutions Diagnostics, Los Angeles, CA, USA; Cruinn Diagnostics Ltd, Dublin, Ireland). Inter- and intra-assay coefficients of variation (CV) were $<13\%$, and limit of detection was 0.03 ng/ml.

Concentrations of androstenedione, progesterone, and E_2 in FF or media were analyzed using commercially available kits from Siemens Medical Solutions Diagnostics. Intra-assay CV were $<10\%$ for each assay.

mRNA analysis

Total RNA was extracted from thecal cells of each follicle and processed for analysis by real-time PCR as explained previously (Ireland *et al.* 2009). Primers for bovine CYP17A1 were forward 5'-CAAGGATGGCAACCTGAAGT-3' and reverse 5'-GAGAGA-GAGGCTCGGACAGA-3'. Abundance of CYP17A1 mRNA was normalized against levels of the constitutively expressed housekeeping gene *ACTB*, and relative amounts of gene expression were calculated using the formula $2^{-(\Delta\Delta C_t)}$ (Livak & Schmittgen 2001).

Statistical analyses

Testosterone concentrations in serum of intact animals before and after ovariectomies were analyzed using Proc Mixed procedure of SAS (1995). Data were examined for normality of distribution with the Shapiro–Wilk test, and when heterogeneity of variance was detected, data were natural log-transformed before analyses, but actual values are reported. The model included the effects of group (low versus high), day of estrous cycle, and their interaction. Day of blood sampling was treated as a repeated measure across individual animals. When main effects were significant, a Bonferroni's *t*-test was used to determine if statistically significant ($P \leq 0.05$) differences existed among individual means.

Concentrations of steroids in FF or media and abundance of thecal mRNA for CYP17A1 were statistically analyzed using the Proc Mixed model of SAS (1995). Data were examined for normality of distribution as described above. Main effects included animal groups (low versus high). When main effects were significant ($P < 0.05$), a Bonferroni's *t*-test was used to determine if statistically significant ($P < 0.05$) differences existed among individual means.

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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