Elevated free fatty acids affect bovine granulosa cell function: a molecular cue for compromised reproduction during negative energy balance

Arpna Sharma, Vijay Simha Baddela, Frank Becker, Dirk Dannenberger, Torsten Viergutz and Jens Vanselow
Leibniz Institute for Farm Animal Biology (FBN), Dummerstorf, Germany

Correspondence should be addressed to J Vanselow: vanselow@fbn-dummerstorf.de

Abstract

High-yielding dairy cows postpartum face the challenge of negative energy balance leading to elevated free fatty acids levels in the serum and follicular fluid thus affecting the ovarian function. Here, we investigated effects of physiological concentrations of palmitic acid (PA), stearic acid (SA) and oleic acid (OA) on the viability, steroid production and gene expression in a bovine granulosa cell (GC) culture model. Treatment with individual and combined fatty acids increased the CD36 gene expression, while no significant apoptotic effects were observed. Both PA and SA significantly upregulated the expression of FSHR, LHCGR, CYP19A1, HSD3B1, CCND2 and increased 17β-estradiol (E2) production, while OA downregulated the expression of these genes and reduced E2. Interestingly, STAR was equally downregulated by all fatty acids and combination treatment. E2 was significantly reduced after combination treatment. To validate the effects of OA, in vivo growing dominant follicles (10–19 mm) were injected with bovine serum albumin (BSA) with/without conjugated OA. The follicular fluid was recovered 48 h post injection. As in our in vitro model, OA significantly reduced intrafollicular E2 concentrations. In addition, expression of CD36 was significantly up- and that of CYP19A1 and STAR significantly downregulated in antral GC recovered from aspirated follicles. The ovulation rates of OA-injected follicles tended to be reduced. Our results indicate that elevated free fatty acid concentrations specifically target functional key genes in GC both in vitro and in vivo. Suggestively, this could be a possible mechanism through which elevated free fatty acids affect folliculogenesis in dairy cows postpartum.

Key Words
- palmitic acid
- stearic acid
- oleic acid
- estradiol
- progesterone

Introduction

High-yielding dairy cows suffer post-parturition from ovarian dysfunctions such as prolonged calving intervals and delayed cyclic resumption due to various factors (1). One of such risk factor is negative energy balance (NEB). Catabolism and mobilization of stored triglycerides lead to increased systemic concentrations of non-esterified fatty acids (NEFA) like palmitic acid (PA, C16:0), stearic acid (SA, C18:0), oleic acid (OA, C18:1) and of β-hydroxybutyric acid (BHBA) (2). Such excessive lipid mobilization has been associated with metabolic and reproductive disorders (3) affecting both the cumulus oocyte complex morphology and embryo viability (4). Elevated plasma levels of PA, SA, OA and of linoleic acid have been shown in dairy cows postpartum as well as their infiltration into the follicular fluid (5). Since granulosa cells (GC) are the most important somatic constituents
of the ovarian follicle regulating follicle development along with the production of follicular fluid and plasma estradiol (6). Elevated NEFA levels have detrimental effects on bovine GC (7). In human GC, saturated fatty acids (PA and SA) are known to induce apoptosis, causing potential reproductive abnormalities such as amenorrhea, which is often observed in obese women (8). However, on the contrary, OA (monounsaturated fatty acid) supplementation has shown to protect exocrine pancreatic cells from PA-induced apoptosis thus reducing the rate of pancreatic diseases caused by obesity (9). Also, OA co-supplementation diminishes pro-inflammatory and cytotoxic effects of SA accumulation in human aortic endothelial cells (10). In vitro exposure of COCs to PA and SA induces endoplasmic reticulum stress, mitochondrial damage and increased apoptosis in cumulus cells thus impairing oocyte developmental competence (11), while OA is harmless even at high concentrations (12, 13, 14). However, studying the effects of these major NEFAs, particularly of PA, SA and OA both individually and in combination on steroidogenesis, cell proliferation and apoptosis in an in vitro bovine GC culture model might contribute to our understanding of the collective effects of elevated free fatty acid concentrations during NEB on bovine reproduction. Thus, in the present study, we examined the effects of these fatty acids on GC functionality at elevated, but physiological concentrations as observed in follicular fluid during NEB. For solubilization, fatty acids were conjugated with bovine serum albumin (BSA) to mimic the physiological conditions as close as possible. Specifically, we studied the individual and combined effect of PA, SA and OA on proliferation, apoptosis and steroidogenesis in our well-established serum-free, estrogen-producing GC culture model and validated effects of OA in a bovine in vivo animal model, which allows to test the effects of individual fatty acids on the differentiation of individual follicles while largely avoiding superimposed systemic influences.

Materials and methods

In vitro primary GC culture

Primary GC culture was performed as previously described (15). For testing the effects of fatty acids, the media (supplemented α-MEM) were replaced after 48h with the same media containing different concentrations of PA (50, 100 and 200 µM), SA (50, 100, 200 µM) or OA (100, 200 and 400 µM) as BSA conjugates or BSA as vehicle control (fatty acids and BSA from Sigma-Aldrich). For BSA conjugation, fatty acids were dissolved in 1–2 mL chloroform/methanol solution (v/v = 2/3) and evaporated to dryness under nitrogen atmosphere. Subsequently, a solution of fatty acid-free BSA (w/v = 10% in PBS) was prepared and added. The BSA/fatty acid mixtures were then vortexed, sonicated and subjected to a mild heat treatment (<40°C) until a clear solution was formed. Subsequently, BSA/fatty acid solutions were sterilized by filtration (0.02 µm) and kept at −20°C until addition to the cell culture media (v/v = 1/10). All control wells received the same volume of BSA as the test groups with highest fatty acid concentrations.

Steroid hormone estimation

Concentrations of 17β-estradiol (E2) and progesterone (P4) in the conditioned media were determined by competitive 3H radioimmunoassay (RIA) with rabbit-raised antibodies purified by affinity chromatography as described earlier (16).

Cell viability and cell cycle analysis

Attached GCs were thoroughly washed twice with 1× PBS and trypsinized using 250 µL TrypLE solution (Thermo Fisher) followed by incubation (37°C, 20 min). After centrifugation the cells were merged with non-attached cells from spent media. All cells were again centrifuged, washed with 1 mL MEM and analyzed for apoptosis using an Annexin V FITC kit (Miltenyi Biotec). Cell pellets were re-suspended in 100 µL of 1× binding buffer to which 10 µL of Annexin V reagent was added. After gentle pipetting, the tubes were incubated in dark for 15 min followed by washing and resuspension in 500 µL 1× binding buffer. Then, 5 µL of PI (propidium iodide, 500 µg/mL) were added to the cells and gently mixed just prior to flow cytometric analysis. The fluorescence signal was quantified from single cells (10,000 counts) by a flow cytometer (Gallios, Beckman-Coulter) and the data obtained were analyzed using the Kaluza software (Beckman-Coulter).

For cell cycle analysis, the combined (attached and non-attached) cells were washed and dissolved in 300 µL of 1× PBS. Then, 70% ice cold ethanol was added dropwise into the cell suspensions and stored (~20°C, 2 h). Later, cells were pelleted (300 g, 10 min, 4°C), re-suspended in 1 mL RNase solution (1 mg/mL) and incubated (37°C, 5% CO2, 30 min). The PI reagent was added to the cells and further incubated in the dark (37°C, 30 min). Lastly, the fluorescence signal was quantified from single
cells (10,000 counts) by a flow cytometer (EPICS-XL, Beckman-Coulter). Data were analyzed using the MultiCycle software (Phoenix Flow Systems, San Diego, CA, USA).

RNA isolation and cDNA synthesis

Total RNA preparation was done using innuPREP RNA Mini Kit (Analytik Jena, Jena, Germany) according to the manufacturer’s protocol and quantified using NanoDrop1000 Spectrophotometer (Thermo Scientific). Later, cDNA was prepared using SensiFAST cDNA Synthesis Kit (Bioline, Luckenwalde, Germany) from 200 ng RNA as previously described (17).

Quantitative real-time PCR (qPCR) analysis

qPCR was performed using SensiFAST SYBR No-ROX (Bioline, London, UK) with gene-specific primers (Table 1) in a Light Cycler 96 instrument (Roche) as described previously (18). The abundance of transcripts was normalized relative to RPLPO as a suitable reference gene.

Animals, follicle injection and aspiration

Animal experiments were approved by the Landesamt für Landwirtschaft, Lebensmittelsicherheit und Fischerei Mecklenburg-Vorpommern (Aktenzeichen: 7221.3-1.1-068/17). Fourteen Holstein heifers aged 13–14 months, weighing 380–400 kg were kept indoors at our institute and fed a standard diet. Ultrasonographic follicular monitoring (transportable equipment with 5 MHz linear probe, Hitachi CS 9100 Picker) was carried out aside from the normal heat detection to verify the cycle stage. Special attention was given to the development of larger follicles about 2–3 days before intrafollicular injection to recognize growing and regressing follicles within the follicular population. An adapted amount of BSA without/with 400 µM conjugated OA was injected into the selected growing dominant follicle (10–19 mm). A sector scanner with a 6.5 MHz puncture transducer and a special designed carrier (VETEC GmbH, Rostock, Germany) to guide a needle with an outer diameter of 0.5 mm was used. Epidural anesthesia was induced by 5 mL of lidocaine-hydrochloride injectable-2% (Procamidor, WdT, Garbsen, Germany). To monitor effects on the ovulation of injected follicles 14 animal were further monitored for cycling activity. Sizes of the injected dominant follicles were measured and sample collection was done using transvaginal ultrasonographically guided follicle aspiration 48h after injection. Recovered follicular fluid was immediately stored on ice (<15 min) and taken to the laboratory. Further, the follicular fluid was centrifuged for 3 min at 15,000 g to obtain clear follicular fluid and GC pellets. The GC pellets were instantly frozen in liquid nitrogen followed by storage at −80°C for gene expression analysis while the obtained follicular fluid was stored at −20°C for hormone estimation.

Lipid analysis and fatty acid analysis

Follicular fluid samples of approximately 200 µL were dropwise added to 8 mL chloroform/methanol (2:1, v/v) at room temperature. The solution contained C19:0 as an internal standard. The general sample preparation procedure is as earlier described (19). The fatty acid analysis of the follicular fluid was performed using capillary gas chromatography with a CP-Sil 88 CB column (100 m × 0.25 mm, Agilent) that was installed in

Table 1  List of primers used for gene quantification by q-PCR.

| Gene   | Sequence                   | Size (bp) | Accession no.   |
|--------|----------------------------|-----------|-----------------|
| CD36   | For: GCTCTTTAAGGCAATGGAT   | 151       | NM_001278621.1  |
|        | Rev: CACCGGTTTGCAACGCATT   |           |                 |
| CYP19A1| For: GCTTTTGAAGCTGAAACCAAGG| 172       | NM_174305       |
|        | Rev: GGGGCAATTTCCAGAAAGTAGCTG|          |                 |
| FSHR   | For: TACCAAAGTCAGTGATCTCAAA| 189       | NM_174061       |
|        | Rev: TCTGGAGAACATCAGGTCGATGTA|         |                 |
| CCND2  | For: CGCAGGGGCCGTCCAGGACCAAC| 279       | NM_001076372    |
|        | Rev: CACGGCGCCACAGCGTCAAGG|           |                 |
| PCNA   | For: GTGAACCTCAGAGGATGACTCTG| 205       | NM_001034494    |
|        | Rev: CGTGTCCGTTATCTCATCTCTT|           |                 |
| LHCGR  | For: GCATGAAGACCTCCAGGATTTACGA| 208     | NM_174381       |
|        | Rev: GGAGATACGGATGTCCCATAGGA|          |                 |
| HSD3B1 | For: TTGGTGAGCAGGAAAGATCTG| 142       | NM_174343       |
|        | Rev: GCATTTCAAGCTCCAGTCTGTA|           |                 |
| RPLP0  | For: TGGTTACCAACCAGCTCCAGGAAGT| 142    | NM_001012682    |
|        | Rev: CACAAAGCCAGATGATCCAGGAAG|         |                 |
a PerkinElmer gas chromatograph CLARUS 680 with a flame ionization detector and split injection (PerkinElmer Instruments). The detailed gas chromatography conditions were set as described (20).

**Statistical analysis**

All experiments were carried out in triplicates with different GC preparations. Data of tested groups were analyzed using one-way ANOVA (all pair wise multiple comparison procedures with Holm-Sidak method) or unpaired t test using the Sigma Plot 11.0 Statistical Analysis System (Jandel Scientific, San Rafael, CA, USA). Differences were considered significant at $P < 0.05$.

**Results**

**Effects of individual fatty acids on GC functionality in vitro**

**Cell morphology**

As evident from the light microscopic photographs, treatment with increased PA, SA and OA concentrations resulted in morphological changes. GC changed their appearance from a regular fibroblast-like appearance under BSA vehicle control treatment to an increasingly round-shaped morphology with increased accumulation of intracellular vesicles at highest PA, SA or OA concentrations (Fig. 1).

**Steroid hormone concentrations**

Estimations of P4 and E2 concentrations within conditioned media after the 8-day culture period revealed that PA and SA significantly stimulated E2 production as compared to the BSA control, whereas OA strongly reduced E2 concentrations. In contrast, levels of P4 remained unaltered after PA and SA treatment, but were significantly reduced only at highest OA concentration (Fig. 2).

**Gene expression analysis**

qPCR data showed that transcript abundance of the cell cycle regulator $CCND2$ was significantly up-regulated by PA and SA at high concentrations. In contrast, OA significantly reduced the $CCND2$ transcripts at all concentrations. Transcript abundance of the proliferation marker PCNA however, was only affected by PA showing significant upregulation at the highest concentration (Fig. 3). Interestingly, we also found differential, in part opposing regulation of other functional key genes. Whereas the transcript abundance of the fatty acid transporter $CD36$ was strongly upregulated by all three fatty acids (Fig. 4), key transcripts of estradiol and progesterone production, $CYP19A1$ and $HSD3B1$ as well as those of gonadotropin receptors, $FSHR$ and $LHCGR$, showed increased upregulation after PA (Fig. 5A) and SA treatment (Fig. 5B), but clearly reduced expression in OA-treated cells (Fig. 6). In contrast, all fatty acids reduced transcript abundance of $STAR$, the rate-limiting factor of steroidogenesis (Fig. 7).

**Cell viability and proliferation**

Flow cytometry analysis of Annexin V-FITC/PI-stained cells showed no statistically significant differences in the proportions of apoptotic cells after treatment with highest concentrations of PA, SA or OA (200 µM, 200 µM and 400 µM, respectively) as compared to vehicle control. However, the percentage of apoptotic cells tended to be higher in the PA and SA treated groups,
but lower with OA (Table 2). The proportion of viable cells was virtually unchanged in all treatment groups, whereas the number of dead cells was significantly higher in OA group. Further, none of the fatty acids induced a significant shift of cell cycle phases in cultured GC. Flow cytometric analysis revealed similar proportions of cells in SubG0/G1, G0/G1, S and G2/M phases (Table 3).

**Effects of fatty acid combinations**

To test combined effects of PA, SA and OA, highest effective concentrations of these fatty acids (200 µM, 200 µM and 400 µM) were mixed and added to cultured GC. As in case of individual fatty acids the morphology of the cells dramatically changed as compared to BSA vehicle controls (Fig. 8A). The results also revealed that co-supplementation had no influence on P4 production, but significantly reduced E2 levels (Fig. 8B). qPCR quantification of key transcripts showed no significant regulation of CYP19A1, HSD3B1, FSHR, LHCGR CCND2 and PCNA, but highly upregulated CD36 transcripts and significantly downregulated STAR (Fig. 9). Results from flow cytometry by Annexin V-FITC/PI staining revealed that the percentage of apoptotic cells was unchanged, whereas that of viable cells was significantly reduced by the combined fatty acid treatment. In particular, the proportion of dead cells was remarkably increased by the combined treatment as compared to the BSA controls (Table 4). However, we did not find significant difference in cell cycle stages (Table 5).

**Effect of intrafollicular OA injection on GC functionality in vivo**

To validate the data obtained from cultured GC, we tested effects of OA in an animal model by directly injecting growing dominant follicles. This approach will expose antral GCs directly to increased OA concentrations while largely avoiding systemic effects. In some animals, post-injection follicle differentiation and ovulation rate was also monitored, whereas in other animals injected follicles were aspirated 48 h later in order to collect follicular fluid and cells therein for hormone production and gene expression analysis.
Ovulation rates

From 14 injected growing dominant follicles (one per animal) that were kept for monitoring the ovulatory competence, nine were injected with BSA, out of which seven follicles advanced to the ovulatory stage (77%), while only one out of five follicles injected with OA ovulated (20%) within 96 h post injection. Although not significant yet the ovulatory competence tended to be reduced following the OA as compared to the BSA injections.

Fatty acid analysis by gas chromatography

Gas chromatography analysis of follicular fluid samples obtained 48 h post intrafollicular injections (BSA with/without conjugated OA) revealed varying proportions of different fatty acids. However, the percentage of OA (46.51 ± 5.8%) in OA-injected follicles was significantly higher as compare to BSA (13.59 ± 2.5%) injected follicles (Supplementary Table 1, see section on supplementary data given at the end of this article).

Steroid hormone estimation

Follicular fluid obtained from aspirated follicles was subjected to RIA for estimating the concentration of P4 and E2, respectively. The results revealed that intrafollicular OA injection remarkably reduced the production of E2 compared to the vehicle control (BSA), while P4 level remained largely unaffected (Fig. 10A).

Gene expression analysis

qPCR analysis showed significantly higher levels of CD36 and lower levels of CYP19A1 and STAR transcripts in GC recovered after intrafollicular OA injection. However, the transcript level of CCND2 remained unaltered throughout (Fig. 10B).

Discussion

Considerable changes in the serum metabolite profile of post-partum dairy cows are approximately mirrored in the follicular fluid of dominant follicles to which pre-ovulatory
oocytes and GC are exposed (21). Thus, to determine if elevated intrafollicular concentrations of free fatty acids affect GC functionality and thus may contribute to declined fertility rates as experienced by cows during NEB, in the present study, we analyzed the in vitro effects of major free fatty acids both separately and in combination on GC functionality. The tested concentrations of PA, SA and OA were based on earlier in vivo experiments which provided estimated physiological concentrations of free fatty acids in the follicular fluid of short-term fasted cows (22). Additionally, we knew from earlier reports that concentrations of OA is highest in the follicular fluid of cows and the present data showed that GC functionality is adversely modulated in vitro; thus, we specifically selected OA for intrafollicular injection to validate whether the effects of OA observed in our GC culture model can be transferred to the in vivo situation. In contrast to our previous studies (23) and to those of others (11), where the fatty acids had been solubilized by using ethanol, we solubilized the fatty acids by conjugation with BSA as previously published (24). On one hand, this certainly mirrors the physiological situation in the follicular fluid more closely, and on the other hand, it enabled us to reproducibly solubilize not only the monounsaturated fatty acid OA, but also the saturated fatty acids PA and SA. According to our previous (unpublished) observations, the effects of ethanol solubilized PA or SA, but not those of OA, on cultured GC showed extreme variability. Very likely, this might be due to different physicochemical properties of saturated vs unsaturated fatty acids. In any case, this let us eventually change our experimental procedure. However, it is also obvious from our present data that the effects of OA on GC were very similar independent of the method of solubilization.

PA, SA and OA alter cell morphology of cultured bovine GC by inducing lipid accumulation

In vitro supplementation of fatty acids separately and in combination induced significant alterations in the morphology and physiological activity of cultured GC. Their morphology closely resembled the morphology of foam cells as observed in OA-treated smooth muscle cells and macrophages (25, 26, 27). In addition, these morphological changes were associated with a highly induced expression of fatty acid translocase CD36 (FAT) known to mediate fatty acid uptake (28). This clearly suggests that all three fatty acids induce their own uptake by the cells. Our results are quite consistent with other studies in primary bovine mammary epithelial cells where mRNA abundance of CD36 was markedly increased when the cells were cultured with PA, SA, OA, linoleate or an conjugated linoleic acid isomer (trans-10, cis-12 18:2) (29, 30) and in rat type II pneumocytes, where FAT/CD36 is expressed mediating the uptake of PA (31). This is well in line with data of the present study showing that PA, SA and OA induce massive accumulation of intracellular vesicles previously (23) identified as lipid droplets in cultured bovine GC.
Effects of free fatty acids on granulosa cells

A Sharma et al.

Table 2  Cell viability was determined by Annexin V-FITC staining assay as described in the Materials and methods section.

| Tested groups   | Parameters       | Viable cells (%) | Apoptotic cells (%) | Dead cells (%) |
|-----------------|------------------|------------------|---------------------|----------------|
| (I) Control     | Viable cells (%) | 79.27 ± 1.2      | 13.72 ± 2.4         | 5.9 ± 1.2 a    |
| (II) Palmitic acid (200 µM) | Viable cells (%) | 74 ± 2.9        | 19.2 ± 3           | 5.8 ± 0.2 a    |
| (III) Stearic acid (200 µM) | Viable cells (%) | 76.9 ± 4.2      | 15.8 ± 2.6         | 6% ± 1.9 a     |
| (IV) Oleic acid (400 µM)   | Viable cells (%) | 67.8 ± 3.5      | 11.3 ± 0.8         | 20.4 ± 3.6 b   |

The data are shown as means ± s.e.m. (Holm–Sidak test: n = 3). Significant differences are indicated by letters a and b (P < 0.05).

**In vitro cell viability and proliferation**

In spite of dramatic morphological alteration of cultured GC under PA and SA supplementation, even at their highest concentrations these fatty acids did not elicit significant apoptotic effects. As mentioned earlier saturated fatty acids, particularly PA and SA, induce apoptosis in human GC (8) and lipooapoptosis in human β-cells, while the unsaturated fatty acids OA and linoleate have no such effects (32). But it is equally important to consider the fact that in cows, follicles that secrete higher E2 levels are selected for continued growth up to the ovulatory stage (33, 34) and higher E2 level is known to increase both GC proliferation and follicle cell differentiation (35, 36, 37). A positive correlation between elevated E2 levels and GC survival has been reported, where higher E2 levels not only protected GC from Fas ligand-induced apoptosis, but also increased the percentage of cells entering from G1 to S phase of the cell cycle, in addition to an increased cyclin D2 protein expression (38). In the present study, both PA and SA stimulated the E2 production significantly along with upregulation of the CCND2 gene. This could effectively mediate the protective effect of high estradiol levels in PA- and SA-treated GC despite of their apoptotic impacts in other mammalian cells as described earlier. As per OA, there were no signs of apoptosis in cultured GC even at its highest tested concentration. OA has been previously reported to have no apoptotic effects on CHO cells and β-islet cells even at high concentrations.

This is in line with the observation that OA causes lipid accumulation more effectively as compared to saturated fatty acids such as PA (39). The cells ability to effectively incorporate fatty acids into cytoplasmic triglycerides (TG) might serve as a protection against their pro-apoptotic effects. OA is more readily incorporated into triglyceride and is therefore considered less apoptotic as compared to saturated fatty acids (40), which are poor substrates for TG synthesis. In addition, OA is known to even mediate protection against PA-induced apoptosis (41). In response to FSH, CCND2 mRNA expression rapidly increased in GC (42, 43), while FSH β-null mutant mice display decreased CCND2 mRNA levels (44). This is well in line with our observation that PA and SA increased the FSHR transcript levels, and as a consequence probably increased the responsiveness of the GC toward this gonadotropin, which in turn may have indirectly induced elevated CCND2 transcript abundance. In contrast, OA steadily decreased the FSHR expression, thus leading to reduced FSH responsiveness. The observed steep downregulation of CCND2 transcript abundance might be therefore a consequence of reduced FSH signaling under OA treatment. Both observations thus are indicative for the important role of FSH signaling for GC viability and proliferation.

Our results showed that combined PA, SA and OA treatment did also not induce apoptotic effects and more interestingly, significantly lowered the E2 production, thus suggesting that OA effects seem to dominate those of PA and SA in the mixture. This could be partially due to the observation that OA effectively rescues saturated fatty acid-induced apoptosis under the activity of the enzyme stearoyl-CoA desaturase (SCD1), catalyzing the conversion of saturated fatty acid into monounsaturated fatty acids, thus directing the saturated fats into TG synthesis and away from pathways leading to apoptosis.

**Steroidogenesis is modulated differentially in vitro by saturated and monounsaturated fatty acid**

To determine the physiological effects of PA, SA and OA on GC we looked upon the transcriptional activity of key

Table 3  Cell proliferation was determined by flow cytometer analysis.

| Tested groups | SubGO/G1 (%) | GO/G1 (%) | S Phase (%) | G2/M (%) |
|---------------|--------------|-----------|-------------|----------|
| (I) Control   | 13.23 ± 6.4  | 91.2 ± 1.5| 6.8 ± 1.2  | 1.9 ± 0.25|
| (II) PA 200 µM| 10.16 ± 3.2  | 88.6 ± 1.0| 8.1 ± 1.4  | 3.2 ± 0.4 |
| (III) SA 200 µM| 24.3 ± 9    | 89.2 ± 1.3| 6.8 ± 0.4  | 3.8 ± 1  |
| (IV) OA 400 µM| 11.9 ± 4.07 | 92 ± 2.9  | 4.1 ± 1.7  | 3.8 ± 1.25|

PA, SA, OA represent palmitic acid, stearic acid and oleic acid, respectively. The data are shown as means ± s.e.m., Holm–Sidak test, n = 3.

https://ec.bioscientifica.com
https://doi.org/10.1530/EC-19-0011
© 2019 The authors
Published by Bioscientifica Ltd
This work is licensed under a Creative Commons Attribution-NonCommercial 4.0 International License.
genes involved in gonadotropin signaling such as FSHR and LHCGR and in steroidogenesis such as CYP19A1, STAR and HSD3B1. Since both folliculogenesis and steroidogenesis are closely dependent on the coordinated actions of FSH and LH with their receptors on GC and thecal cells of ovarian follicles (45). CYP19A1 expression has been found to be elevated by SA supplementation in murine GC (46). This is in line with our data showing that both PA and SA increased the expression of FSHR, LHCGR, CYP19A1 and HSD3B1, and thus, stimulated E2 production. In contrast, all these genes and E2 production were significantly downregulated by OA. Interestingly, the expression of STAR was significantly downregulated by all three fatty acids and by the combined treatment. However, only OA and the combined treatment also resulted in a significantly reduced E2 production, whereas E2 levels were increased by PA and SA treatment. Most likely, in case of OA treatment E2 reduction could be largely due to the reduced transcription of HSD3B1 and CYP19A1, whereas higher levels of E2 in PA and SA treated GC can be attributed to upregulation of CYP19A1 (47). Nevertheless, a clear effect of reduced transcription of STAR that is considered as the rate-limiting factor of steroid hormone synthesis (48) might be obscured by the presence of androstenedione that is a preferred substrate for CYP19A1 to produce E2 in GC (49) in our cell culture medium.

The presented results on OA effects are in accordance with our previous study (23), but are contradictory to data reported by others (7). These authors concluded that OA despite of inhibiting proliferation stimulates the production of E2 in cultured bovine GC. However, our results on stimulatory effects of PA and SA on E2 production are in line with the same study and also with other reports showing that exposure of

Figure 8
(A) Effects of combination treatment with PA + SA + OA (200 µM + 200 µM + 400 µM) on in vitro cultured GC. Photomicrographs were obtained from Nikon TMS-F inverted microscope. Arrows indicate lipid droplet formation in the cells. (B) Effects of combination treatment with PA + SA + OA (200 µM + 200 µM + 400 µM) on steroid hormone concentration in conditioned media of cultured bovine GC. The data are shown as means ± SEM. Different letters indicate significantly different means (unpaired t test; n = 3, P < 0.05).

Figure 9
Effects of combination treatment with PA + SA + OA (200 µM + 200 µM + 400 µM) on the abundance of key transcripts of estradiol, progesterone production (CYP19A1, HSD3B1), gonadotrophin receptor genes (FSHR, LHCGR), cell proliferation markers (CCND2, PCNA), CD36 and STAR. Transcript abundance was normalized to the reference gene RPLPO. The data are shown as means ± S.E.M. Different letters indicate significantly different means (unpaired t test: n = 3, P < 0.05).
Effects of free fatty acids on granulosa cells

Palmitic acid (PA, 200 µM), stearic acid (SA, 200 µM) and oleic acid (OA, 400 µM) were co-supplemented to cultured GC and were compared to control. The data are shown as means ± s.e.m., unpaired t test: (n = 3). Significant difference is indicated by letters a and b (P < 0.05).

Table 4  Cell viability was determined by Annexin V-FITC staining assay as described in the Materials and methods section.

| Tested groups       | Viable cell (%) | Apoptotic cells (%) | Dead cells (%) |
|---------------------|-----------------|---------------------|---------------|
| (I) Control         | 55.7 ± 9.5a     | 34 ± 8.3            | 9 ± 0.9a      |
| (II) PA + SA + OA   | 29.7 ± 1.09b    | 32.3 ± 3.2          | 36.9 ± 4.1b   |

Palmitic acid (PA, 200 µM), stearic acid (SA, 200 µM) and oleic acid (OA, 400 µM) were co-supplemented to cultured GC and were compared to control. The data are shown as means ± s.e.m., unpaired t test: (n = 3).

androgen-producing cells to saturated fatty acids significantly increased androgen production (50). In this context, it is important to point out that PA and SA clearly increase the expression of gonadotropin receptors FSHR and LHGR, whereas OA showed opposite effects. FSH is known to induce the expression of LHGR via protein kinase A (PKA) and phosphoinositide3-kinase (PI3K) pathways in rat GC (51). LHGR is highly expressed in GC during the pre-ovulatory stage to enable responsiveness to the LH surge thus leading to ovulation, oocyte maturation and corpus luteum formation (52). The downregulation of both FSHR and LHGR might therefore affect the FSH signaling pathway leading to compromised GC functionality due to elevated NEFAs specifically by OA. Both PA and SA upregulated CYP19A1 expression with subsequent increase of the E2 production, while OA downregulated the CYP19A1 expression while reducing E2 production. These data, but in particular, our results on the effects of combined fatty acids (PA+SA+OA) may give some hints to better understand the in vivo situation. STAR expression is downregulated by all three fatty acids as well as by the combined treatment. This suggests that elevated in vivo concentrations of fatty acids during NEB might cause cumulative effects on STAR expression in steroiogenic cells similar to our in vitro tested combination. However, it has to be considered as well that about 10–15% of steroid biosynthesis is known to occur via STAR independent mechanisms (53, 54) as some other proteins like MLN4 have been known to possess STAR-like activity in promoting cholesterol flux into mitochondria (55). From our data, it became very clear that the saturated fatty acids tested (PA, SA) elicited very different, partly opposing effects in GC as compared with effects of OA as a monounsaturated fatty acid.

Table 5  Cell proliferation was determined by flow cytometer analysis.

| Tested groups       | SubG0/G1 (%) | G0/G1 (%) | S phase (%) | G2/M (%) |
|---------------------|--------------|-----------|-------------|----------|
| (I) Control         | 13.36 ± 3.7  | 94.8 ± 0.5| 3.8 ± 0.8   | 1.5 ± 0.2|
| (II) PA + SA + OA   | 14 ± 3.1     | 94.5 ± 0.5| 3.9 ± 0.8   | 1.75 ± 0.45|

Palmitic acid (PA, 200 µM), stearic acid (SA, 200 µM) and oleic acid (OA, 400 µM) were co-supplemented to cultured GC and were compared to control. The data are shown as means ± s.e.m., unpaired t test: n = 3.

Intrafollicular OA injection modulates GC function in vivo and impedes ovulation

To validate the in vitro data obtained from our GC culture model we used an in vivo approach by directly injecting the antral cavity of growing dominant follicles. This approach will expose antral GC directly to the injected agent while largely avoiding superimposing systemic effects. In vivo effects of Insulin-like Growth Factor 1 (IGF-1) has been also studied by intrafollicular injection to reveal the mechanisms underlying follicle deviation in heifers and mares (56). However, to our knowledge no functional in vivo study on the role of fatty acids on GC function and hormone concentration by direct injection of follicles in heifers has been carried out so far. Our in vivo data clearly provided an affirmation of the results obtained in vitro. As discussed above in vitro data indicated that OA has a dominant effect as compared to other fatty acids in vitro, thus, we specifically selected this fatty acid for intrafollicular injection. Fatty acid analysis of follicular fluid samples by gas chromatography revealed significantly higher proportions of OA in OA injected as compared to BSA injected follicles even 48 h post-injection, but also demonstrated that proportions of most of the other fatty acids was not markedly altered.

The results are consistent with those reported in vitro, with a reduced E2 concentration detected in follicular fluid recovered from OA injected follicles. Additionally, only 22% of OA injected follicles reached the ovulatory stage as compared to 77% of BSA injected follicles achieving ovulation. This indicates a tendency of OA to inhibit ovulation. Since, dominant follicles secrets sufficient E2 to induce the pre-ovulatory LH surge for successful ovulation (57), reduced E2 concentrations as observed in
In the present study, we showed that PA and SA in contrast to OA induced the expression of key genes of steroidogenesis and E2 production. These differential effects might be attributed to differential saturation or stereo metric positions of double bonds in fatty acids. Combined effects of fatty acids are largely dominated by mostly negative effect of OA on both hormone production and transcript abundance. Suggestively, this might mirror the in vivo situation, where OA is the most up-regulated fatty acid during NEB in the follicular fluid. Additionally, lower CYP19A1 and STAR expression followed by lower E2 production after intrafollicular OA injection confirms the assertion that this fatty acid impedes steroidogenesis both in vitro and in vivo in bovine GC. Collectively, these effects of NEFA might therefore be responsible for mechanism through which the NEB influences the reproductive performance of high-yielding dairy cows during the early postpartum period. Finally, we suggest that these findings may partly be also applicable to obese women with increased serum concentrations of free fatty acids suffering from impaired fertility.

**Conclusion**

The follicular fluid recovered from OA injected follicles might be responsible for the observed lower ovulation rate. A similar decrement of E2 concentrations by OA was also observed in the in vitro experiments both in independent as well as in combination treatments. Also, further investigation revealed that gene expression of CD36 was significantly up-regulated while both CYP19A1 and STAR were significantly down-regulated in GC recovered from OA injected follicles. These results evidently indicate that following intrafollicular injection, OA was actively transported into the GC, with further down-regulation of both CYP19A1 and STAR. Since CYP19A1 in bovine is the key enzyme for E2 synthesis, the resulting lower concentration of E2 in follicular fluid is clearly in line with suppressed CYP19A1 expression. Thus, considering the in vivo data we anticipate that OA effects might dominate among the mixed fatty acids that are mobilized during the period of NEB in postpartum dairy cattle.

**Supplementary data**

This is linked to the online version of the paper at [https://doi.org/10.1530/EC-19-0011](https://doi.org/10.1530/EC-19-0011).

**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.

**Funding**

The project is funded by Federal Ministry of Food and Agriculture (BMEL) based on a decision of the Parliament of the Federal Republic of Germany via the Federal Office for Agriculture and Food (BLE) under the innovation support program.

**Acknowledgements**

Authors appreciate Veronika Schreiter, Maren Anders, Swanhild Rodewald and Christian Plinski, for their technical support throughout the experiments. They also thank the Argrar-Milchhof-Stiewe GbR in 18279 Lalendorf, Germany for providing the animals and supporting our study.

**References**

1. Opsomer G, Coryn M, Deluyker H & Kruif A. An analysis of ovarian dysfunction in high yielding dairy cows after calving based on progesterone profiles. Reproduction in Domestic Animals 1998 33 193–204. (https://doi.org/10.1111/j.1439-0531.1998.tb01342.x)
2. Rukkwamsuk T, Geelen MJH, Kruij TAM & Wensing T. Intereaction of fatty acid composition in adipose Tissue, serum, and liver of dairy

---

**Figure 10**

(A) Effects of intrafollicular oleic acid (C18:1 cis-9) injection on the steroid hormone concentration in follicular fluid recovered 48 h post injection. The data are shown as means ± s.e.m. Different letters indicate significantly different means (unpaired t test; n = 3, P < 0.05). (B) Effects of intrafollicular oleic acid (C18:1 cis-9) injection on transcript abundance of CD36, CYP19A1, STAR and CCND2. BSA and OA (400 µM) indicate vehicle control and tested fatty acid respectively. Transcript abundance was normalized to the reference gene RPLPO. The data are shown as means ± s.e.m. Different letters indicate significantly different means (unpaired t test; n = 3, P < 0.05).
Effects of free fatty acids on granulosa cells

A Sharma et al. Effects of free fatty acids on granulosa cells –14

14 Coll T, Eyre E, Rodríguez-Calvo R, Palomer X, Sánchez RM, Merlos M, Mishra R & Simonson MS. Saturated free fatty acids and apoptosis
Sutton-McDowall ML, Wu LL, Purdey M, Abell AD, Goldys EM, Mu YM, Yanase T, Nishi Y, Tanaka A, Saito M, Jin CH, Mukasa C, Vanholder T, Leroy JLMR, Soom AV, Opsomer G, Maes D, Coryn M
6 Kulick LJ, Kot K, Wiltbank MC & Ginther OJ. Follicular and signaling involving caspase 9 and mitochondrial release of complexes alters cell metabolism and developmental competence.
MacMillan KL, Thompson JG & Robker RL. Nonesterified fatty acids induce ceramide-accumulation and lipid droplet formation in primary cultured aortic endothelial cells.
Zaloga GP & Siddiqui RA. Oleic acid inhibits stearic acid-induced inhibition of cell growth and pro-inflammatory responses in human aortic endothelial cells.

2013 534 486-495. (https://doi.org/10.1186/s12958-018-0332-3)

16 Ratts VS & Moley KH. Associations between free fatty acids, cumulus expansion, cumulus thickness and ovarian response to controlled ovarian hyperstimulation in dairy cows during the development of fatty liver postpartum 1.
15. (https://doi.org/10.1186/1475-2840-4-2)

2010 5470–5480. (https://doi.org/10.1086/558938)

17 Baufeld A & Vanselov J. Increasing cell plating density mimics an early post-LH stage in cultured bovine granulosa cells. Cell and Tissue Research 2013 354 869–880. (https://doi.org/10.1007/s00441-013-1712-9)

128 56535. (https://doi.org/10.3791/56535)

20 Zhang Y, Hao J, Sun Y & Li B. Saturated fatty acids induce smooth muscle cell dysfunction and enhances atherosclerotic lesion development via CD36. Lipids in Health and Disease 2011 10 53. (https://doi.org/10.1186/1476-511X-10-53)

2012 134–144. (https://doi.org/10.1016/j.jygenec.2016.04.020)

2011 2013 961311–1134. (https://doi.org/10.1016/j.theriogenology.2003.12.017)

2013 2590–2597. (https://doi.org/10.1016/j.tet.2009.03.031)

18 Baddela VS, Sharma A, Viergutz T, Koczan D & Vanselow J. Low oxygen levels induce early luteinization associated changes in bovine granulosa cells. Frontiers in Physiology 2018 9 1066. (https://doi.org/10.3389/fphys.2018.01066)

19 Dannenberger D, Nuerenberg G, Nuerenberg K, Will K, Schauer N & Schmieke M. Effects of diets supplemented with n-3 or n-6 PUFA on pig muscle lipid metabolites measured by non-targeted LC-MS lipidomic profiling. Journal of Food Composition and Analysis 2017 56 47–54. (https://doi.org/10.1016/j.jfca.2016.11.015)

20 Dannenberger D, Nuerenberg K, Nuerenberg G & Priepke A. Different dietary protein and PUFA interventions alter the fatty acid concentrations, but not the meat quality, of porcine muscle. Nutrients 2012 4 1237–1246. (https://doi.org/10.3390/nu40401237)

18 Baddela VS, Sharma A, Viergutz T, Koczan D & Vanselow J. Low oxygen levels induce early luteinization associated changes in bovine granulosa cells. Frontiers in Physiology 2018 9 1066. (https://doi.org/10.3389/fphys.2018.01066)

2011 2013 961311–1134. (https://doi.org/10.1016/j.theriogenology.2003.12.017)

2013 2590–2597. (https://doi.org/10.1016/j.tet.2009.03.031)

18 Baddela VS, Sharma A, Viergutz T, Koczan D & Vanselow J. Low oxygen levels induce early luteinization associated changes in bovine granulosa cells. Frontiers in Physiology 2018 9 1066. (https://doi.org/10.3389/fphys.2018.01066)

20 Dannenberger D, Nuerenberg G, Nuerenberg K, Will K, Schauer N & Schmieke M. Effects of diets supplemented with n-3 or n-6 PUFA on pig muscle lipid metabolites measured by non-targeted LC-MS lipidomic profiling. Journal of Food Composition and Analysis 2017 56 47–54. (https://doi.org/10.1016/j.jfca.2016.11.015)

20 Dannenberger D, Nuerenberg K, Nuerenberg G & Priepke A. Different dietary protein and PUFA interventions alter the fatty acid concentrations, but not the meat quality, of porcine muscle. Nutrients 2012 4 1237–1246. (https://doi.org/10.3390/nu40401237)

18 Baddela VS, Sharma A, Viergutz T, Koczan D & Vanselow J. Low oxygen levels induce early luteinization associated changes in bovine granulosa cells. Frontiers in Physiology 2018 9 1066. (https://doi.org/10.3389/fphys.2018.01066)

20 Dannenberger D, Nuerenberg G, Nuerenberg K, Will K, Schauer N & Schmieke M. Effects of diets supplemented with n-3 or n-6 PUFA on pig muscle lipid metabolites measured by non-targeted LC-MS lipidomic profiling. Journal of Food Composition and Analysis 2017 56 47–54. (https://doi.org/10.1016/j.jfca.2016.11.015)

20 Dannenberger D, Nuerenberg G, Nuerenberg K, Will K, Schauer N & Schmieke M. Effects of diets supplemented with n-3 or n-6 PUFA on pig muscle lipid metabolites measured by non-targeted LC-MS lipidomic profiling. Journal of Food Composition and Analysis 2017 56 47–54. (https://doi.org/10.1016/j.jfca.2016.11.015)

20 Dannenberger D, Nuerenberg G, Nuerenberg K, Will K, Schauer N & Schmieke M. Effects of diets supplemented with n-3 or n-6 PUFA on pig muscle lipid metabolites measured by non-targeted LC-MS lipidomic profiling. Journal of Food Composition and Analysis 2017 56 47–54. (https://doi.org/10.1016/j.jfca.2016.11.015)

20 Dannenberger D, Nuerenberg G, Nuerenberg K, Will K, Schauer N & Schmieke M. Effects of diets supplemented with n-3 or n-6 PUFA on pig muscle lipid metabolites measured by non-targeted LC-MS lipidomic profiling. Journal of Food Composition and Analysis 2017 56 47–54. (https://doi.org/10.1016/j.jfca.2016.11.015)

20 Dannenberger D, Nuerenberg G, Nuerenberg K, Will K, Schauer N & Schmieke M. Effects of diets supplemented with n-3 or n-6 PUFA on pig muscle lipid metabolites measured by non-targeted LC-MS lipidomic profiling. Journal of Food Composition and Analysis 2017 56 47–54. (https://doi.org/10.1016/j.jfca.2016.11.015)
Effects of free fatty acids on granulosa cells

A Sharma et al.

32 Eitel K, Staiger H, Brendel MD, Brandhorst D, Bretzel RG, Häring HU & Kellerer M. Different role of saturated and unsaturated fatty acids in β-cell apoptosis. *Biochemical and Biophysical Research Communications* 2002 299 853–856. (https://doi.org/10.1016/S0006-291X(02)02752-3)

33 Evans AC & Fortune JE. Selection of the dominant follicle in cattle occurs in the absence of the expression in messenger ribonucleic acid for gonadotropin receptors. *Endocrinology* 1997 138 2963–2971. (https://doi.org/10.1210/endo.138.7.5245)

34 Mihm M, Austin EJ, Good TE, Ireland JL, Knight PG, Roche JF & Ireland JJ. Identification of potential intrafollicular factors involved in selection of dominant follicles in heifers. *Biology of Reproduction* 2000 63 811–819. (https://doi.org/10.1095/biolreprod.6.3.811)

35 Couse JF, Yates MM, Deroo BJ & Korach KS. Estrogen receptor-β is critical to granulosa cell differentiation and the ovulatory response to gonadotropins. *Endocrinology* 2005 146 3247–3262. (https://doi.org/10.1210/en.2005-0213)

36 Rosenfeld CS, Wagner JS, Roberts RM & Lubahn DB. Intracellular actions of oestrogen. *Reproduction* 2001 122 215–226. (https://doi.org/10.1530/rep.0.1220152)

37 Drummond AE, Brit KL, Dyson M, Jones ME, Kerr JB, O’Donnell L, Simpson ER & Findlay JK. Ovarian steroid receptors and their role in ovarian function. *Molecular and Cellular Endocrinology* 2002 191 23–37. (https://doi.org/10.1016/S0303-7207(02)00052-7)

38 Quirk SM, Cowan RG & Harman RM. The susceptibility of granulosa cells to apoptosis is influenced by oestradiol and the cell cycle. *Journal of Endocrinology* 2006 189 441–453. (https://doi.org/10.1677/joe.1.06549)

39 Ricchi M, Odoardi MR, Carulli L, Anzivino C, Balestrieri S, Pinetti A, Fantoni LJ, Manara F, Bertolotti M, Banni S, et al. Differential effect of oleic and palmitic acid on lipid accumulation and apoptosis in cultured hepatocytes. *Journal of Gastroenterology and Hepatology* 2009 24 830–840. (https://doi.org/10.1111/j.1440-1746.2008.05733.x)

40 Listenberger HL, Han X, Lewis SE, Cases S, Farese RV, Ory DS & Schaffer JE. Triglyceride accumulation protects against fatty acid-induced lipotoxicity. *PNAS* 2003 100 3077–3082. (https://doi.org/10.1073/pnas.0630588100)

41 Chen X, Li L, Liu X, Luo R, Liao G, Li L, Liu J, Cheng J, Lu Y & Chen Y. Oleic acid protects saturated fatty acid mediated lipotoxicity in hepatocytes and rat of non-alcoholic steatohepatitis. *Life Sciences* 2018 203 291–304. (https://doi.org/10.1016/j.lfs.2018.02.023)

42 Szczesny P, Donaher JL, Geng Y, Parker SB, Gardner H, Park MY, Robker RL, Richards JS, McGinnis LK, Biggers JD, et al. Cyclin D2 is an FSH-responsive gene involved in gonadal cell proliferation and oncogenesis. *Nature* 1996 384 470–474. (https://doi.org/10.1038/384470a0)

43 Robker RL & Richards JS. Hormone-induced proliferation and differentiation of granulosa cells: a coordinated balance of the cell cycle regulators cyclin D2 and p27Kip1. *Molecular Endocrinology* 1998 12 924–940. (https://doi.org/10.1210/mend.12.7.0138)

44 Dierich A, Sairam MR, Monaco L, Fimia GM, Gansmuller A, LeMeur M & Sassone-Corsi P. Impairing follicle-stimulating hormone (FSH) signaling in vivo: targeted disruption of the FSH receptor leads to aberrant gametogenesis and hormonal imbalance. *PNAS* 1998 95 13612–13617. (https://doi.org/10.1073/pnas.95.23.13612)

45 Bao B & Garverick HA. Expression of steroidogenic enzyme and gonadotropin receptor genes in bovine follicles during ovarian follicular waves: a review. *Journal of Animal Science* 1998 76 1903–1921. (https://doi.org/10.2527/1998.7671903x)

46 Valck SDM, Van Hoeck V, Arias-Alvarez M,aillo V, Lopez-Cardona AP, Gutierrez-Adan A, Berth M, Cortvriendt R, Bols PEJ & Leroy JLMM. Elevated non-esterified fatty acid concentrations during in vitro murine follicle growth alter follicular physiology and reduce oocyte developmental competence. *Fertility and Sterility* 2014 102 1769.e1–1776.e1. (https://doi.org/10.1016/j.fertnstert.2014.08.018)

47 Goldstone JV, Sundaramoorthy M, Zhao B, Waterman MR, Stegeman JJ & Lamb DC. Genetic and structural analyses of cytochrome P450 hydroxylases in sex hormone biosynthesis: sequential origin and subsequent coevolution. *Molecular Phylogenetics and Evolution* 2016 94 676–687. (https://doi.org/10.1016/j.ympev.2015.09.012)

48 Stocco DM. STAR protein and the regulation of steroid hormone biosynthesis. *Annual Review of Physiology* 2001 63 193–213. (https://doi.org/10.1146/annurev.physiol.63.1.193)

49 Rio ML, Dau AMP, Glanzner WG, Martins M, Zanella R, Rizzetti TM, Comin FV & Gonçalves PBD. Steroidogenic enzymes mRNA expression profile and steroids production in bovine theca cells cultured in vitro and stimulated with angiotensin II. *Ciência Rural* 2015 45 704–710. (https://doi.org/10.1590/0103-8478cr20240254)

50 Bellanger S, Battista M-C, Fink GD & Baillargeon J-P. Saturated fatty acid exposure induces androgen overproduction in bovine adrenal cells. *Steroids* 2012 77 347–353. (https://doi.org/10.1016/j.steroids.2011.12.017)

51 Law NC, Weck J, Kyrris B, Nilsen JH & Hunkziker-Dunn M. Lhnr expression in granulosa cells: roles for PKA-phosphorylated β-catenin, TCF3, and FOXO1. *Molecular Endocrinology* 2013 27 1295–1310. (https://doi.org/10.1210/me.2013-1025)

52 Zhang F-P, Poutanen M, Wilbertz J & Huhtaniemi I. Normal prenatal but arrested postnatal sexual development of luteinizing hormone receptor knockout (LuKKO) mice. *Molecular Endocrinology* 2001 15 172–183. (https://doi.org/10.1210/mend.15.1.0582)

53 Clark BJ, Combs R, Hales KH, Hales DB & Stocco DM. Inhibition of transcription affects synthesis of steroidogenic acute regulatory protein and steroidogenesis in MA-10 mouse Leydig tumor cells 1. *Endocrinology* 1997 138 4893–4901. (https://doi.org/10.1210/endo.138.11.5535)

54 Cooke BA. Signal transduction involving cyclic AMP-dependent and cyclic AMP-independent mechanisms in the control of steroidogenesis. *Molecular and Cellular Endocrinology* 1999 151 25–35. (https://doi.org/10.1016/S0303-7077(98)00255-X)

55 Bose HS, Whittall RM, Huang M-C, Baldwin MA & Miller WL. N-218 MNLN4, a protein with STAR-like steroidogenic activity, is folded and cleaved similarly to STAR. *Biochemistry* 2000 39 11722–11731. (https://doi.org/10.1021/bi000911i)

56 Ginther OJ, Bergfelt DR, Beg MA, Meira C & Kot K. In vivo effects of an intrafollicular injection of insulin-like growth factor I on the mechanism of follicle deviation in heifers and mares. *Biology of Reproduction* 2004 70 99–105. (https://doi.org/10.1095/ biologyreprod.03.021949)

57 Fortune JE. Ovarian follicular growth and development in mammals. *Biology of Reproduction* 1994 50 225–232. (https://doi.org/10.1095/biolreprod50.2.225)

Received in final form 10 January 2019
Accepted 29 March 2019
Accepted Preprint published online 29 March 2019