KIRREL is differentially expressed in adipose tissue from “fertil+” and “fertil-” cows: in vitro role in ovary?

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Short title: KIRREL in bovine adipose tissue and ovary

Abstract

We have previously shown that dairy cows carrying the "fertil-" haplotype for one quantitative trait locus affecting female fertility located on the bovine chromosome three (QTL-F-Fert-BTA3) have a significantly lower conception rate and body weight after calving than cows carrying the "fertil+" haplotype. Here, we compared by tiling array the expression of genes included in the QTL-F-Fert-BTA3 in "fertil+" and "fertil-" adipose tissue one week after calving when plasma non esterified fatty acid concentrations were greater in "fertil-" animals. We observed that thirty-one genes were over-expressed whereas twelve were under-expressed in "fertil+" as compared to "fertil-" cows ($P<0.05$). By quantitative PCR and immunoblot we confirmed that adipose tissue *KIRREL* mRNA and protein were significantly greater expressed in "fertil+" than in "fertil-". *KIRREL* mRNA is abundant in bovine kidney, adipose tissue, pituitary, and ovary and detectable in hypothalamus and mammary gland. Its expression (mRNA and protein) is greater in kidney of "fertil+" than "fertil-" cows ($P<0.05$). KIRREL (mRNA and protein) is also present in the different ovarian cells with a greater expression in granulosa cells of "fertil+" than "fertil-" cows. In cultured granulosa cells, recombinant KIRREL halved steroid secretion in basal state ($P<0.05$). It also decreased cell proliferation ($P<0.05$) and *in vitro* oocyte maturation ($P<0.05$). These results were associated to a rapidly increase in MAPK1/3 and MAPK14 phosphorylation in granulosa cells and to a decrease in MAPK1/3 phosphorylation in oocyte. Thus, *KIRREL* could be a potential metabolic messenger linking body composition and fertility.

Introduction

We have previously shown that primiparous cows carrying “fertil+” haplotype for one quantitative trait locus affecting female fertility located on the bovine chromosome 3 (QTL-F-Fert-BTA3) had a greater conception rate 35 days after the first artificial insemination than those carrying “fertil-” haplotype (Coyral-Castel *et al.* 2011). This QTL-F-Fert-BTA3, finely mapped (Druet *et al.* 2008), was described to affect early reproductive events (Guillaume *et al.* 2007) and explained 14% of the total genetic variance (Ben Jemaa *et al.* 2008).

We observed no differences in ovarian activity (number of follicles and follicular waves, length of oestrus cycle...) in “fertil+” and “fertil-” heifers and cows (Coyral-Castel *et al.* 2011). However, we have demonstrated that the lower fertility of “fertil-” females could be partially due to a lowest quality of the oocytes and consequently of pre-implantation embryo development (Coyral-Castel *et al.* 2012). We have also characterized “fertil+” and “fertil-” cows for food intake and eating behaviour, milk production, live weight and plasma metabolites during the first lactation. Interestingly, the body weight of “fertil-” cows in the first eight weeks *post partum* was significantly lower than “fertil+” cows (Coyral-Castel *et al.*, 2013) suggesting a greater fat mobilization in “fertil-” animals. During early lactation in cow, it is well known that energy expenditures for physiological functions, such as milk production, locomotion, maintenance or reproduction, are greater than the energy provided by feed intake. The maximum dry matter intake is reached about four to ten weeks after peak milk (Coppock 1985). So, high-yielding dairy cows assume a period of negative energy balance, but its magnitude and duration are quite variable (Butler *et al.* 1981). As a result of the energy deficit, body reserves are mobilized (by increased lipolysis) (Bauman & Bruce Currie 1980, Schröder & Staufenbiel 2006) and cows may lose body weight and body condition. In early lactation, cows may mobilize about 50 kg of lipid (Bauman & Bruce Currie 1980) to support lactation. The use of body reserves accounts energetically for about

33% of the milk produced in the first month of lactation (Bauman & Bruce Currie 1980). Mobilization of fat results in release of non-esterified fatty acids (NEFA) in blood, which were reviewed as indicators of energy status of ruminants (Bowden 1971). It is now well established that negative energy balance impact reproductive traits at various levels of the hypothalamo-pituitary-gonadal axis (Beam & Butler 1999, Roche *et al.* 2000, Leroy *et al.* 2008, Roche *et al.* 2009). Adipose tissue is not only an energy storage organ but it is also able to secrete a number of hormone-like compounds that regulates adipocyte development and metabolic function (Ouchi *et al.*) but also fertility (Campos *et al.* 2008, Tersigni *et al.* 2011).

In order to better understand the molecular mechanisms involved in the lower fertility and greater fat mobilization of "fertil-" cows, we compared by Tiling array the expression of genes included in QTL-F-Fert-BTA3 in the adipose tissue of "fertil+" and "fertil-" females one week after calving. We then studied the distribution in bovine tissues of one candidate gene, Kin of IRRE like (Drosophila)-like (*KIRREL*), significantly greater expressed in "fertil+" adipose tissue. Finally, we localized KIRREL by immunohistochemistry in bovine ovarian cells and investigated more precisely its *in vitro* effects on the granulosa cell steroidogenesis and proliferation and oocyte maturation by using recombinant KIRREL.

Materials and methods

Ethics

An ethics committee ("Comité d'Ethique en Expérimentation Animale Val de Loire (CEEA VdL")), protocol registered under ref. n° 2012-10-4) approved all experimental protocols, which were consistent with the guidelines provided by the French Council for Animal Care.

Animals

Thirty-six Holstein dairy cows (n=18 fertil+ and =18 fertil- animals), born in 2006, were monitored during their second lactation. Dairy cows were managed in straw-bedded yards and fed *ad libitum* with a total mixed ration composed of 64.5% maize silage, 10% soybean, 15% concentrate, 10% dehydrated alfalfa, and 0.5% calcium oxide (CaO). After each milking, cows were automatically weighted (software RIC version RW1.7). Only the morning live body weight was used for weight analyses, because the afternoon body weight was more variable. Animals were artificially inseminated from 55-60 d postpartum 12 h after heat detection with the semen of the same bull. Blood samples were taken from the tail before diet distribution, one week after calving and 5 months of pregnancy (about 7 and 8 months after calving). Plasmas were stored at 20°C until assay. NEFA plasma concentrations were determined by enzymatic colorimetry on a multiparameter analyser (KONE instruments corporation, Espoo, Finland). Energy balance (EB, expressed in Mcal/d) was calculated one week after calving when the adipose biopsy was performed as described below. It was calculated per wk according to the INRA feeding systems (INRA, 2007) as the difference between the energy intake and the energy requirements for maintenance, milk production, and pregnancy. According to the INRA system, the daily requirement for maintenance is 1.1 *

115 0.041 * $kg^{0.75}$, and the requirement for milk production is 0.44 * milk production. EB is
116 expressed in Mcal/d, where $kg^{0.75}$ indicates metabolic body weight (INRA, 2007).
117
118 ***Biopsy of subcutaneous adipose tissue***

119 During the second lactation, biopsies of adipose tissue were collected from the same
120 animals at 1wkpp (one week postpartum) and 5mpg (5 months of gestation). Cows were
121 fasted for 12 h before surgery. Anesthesia was induced with injections of 12 to 14 mg of
122 Xylazine i.v. (Rompun; Bayer AG, Leverkusen, Germany) and an injection of 200 mg of
123 Lidocaine s.c. (Lurocaïne; Vétoquinol SA, Lure, France). Subcutaneous fat was collected
124 from the dewlap.

125
126 ***Bovine fertility Tiling Array design***

127 The 385k bovine fertility Tiling Array was designed in both orientations to cover the
128 QTL-F-Fert-BTA3. The sequence from position 9 887 417 to 13 515 249 on chromosome 3
129 was got from UCSC database on Oct. 2007 release bosTau4 including 3627832 nt. The
130 fertility Tiling Array was designed and produced by Roche NimbleGen Inc. (Madison, USA).
131 Highly repeated elements in the genome were repeat-masked. Concerning uniqueness, probes
132 having a unique genome sequence match were selected with SSAHA (Ning *et al.* 2001). An
133 isothermal format (Tm=76°C) and probe length constraint between 50 and 75 bp were used
134 for probe synthesis. Each probe overlapped its neighbour by about 40 bases. The arrays were
135 manufactured by maskless array synthesis technology and the oligonucleotides were
136 synthesized on the arrays by photolithography (Singh-Gasson *et al.* 1999, Nuwaysir *et al.*
137 2002). NimbleGen synthesized the oligonucleotide probes *in situ* using a photo-mediated,
138 maskless process in which the synthesis of each probe is directed by a digital light processor.

The array contained 343 162 50-75-mer oligonucleotides designed on both strands and tiled on average every eleven bases and 45 961 randomly generated probes. All information of bovine fertility Tiling Array platform has been submitted to the Gene Expression Omnibus (GEO) repository and the accession number is GPL15186. Annotation of probes was obtained by aligning probe coordinates with annotation data from Ensembl database (release 56). The loci are classified three types: (1) known protein coding gene, known gene has at least one transcript with a sequence match in a sequence repository external to Ensembl for the same species. (2) Known by projection protein coding gene, refers to genes that are homologous, based on Ensembl comparative analysis, to genes with known status in another species (usually human genes). (3) Putative protein coding gene refers to genes where the Ensembl genebuild transcript and the Vega manual annotation have the same sequence, for every base pair.

Tiling Array data analysis

We developed a new model to perform Tiling array analysis taking advantage that several probes are available per exon and per gene. The model proposed is a mixed model including a fixed exon and a random probe effects. In this study, our aim was to detect differentially expressed genes between “fertil+” and “fertil-” samples.

A hierarchical mixed model with an exon within gene effect and a random probe within exon effect has been considered for each gene $i\,(i = 1, \ldots, I)$. For simplicity the index i will be omitted here as it is a gene-by-gene model:

$$y_{jkrc} = \mu + \alpha_c + \beta_j + (\alpha\beta)_{jc} + \gamma_{jk} + e_{jkrc}$$

where α_c corresponds to a condition effect with two levels (c=1.2 for "fertil+" and "fertil-"), β_j corresponds to an exon effect j within gene i ($j = 1, \ldots, n_i$), and $(\alpha\beta)_{jc}$ is the interaction term between exon and condition. Parameter γ_{jk} corresponds to the probe effect k within

exon j ($k = 1, \ldots, n_j$). The probe effect is assumed to be a random effect such that: $\gamma_{jk} \sim \mathcal{N}(0, \tau_i^2)$, with gene-by-gene variances τ_i^2. Residuals e_{jkrc} are also assumed independent and normally distributed such that: $e_{jkrc} \sim \mathcal{N}(0, \sigma_i^2)$, with gene-by-gene variances σ_i^2. Index r represents the biological replicates $(r = 1, \ldots, R)$.

In this model, testing for differentially expressed genes is equivalent to testing the null hypothesis $H_0 : \mu_1 = \mu_2$, where $\mu_1 = \mu + \alpha_1$ and $\mu_2 = \mu + \alpha_2$ for two conditions (c=1.2 for "fertil+" and "fertil-"). Taking into account multiple testing, *P*-values were adjusted by Benjamini-Hochberg's procedure to control the False Discovery Rate (Benjamini & Hochberg 1995). This model was applied on two datasets containing annoted probes with gene and exon information: 5 822 probes matching to 62 genes and 449 exons in the analysis for the forward strand, and 4 379 probes matching to 62 genes and 352 exons for the reverse strand.

Our model could also be used to detect differentially expressed exons and genes with alternative splicing. R functions implementing this model are available upon request from F. Jaffrézic. We focused our study on known and known by projection protein coding genes. Genes were classified according to the Gene Ontology using NCBI, Ensembl, DAVID and the Gene Ontology website (AmiGO release 1.8).

Total RNA extraction

Subcutaneous adipose tissue was sampled at the dewlap of 36 second lactation cows (18 "fertil+" and 18 "fertil-") one week after parturition and at 5 months of pregnancy (16 "fertil+" and 14 "fertil-"), frozen in liquid nitrogen and stored at -80°C until use. Total RNA was extracted on ice from 250 mg of tissue with an ultraturax homogenizer using 8 ml of QIAzol lysis reagent (Qiagen, Courtaboeuf, France). Chloroform (0.2 ml) was added to each sample. Tubes were waved for 15 seconds and left at room temperature for 5 minutes before

centrifugation (5000 g, 15 minutes, 4°C). Each aqueous phase was mixed to equal volume of ethanol 70% (v:v). Then total RNA was purified using a RNeasy® Midi Kit (Qiagen) according to the manufacturer's recommendations. During purification, a treatment with a RNase-free DNaseI (Qiagen) was performed. After elution with RNase free water, samples were evaporated without heating during 1.5 hours in a Thermo Savant SPD1010 SpeedVac® System and stored at -80°C until cDNA synthesis. RNA quantity was assessed with a NanoDrop Spectrophotometer (Nyxor Biotech, Paris, France) and RNA quality with an Agilent 2100 Bioanalyzer using a RNA 6000 Nano assay protocol (Agilent Technologies, Massy, France). The RNA integrity number (RIN) for each RNA sample is shown in the Table S1.

For RT-PCR, total RNA from bovine tissues (Liver, mammary gland, heart, adipose tissue, kidney, pituitary, lung, skeletal muscle, ovary, hypothalamus, small follicles (< 6 mm), large follicles (> 7 mm), corpus luteum, ovarian cortex and granulosa cells) from slaughterhouse was extracted on ice with an ultraturax homogenizer in TRIzol® reagent according to manufacturer's recommendation (Invitrogen™ by Life technologies™, Villebon sur Yvette, France). A treatment with DNaseI using the DNA-*free*™ Kit (Ambion® by Life technologies™) was performed on the total RNAs. Total RNA from granulosa cells in culture was extracted using 1 ml of TRIzol® reagent by scratching wells. RNA quantity was assessed with a NanoDrop Spectrophotometer.

cDNA synthesis and labeling, array hybridization, washing and scanning

Array hybridation was performed using cDNA of adipose tissue from eighteen animals (nine "fertil+" and nine "fertil-") one week after calving that is a stage of intense adipose tissue mobilization. Samples were prepared, labelled and hybridized according to the

NimbleGen Arrays User's Guide: Gene Expression Analysis v3.2. cDNAs were synthesized using an Invitrogen Superscript Double-Stranded cDNA Synthesis Kit (Invitrogen™ by Life Technologies™). They were then purified using a MinElute Reaction Cleanup Kit (Qiagen). Samples were labelled with Cy3 with a NimbleGen One-Color DNA Labeling Kit (Roche NimbleGen, Inc.). Hybridization solution was prepared from the NimbleGen Hybridization Kit (Roche NimbleGen, Inc.) and Cy3-labeled samples were hybridized on the 385K array at 42°C for 18 hours. Finally, arrays were washed with solutions of the NimbleGen Wash Buffer Kit (Roche NimbleGen, Inc.). Arrays were scanned with a GenePix 4000B Scanner at 532 nm. Data were extracted with the Roche NimbleScan software (Roche NimbleGen, Inc.).

Reverse Transcription and Polymerase Chain Reaction

Reverse transcription (RT) of total RNA (1 µg) was performed for 1 hour at 37°C in a 20 µl mixture as previously described (Coyral-Castel *et al.* 2010). Single-strand cDNAs of *KIRREL* and *ACTR3* were amplified with specific primers (Invitrogen™ by Life technologies™, Table 2). Polymerase chain reaction (PCR) was carried out in a previously described mixture (Coyral-Castel *et al.* 2010) for 30 (*ACTR3*) or 40 (*KIRREL*) PCR cycles (1 minute at 94°C, 1 minute at 58°C, 1 minute at 72°C), with a final extension step of 7 minutes at 72°C. PCR products were visualized in a 1.5% (w:v) agarose gel stained with ethidium bromide. *ACTR3* was used as positive control. Finally, DNA was extracted from the agarose gel using the EZNA microelute Gel Extraction kit (VWR, Fontenay-sous-Bois, France) according to the manufacturer's procedure. DNA was sequenced by Beckman Coulter Genomics (Grenoble, France). RT and PCR consumables were purchased from Promega (Charbonnières-les-Bains, France).

Real-time quantitative PCR (qPCR)

239 Targeted cDNAs were quantified by real-time PCR using SYBR Green Supermix
240 (Bio-Rad, Marnes la Coquette, France) and 250 nM of specific primers (Invitrogen™ by Life
241 technologies™, Table 1) in total volume of 20 µl in a MyiQ Cycle device (Bio-Rad). For
242 adipose tissue, samples from thirty six animals (n=18 "fertil+" and =18 "fertil-" animals) at 1
243 wkpp and thirty animals (n=16 "fertil+" and =14 "fertil-" animals) at 5 months of pregnancy
244 were tested in duplicate on the same plate and the CVs was less than 5% . PCR amplification
245 with water, instead of cDNA, was performed systematically as a negative control. After
246 incubation for 2 minutes at 50°C and a denaturation step of 10 minutes at 95°C, samples were
247 subjected to 40 cycles (30 seconds at 95°C, 30 seconds at 60°C, 30 seconds at 72°C),
248 following by the acquisition of the melting curve. Primers' efficiency (E) was performed from
249 serial dilutions of a pool of obtained cDNA and ranged from 1.80 to 2.16. For normalization,
250 the expressions of four housekeeping genes – PPIA (cyclophilin A), RPL19, ACTR3 and
251 EEF1A1– were examined. For each gene, expression was calculated according to primer
252 efficiency and Cq : expression=E^{-Cq}. These four housekeeping genes showed expressional
253 changes between « fertil+ » and « fertil-» tissues or cells. Therefore, the data were normalized
254 to the geometric mean of PPIA and EEF1A1 (the most stable combination) following the
255 report that suggests the geometric mean of multiple housekeeping genes as an accurate
256 normalization factor (Vandesompele *et al.* 2002).
257
258 ***Granulosa cell collection and primary culture***
259 Bovine ovaries were collected at the slaughterhouse and transported in physiological
260 saline up to the laboratory. Granulosa cells were isolated by puncturing small follicles (< 6
261 mm) in McCoy's 5A culture medium enriched with bovine serum albumin (BSA 0.1% (w:v),
262 Euromedex, Souffelweyersheim, France), L-glutamine (3 mM, Eurobio, Courtaboeuf,
263 France), penicillin (100 UI/ml, PAA laboratories, Les Mureaux, France), streptomycin (0.1

mg/ml, PAA laboratories), Hepes (20 mM pH = 7.6), bovine apo-transferrin (5 µg/ml, Sigma-Aldrich, Saint-Quentin-Fallavier, France) and androstenedione (0.1 µmol/l, Sigma-Aldrich, Saint-Quentin-Fallavier, France). Cells were centrifuged at 200 g for 5 minutes, washed with fresh enriched McCoy's 5A and the pellet was resuspended in enriched McCoy's 5A supplemented with 10% (v:v) fetal bovine serum (FBS, PAA laboratories, Les Mureaux, France) and amphotericin B (5 µg/ml, PAA laboratories). Approximately 2 x 10^5 live cells were seeded per well of a 24-well culture plate. After 24 hours of culture, cells were serum starved for 18 hours before treatment with a recombinant mouse (rm) KIRREL (R&D Systems®, Lille, France), human recombinant IGF1 (Sigma) and/or ovine recombinant FSH (NIDDK, NIH Bethesda, USA). Cultures were performed at 37°C in a humidified air containing 5% CO_2.

Cell viability

Cell viability was determined by Blue Trypan staining. Live (normal cells) and dead cells (blue cells) were counted using a hemocytometer.

Thymidine incorporation into granulosa cells

After 18 hours of serum starvation, culture medium was removed and 1 µCi/ml of [^{3}H]-thymidine (Perkin-Elmer, Courtaboeuf, France) was added in the presence or absence of rm KIRREL (10 ng/ml or 100 ng/ml) in enriched McCoy's 5A. After 24 hours of culture, excess of thymidine was removed by washing cells twice using PBS 1X. Then cells was fixed using cold 50% (v:v) trichloroacetic acid for 10 minutes and lysed by 0.5 N NaOH. The radioactivity was determined in scintillation fluid by counting in a β-photomultiplier. The values, expressed as count per min (CPM), are representative of five independent cultures with each condition in quadruplate.

289

290 ***Progesterone and oestradiol assay***

291 Granulosa cells were cultured for 48 hours, after 18 hours of serum starvation, in the
292 presence or absence of rm KIRREL (10 ng/ml or 100 ng/ml), IGF1 (10^{-8}M) and/or FSH (10^{-}
293 ^{8}M) in enriched McCoy's 5A. The concentration of progesterone and oestradiol in the culture
294 medium was measured by a radioimmunoassay protocol as previously described (Tosca *et al.*
295 2005). The limit of detection of progesterone was 12 pg/tube and the intra- and inter-assay
296 coefficients of variation were less than 10% and 11%, respectively. The limit of detection of
297 oestradiol was 25 pg/tube and the intra- and inter-assay coefficients of variation were less than
298 12% and 10%, respectively. Results were expressed as the concentration of steroids/cell
299 protein concentration/well. Results are presented as mean ± S.E.M of four independent
300 cultures, in which each condition was analyzed in quadruplate.

301

302 ***Protein extraction and western-blot***

303 Lysates of tissues (adipose tissue and kidney) or cells were prepared on ice with an
304 ultraturax homogenizer (tissues) or by scratching wells (primary-cultured cells) in lysis buffer
305 as previously described (Coyral-Castel *et al.* 2010). Proteins extracts (80 µg) were
306 denaturated, submitted to electrophoresis in a 12% (w:v) SDS-polyacrylamide gel, transferred
307 onto nitrocellulose membrane and incubated with specific antibodies as previously described
308 (Coyral-Castel *et al.* 2010). Rabbit polyclonal antibodies to AKT1, phospho-PRKAA
309 (Thr172), PRKAA, phospho-MAPK1/3 (Tyr204/Thr202), phospho-MAPK14
310 (Thr180/Tyr182) were purchased from Cell signalling Technology (Ozyme, Saint Quantin en
311 Yveline, France). Rabbit polyclonal antibodies to phospho-AKT1 (Ser473), MAPK1,
312 MAPK14 and KIRREL were obtained from Santa Cruz Biotechnology (Euromedex,
313 Souffelweyersheim, France). Mouse monoclonal antibodies to Vinculin (VCL) and PCNA

(proliferating cell nuclear antigen) were purchased from Sigma-Aldrich and Ozyme, respectively. Antibodies were used at 1:1000. Horseradish peroxidase-conjugated anti-rabbit and anti-mouse IgG were purchased from Eurobio (Les Ulis,France). Proteins were detected by enhanced chemiluminescence (Western Lightning *Plus*-ECL, Perkin Elmer) using a G:Box SynGene (Ozyme) with the GeneSnap software (release 7.09.17). Signals detected were quantified with the GeneTools software (release 4.01.02). The results are expressed as the intensity signal in arbitrary units after normalization allowed by the presence of MAPK3, MAPK14, AKT1, PRKAA total (for MAPK1/3, MAPK14, AKT1 and PRKAA phosphorylation, respectively) and vinculin (for KIRREL) as an internal standard.

Immunohistochemistry

Bovine ovaries embedded in paraffin were serially sectioned at a thickness of 7 µm. Immunohistochemistry was performed as previously described (Tosca *et al.* 2005). Sections were incubated overnight with antibodies against KIRREL (1:100, Santa Cruz biotechnology) or rabbit IgG as negative controls. Ovaries from 3 different cows were studied.

Bovine Oocyte Collection and In Vitro Maturation

Bovine ovaries were collected from a slaughterhouse in sterile NaCl solution and maintained at 37°C until aspiration. The cumulus-oocyte complexes (COCs) were aspirated from follicles 3–8 mm in diameter using an 18-gauge needle connected to a sterile test tube and to a vacuum line (100mmHg) as previously described (Reverchon et *al.* 2014). COCs were then selected under a dissecting microscope. Expanded or nonintact COCs were eliminated: only intact COCs were washed in TCM Hepes 199 (Sigma) supplemented with 0.4% BSA and gentamycine (2.5ml/L) under mineral oil (Sigma). The COCs were cultured in TCM 199 (Sigma) with 4 mg/ml BSA supplemented or not with different concentrations of

rm KIRREL (10 and 100 ng/ml) for 22 h at 39°C in 5% CO2 in air with saturated humidity. Each oocyte group contained at least 50 oocytes. After maturation, COCs were denuded by pipetting with 0.5% hyaluronidase (Sigma), and the DNA was colored with Hoechst before mounting.

Statistical analysis

All statistical analyses were conducted using the SAS software (SAS Institute INC, 2009). The MIXED procedure for linear mixed models was used to determine the changes of : i) the live body weight; ii) the energy balance ; iii) the plasma NEFA concentrations ; iv) adipose tissue KIRREL expression. The initial model included time after calving (1 wkpp, 5 mpg), haplotype (fertil +, fertil-) and time after calving×haplotype interaction.

The protein amount of KIRREL in adipose tissue, kidney, granulosa cells and various tissues (kidney, hypothalamus, pituitary and mammary), the KIRREL mRNA expression in various ovarian compartments and in granulosa cells, the effect of rm KIRREL on progesterone and oestradiol secretion by bovine granulosa cells in basal state or in response to IGF1 and FSH, the effect of rm KIRREL on the amount of 3H thymidine incorporated into granulosa cells and on the amount of PCNA, the amount of oocyte at the GV stage, and the progesterone concentration in the in vitro maturation medium and the level of phospho-MAPK1/3 in oocyte were assessed using one-way ANOVA. Numerical data are expressed as means±SEM and results were considered statistically significant at $P<0.05$.

Results

NEFA plasma concentrations, Energy Balance and Live Body Weight of animals

One week after calving, "fertil+" cows (n=18) had significant lower concentrations of plasma NEFA than "fertil-" cows (n= 18; 860.6 ± 105.4 µmol/l vs 1247.0 ± 72.7 µmol/l, respectively, $P<0.05$, Fig. 1A.) and a greater energy balance (-10.8±0.7 Mcal/day vs -14.4 ± 0.6 Mcal/day, respectively, $P<0.05$, Fig.1B) and live body weight (666.1± 19.6 kg vs 610.2± 0.7 kg, respectively, $P<0.05$, Fig.1C), suggesting a greater adipose tissue mobilization in "fertil-" than in "fertil+" cows. At 5 months of gestation (mpg) during reconstitution of body reserves, plasma NEFA, energy balance and live body weight were not significant between "fertil+" and "fertil-" animals (n=16 "fertil+" and n=14 "fertil-") (Fig. 1).

Tiling array

To better investigate this difference in mobilization, total adipose tissue RNA from nine "fertil+" and nine "fertil-" was extracted, reverse transcribed, labelled and hybridized on a 385K array containing the sequence of the QTL-F-Fert-BTA3. We observed that 43 known genes were differentially expressed in adipose tissue of "fertil+" and "fertil-" cows ($P<0.05$, Table 2). Thirty-one genes were over-expressed in "fertil+" adipose tissue as compared to "fertil-" cows, with fold change ("fertil+"/"fertil-") ranging from 1.0345 to 1.6612 (Table 2). Twelve were under-expressed in "fertil+" adipose tissue, with fold change varying from 0.7694 to 0.9714 (Table 2). Genes under-expressed in "fertil+" adipose tissue were mainly olfactory receptors (10 on 12 genes, Table 2). We then selected about 10 genes represented in bold in the Table 2 that had the highest fold change to perform expression analysis by quantitative PCR using specific primers (Table 2). Interestingly, we confirmed the results of Tiling array by qPCR for only one gene, named *KIRREL* (kin of IRRE like) also known as *NEPH1*.

387

388 ***Expression of KIRREL in subcutaneous adipose tissue of "fertil+" and "fertil-" cows one***
389 ***week after calving and after five months of pregnancy***

390 Differential adipose tissue mRNA expression of *KIRREL* one week after calving was
391 confirmed in 18 "fertil-" and 18 "fertil+" animals (including the samples of nine animals per
392 genotype used for the tiling array experiment). Indeed, as shown in Fig. 2A and in a good
393 agreement with the Tiling array results, adipose tissue *KIRREL* expression was significantly
394 greater expressed in "fertil+" than in "fertil-" in the first week post partum (P=0.005). This
395 difference was also observed at the protein level by immunoblot (Fig. 2B, P=0.023). On the
396 contrary, the mRNA expression of adipose tissue *KIRREL* was similar between the two
397 haplotypes at 5 months of pregnancy (Fig. 2A), when animals were not in negative energy
398 balance. Moreover, we noted that in "fertil+" but not in "fertil-" adipose tissue, the mRNA
399 expression of *KIRREL* was significantly decreased between one week after calving and 5
400 months of pregnancy (P=0.04).

401

402 ***Expression of KIRREL in bovine tissues***

403 *KIRREL* (also called *NEPH1)* expression has been studied in human and mouse tissues
404 where it has been described highly expressed in kidney (Donoviel *et al.* 2001). However, the
405 mRNA or protein distribution of KIRREL has never been investigated in bovine tissues. By
406 RT-PCR, as shown in Fig. 3A, *KIRREL* was strongly detected in bovine adipose tissue,
407 kidney, pituitary and ovary and less abundantly in mammary gland and hypothalamus. We
408 then compared the expression of *KIRREL* mRNA by quantitative PCR in kidney,
409 hypothalamus, pituitary and mammary gland of "fertil+" and "fertil-" cows slaughtered after
410 their third or fourth lactation. As showed in Fig. 3B, kidney *KIRREL* mRNA expression was
411 about two-fold greater expressed in "fertil+" than in "fertil-" cows (P<0.05). However, the

412 relative expression of this gene was similar between the two haplotypes in hypothalamus,
413 pituitary and mammary gland (Fig. 3B). By immunoblot, we confirmed at the protein level
414 the greater expression of KIRREL in the kidney of "fertil+" cows (P<0.05, Fig. 3C).
415
416 ***Expression of KIRREL in bovine ovary***
417 As shown in Fig. 3A, *KIRREL* is expressed in bovine ovary. So, we examined more
418 precisely its expression in the various compartments of the ovary. By RT-PCR, we showed
419 that *KIRREL* mRNA was present in theca-interstitial cells from small and large follicles (SF
420 and LF), corpus luteum (CL), cortex (Ctx) and granulosa cells of small and large follicles (GC
421 SF and GC LF, Fig. 4A). By qPCR, we have observed that *KIRREL* was significantly greater
422 expressed in granulosa cells from large follicles as compared to the other ovarian
423 compartments or cells (P<0.02, Fig. 4B). As showed in Fig. 4C, we confirmed the presence of
424 KIRREL protein by immunohistochemistry in the ovarian follicle. More precisely, KIRREL
425 was localized in theca and granulosa cells, oocyte, cumulus cells and follicular fluid. We then
426 compared the expression of *KIRREL* mRNA by qPCR in granulosa cells from small follicles
427 of "fertil+" and "fertil-" cows slaughtered after their third or fourth lactation. As shown in the
428 Fig. 4D, *KIRREL* mRNA expression in granulosa cells from small follicles was about twelve-
429 fold greater expressed in "fertil+" than in "fertil-" cows (P<0.002).
430 We next performed primary culture of bovine granulosa cells from small follicles
431 collected from random cows and determined whether the two main hormones involved in the
432 folliculogenesis, FSH and IGF1, were able to regulate mRNA expression of *KIRREL*.
433 Treatment with FSH (10^{-8}M) and IGF1 (10^{-8}M) alone or combined for 24 or 48 hours did not
434 affect *KIRREL* expression as determined by qPCR in cultured bovine granulosa cells (data not
435 shown).
436

Effect of rm KIRREL on primary bovine granulosa cell steroidogenesis and proliferation

In order to elucidate the effects of KIRREL in bovine granulosa cells, we *in vitro* incubated these cells with commercial recombinant mouse KIRREL (rm KIRREL) that shares more than 98% identity with bovine KIRREL. Primary bovine granulosa cells were cultured for 48 hours in serum-free medium supplemented with either different concentrations of rm KIRREL (1, 5, 10 or 100 ng/ml) or with or without rm KIRREL (10 ng/ml) in the presence or absence of IGF1 (10^{-8} M) or FSH (10^{-8}M). As shown in Fig. 5A and B, rm KIRREL reduced in a dose dependent manner (1 to 100 ng/ml) basal progesterone and oestradiol secretion in the culture medium ($P<0.05$) as determined by RIA. As expected, the progesterone and oestradiol secretion was significantly increased by IGF1 and FSH (Fig. 5C and D) compared to the basal state ($P<0.01$). However, no significant effect of rm KIRREL at the 10 and 100 ng/ml (data not shown) concentrations was observed on IGF1- or FSH-induced progesterone or oestradiol secretion by primary bovine granulosa cells (Fig.5C and D). We also investigated whether rm KIRREL affected the basal proliferation of primary bovine granulosa cells. We measured the [^{3}H]-thymidine incorporation into cells after 24 hours of culture in the presence or absence of different concentration of rm KIRREL (1, 5, 10 and 100 ng/ml). We observed that rm KIRREL significantly decreased basal proliferation of granulosa cells, in a dose dependent manner (Fig. 6A, $P<0.04$). These results were confirmed by evaluating the PCNA level by Western blotting (Fig. 6B). However, all these data were observed without any effects of rm KIRREL (10 and 100 ng/ml for 24h and 48h) on the viability of primary bovine granulosa cells as determined by trypan blue incorporation (data not shown).

Effect of rm KIRREL on various signalling pathway in primary bovine granulosa cells

In the literature, KIRREL (NEPH1) has been described to modulate intracellular signaling pathways (Harita *et al.* 2008). Thus, we studied the effects of rm KIRREL on

462 various signalling pathways in primary bovine granulosa cells. rm KIRREL (100 ng/ml) was
463 added to the medium culture for different times (0 to 60 minutes) and we analysed the protein
464 pattern of MAPK1/3, AKT1, PRKAA and MAPK14 phosphorylation. As shown in Fig. 7A,
465 rm KIRREL led to a significant rapid and transient increase of the MAPK1/3 phosphorylation
466 after 5 minutes of stimulation (P=0.0056). In the same way, rm KIRREL has rapidly
467 increased MAPK14 phosphorylation from 1 to 5 minutes of treatment (P<0.05, Fig. 7B).
468 Conversely, rm KIRREL did not affect AKT1 and PRKAA phosphorylation (data not shown).
469
470 ***Effect of rm KIRREL on the nuclear maturation and MAPK1/3 phosphorylation of bovine***
471 ***oocytes in COCs and progesterone secretion by bovine COCs during in vitro maturation***
472 We also studied the effects of different concentrations of rm KIRREL on the meiotic
473 progression of bovine oocytes in COCs during in vitro maturation (IVM). After 22 h of
474 culture in IVM medium, about 90% of oocytes had progressed to the metaphase II stage, with
475 less than 10% remaining at the germinal vesicle (GV) stage (Fig. 8A). Conversely, if COCs
476 matured for 22 h in IVM medium supplemented with 10 or 100 ng/ml of rm KIRREL, 40% to
477 45% of oocytes remained at the GV stage (Fig. 8A). Thus, rm KIRREL treatment of COCs
478 during IVM resulted in meiotic arrest. We studied the molecular mechanisms involved in the
479 effects of rm KIRREL on the nuclear maturation of bovine oocytes in COCs by determining
480 the MAPK3/1 phosphorylation in the presence or absence of rm KIRREL (10 and 100 ng/ml)
481 in COCs allowed to mature *in vitro* for 22 h. As shown in Fig.8B, the level of MAPK3/1
482 phosphorylation increased in the oocyte from COCs during IVM and the addition of rm
483 KIRREL (10 and 100 ng/ml) to the maturation medium for 22 h significantly decreased
484 MAPK3/1 phosphorylation. We also observed that the addition of rmKIRREL to the
485 maturation medium for 22 h significantly decreased progesterone secretion in COCs (Fig.
486 8C).

487

488 **Discussion**

489 In the present study we reported by using Tiling array that “fertil-” cows, exhibiting
490 greater plasma NEFA concentrations one week after calving, had 43 genes coding for known
491 proteins differentially expressed in adipose tissue compared to “fertil+” animals. More
492 precisely, thirty-one genes were over-expressed whereas twelve were under-expressed in
493 “fertil+” compared to “fertil-” cows. We confirmed by RT-qPCR and immunoblot that
494 KIRREL was significantly greater expressed in “fertil+” adipose tissue compared to “fertil-”
495 animals. We showed that *KIRREL* is mainly expressed in bovine kidney, adipose tissue, ovary
496 and pituitary and less abundantly in hypothalamus and mammary gland. Interestingly, we
497 observed that *KIRREL* mRNA expression in kidney was significantly greater expressed in
498 “fertil+” compared to “fertil-” animals. In ovary, we have shown for the first time that
499 KIRREL was expressed in various ovarian cells including oocyte, granulosa and theca cells
500 and its mRNA expression was significantly greater in the granulosa cells of “fertil+”
501 compared to “fertil-” cows. By using recombinant protein, we have demonstrated that
502 KIRREL was able to decrease *in vitro* progesterone secretion and proliferation in granulosa
503 cells but also *in vitro* oocyte maturation suggesting that this protein could be a potential
504 metabolic messenger linking metabolism, body composition and fertility.

505 We have previously shown that Holstein cows selected for their homozygous
506 favourable (“fertil+”) haplotype at one QTL of female fertility located on the chromosome 3
507 (QTL-F-Fert-BTA3) had a 31% and 26% greater success rate at 35 and 90 days after the first
508 artificial insemination, respectively, compared to the unfavourable (“fertil-”) haplotype
509 (Coyral-Castel *et al.* 2011). Furthermore, we have observed slower oocyte maturation
510 dynamics after *in vivo* maturation and lower blastocyst quality after *in vitro* embryo
511 development in “fertil-” compared to “fertil+” heifers (Coyral-Castel *et al.* 2012). In addition

512 to fertility problems, we have shown that “fertil-” cows had a lower body weight in the first
513 eight weeks after calving than “fertil+” cows and a more negative energy balance in the first
514 week *post partum*, suggesting a greater fat mobilization in this haplotype (Coyral-Castel *et*
515 *al.*, 2013). In the present study, we confirmed this hypothesis since plasma NEFA
516 concentration was significantly greater in “fertil-” compared to “fertil+” cows one week after
517 calving. In order to determine if this difference in fat mobilization between the two
518 haplotypes could contribute to explain the difference in fertility, we compared the expression
519 of genes located in the QTL-F-Fert-BTA3 by Tiling array in the adipose tissue of the two
520 haplotypes. Various studies indicate that body fat content is associated with changes in
521 reproductive performance (Randel 1990, Dunn & Moss 1992), although the pathway which
522 mediates such effects has not been clearly established. Some evidence suggests *in vivo* and *in*
523 *vitro* that adipokines regulate the hypothalamus-pituitary-ovary axis in mammals including
524 bovine species (Spicer *et al.* 1993, Williams *et al.* 2002, Maillard *et al.* 2010). In the present
525 study, we have shown that 43 genes coding known proteins out of 124 genes on the array
526 were differentially expressed in adipose tissue between the two haplotypes. Indeed, 31 genes
527 were overexpressed whereas 12 were under-expressed in “fertil+” compared to “fertil-” cows.
528 However, the ratios obtained were low (less than 2) even though difference was statistically
529 significant. One explanation of this result is that several probes were available per exon and
530 per gene on the array. We performed qPCR on 10 genes that had the highest fold change.
531 Unfortunately, we significantly confirmed the results of Tiling array for only one gene, named
532 *KIRREL* (kin of IRRE like) also known as *NEPH1*. *KIRREL* is significantly overexpressed in
533 adipose tissue of “fertil+” compared to “fertil-” cows one week after calving. Similar
534 tendency was observed for two other genes (CADM3, P=0.069 and SLAMF6 P=0.08). These
535 data clearly indicate that the two techniques (Tiling array and qPCR) have not the same
536 sensitivity, mainly due to the design of the array.

KIRREL is a member of the nephrin-like protein family, which also includes KIRREL2 and KIRREL3. It is a molecule identified in mice by a retrovirus-mediated mutagenesis screen (Donoviel *et al.* 2001). KIRREL contains five extracellular immunoglobulin-like domains and is structurally related to nephrin. In human and rodents, *KIRREL* is abundantly expressed in the kidney but also found in some reproductive organs such as brain, placenta and testis (Donoviel *et al.* 2001, Beall *et al.* 2005). However, the presence of KIRREL in the ovary or pituitary had never been investigated. In bovine tissues, we have detected *KIRREL* mRNA expression in kidney, adipose tissue, ovary and pituitary and less abundantly in hypothalamus and mammary gland. Interestingly, we have shown a greater mRNA and protein expression of KIRREL in “fertil+” kidney compared to “fertil-” cows. In mice, the disruption of the *NEPH1* gene results in effacement of glomerular podocytes, heavy proteinuria, and early postnatal death (Donoviel *et al.* 2001). Thus, KIRREL plays a pivotal role for the development and maintenance of the filtration barrier in the kidney (Donoviel *et al.* 2001, Neumann-Haefelin *et al.* 2010). In a previous work, we have observed that the plasma concentrations of urea were significantly greater in “fertil+” compared to “fertil-” cows (unpublished data) suggesting that the greater KIRREL expression in the kidney of “fertil+” could explain a better renal glomerular filtration in this haplotype. In bovine ovary, we detected *KIRREL* in different cells including granulosa and corpus luteum cells. As in adipose tissue one week after calving and in kidney, we have shown a greaterr expression of *KIRREL* in granulosa cells from “fertil+” compared to “fertil-” cows. The function of KIRREL in extra renal organ systems is almost unknown. Recent studies revealed that mammalian KIRREL proteins have similar cell-cell recognition functions. Furthermore, KIRREL has been shown to interact with Nephrin and Tight junction protein zona occludens-1 (ZO-1) (Huber *et al.* 2003, Liu *et al.* 2003). ZO-1 is a protein located on a cytoplasmic membrane surface of intercellular tight junctions. It interacts with the gap junction protein

562 connexin43 (Giepmans & Moolenaar 1998). Thus, KIRREL could be involved in the cell
563 adhesion and in the signal transduction at cell-cell junctions. In this way, KIRREL could
564 contribute to the greater fat mobilization in adipose cells of "fertil-" compared to "fertil+"
565 cows.

566 In order to determine the function of KIRREL in the bovine granulosa cells, we
567 performed primary granulosa cells and incubated them with different concentrations of
568 recombinant KIRREL protein. KIRREL is known to associate Nephrin and ZO-1 and these
569 complexes are found in lipid rafts (Schwarz *et al.* 2001), a microdomain that consists of
570 assemblies of sphingolipids and cholesterol in the outer leaflet of the plasma membrane. In
571 our study, we have observed that recombinant KIRREL was able to increase rapidly
572 MAPK1/3 and MAPK14 phosphorylation and to decrease progesterone and oestradiol
573 secretion suggesting that rm KIRREL is active in bovine cultured granulosa cells. Recently,
574 KIRREL has been considered as a signalling molecule. It has a cytoplasmic domain that
575 contains a large number of tyrosine residues. These residues can be phosphorylated by a Src
576 family tyrosine kinase, Fyn (Verma *et al.* 2003) that has been described in rat granulosa cells
577 (Wayne *et al.* 2007). Once tyrosine are phosphorylated, KIRREL is able to modulate
578 intracellular signaling by binding to Grb2 (Harita *et al.* 2008). Grb2 is a cytosolic adaptor
579 involved in the MAPK1/3 signaling pathways. Thus, KIRREL could activate MAPK3/1
580 signaling pathways through the tyrosine kinase, Fyn, in bovine granulosa cells. In the present
581 work, we have observed that recombinant KIRREL protein decreases steroid secretion.
582 Various studies have showed that MAPK3/1 positively regulates progesterone production by
583 granulosa cells in different species (Gyles *et al.* 2001, Tosca *et al.* 2005, Tosca *et al.* 2007).
584 Thus, it is likely that the inhibitory effect of KIRREL on the progesterone secretion is not
585 mediated by MAPK3/1 in cultured bovine granulosa cells. The involvement of MAPK14
586 remains to be determined. This pathway has been described to be involved in the differential

regulation of steroidogenesis in rat granulosa cells (Yu *et* 494 *al.* 2005). In the present study, we have also shown that rm KIRREL decreases *in vitro* bovine oocyte maturation probably through an inhibition of progesterone secrection by COCs . We observed that *KIRREL* mRNA expression is greater *in vivo* in granulosa cells of "fertil+" compared to "fertil-" cows but we showed that recombinant KIRREL decreases *in vitro* steroid production in bovine granulosa cells and oocyte maturation. So, KIRREL might not explain the better fertility in "fertil+" as compared to "fertil-" animals through its effects on the granulosa cells or oocyte. We have previously shown that progesterone secretion by cultured granulosa cells in basal state or in response to FSH or IGF1 was similar between "fertil+" and "fertil-" heifers submitted to ovarian stimulation (Coyral-Castel *et al.* 2012). Thus, it will be interesting to know the role of KIRREL *in vivo* in ovarian functions in the bovine species.

In conclusion, we have shown that dairy cows selected for one QTL-F-Fert-BTA3, exhibiting difference in fertility, had also a difference in fat mobilization one week after calving and a differential expression of adipose tissue genes located in the QTL as determined by Tiling array. Among these genes differentially expressed by Tiling array, we confirmed the results at both mRNA and protein amount for one gene named KIRREL. This gene highly expressed in granulosa cells could be involved in the interactions between metabolism and reproduction and could explain some infertilities in dairy cows.

609 **Declaration of interest**

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

612

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617

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625

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Figure legends

Figure 1: Plasma NEFA level (A), Energy Balance (EB, B), and Live Body Weight determined on the day of sample collection (1 week postpartum (1 wkpp, n=18 fertil+ and n=18 fertil-) and 5 months of gestation (mpg, n=16 fertil+ and n=14 fertil-) in second lactation)). Results are presented as means ± SEM and were analyzed using the MIXED procedure for linear mixed models in the SAS software. Information about effects of the time after calving (T, 1 wkpp, 5 mpg), haplotype (H, fertil+, fertil-) and Time x haplotype (TxH) interaction on Live Body Weight, EB and plasma NEFA levels are placed above each graph.

Figure 2: Relative expression of *KIRREL* mRNA (A.) and KIRREL protein (B.) in adipose tissue of "fertil+" and "fertil-" dairy cows. A. mRNA of *KIRREL* was analysed by RT-qPCR in adipose tissue, sampled 1 week post partum (wkpp) and at 5 months of pregnancy (mpg). The data were normalized to the geometric mean of PPIA and EEF1A1. Results are presented as means ± SEM and were analyzed using the MIXED procedure for linear mixed models in the SAS software. Information about effects of the time after calving (T, 1 wkpp, 5 mpg), haplotype (H, fertil+, fertil-) and Time x haplotype (TxH) are placed above the graph. B. Protein of KIRREL was studied by western blot in adipose tissue collected the first week post partum. VCL was used as a loading control. Results are represented as mean ± SEM. Bars with different superscripts are significantly different ($P < 0.05$).

Figure 3: Expression of *KIRREL* mRNA in bovine tissues. A. RT-PCR of the mRNA of *KIRREL* in liver (Li), mammary gland (Ma), adipose tissue (AT), kidney (Kid), pituitary (Pit),

skeletal muscle (SM), ovary (Ov) and hypothalamus (Hypo). ACTR3 was used as positive control. B. Relative expression of *KIRREL* mRNA in bovine kidney, hypothalamus, pituitary and mammary gland of "fertil+" and "fertil-" dairy cows. The data were normalized to the geometric mean of PPIA and EEF1A1. C. Protein of KIRREL was studied by western blot in kidney of "fertil+" and "fertil-" dairy cows. VCL was used as a loading control. Results are represented as mean ± SEM. Bars with different superscripts are significantly different ($P < 0.05$).

Figure 4: Expression of *KIRREL* mRNA (A. and B.) and localization (C.) of KIRREL in bovine ovary. A. RT-PCR of *KIRREL* mRNA in theca-interstitial cells from small follicle (SF) and large follicle (LF), Corpus luteum (CL), cortex (Ctx), granulosa cells from SF (GC SF), and granulosa cells from LF (GC LF). *ACTR3* was used as a positive control. B. Relative expression of *KIRREL* mRNA in the different compartments or cell types from bovine ovary. The data were normalized to the geometric mean of PPIA and EEF1A1. Results are represented as mean ± SEM. (n=6). Bars with different superscripts are significantly different ($P < 0.05$). C. Localization of KIRREL by immunohistochemistry. Negative controls included a section incubated with rabbit IgG (n=3). FF, follicular fluid; GC, granulosa cells; TC, theca cells; Oo, oocyte; CC, cumulus cells. D. **Relative expression of *KIRREL* mRNA in bovine granulosa cells from small follicles (< 6 mm) of "fertil+" and "fertil-" dairy cows**. The data were normalized to the geometric mean of PPIA and EEF1A1. Results are represented as mean ± SEM.

Figure 5: Effect of rm KIRREL treatment on basal and FSH- or IGF1-stimulated secretion of progesterone (A,B) and estradiol (C,D) by bovine granulosa cells. Granulosa cells from small bovine follicles were cultured for 48 h in a medium with serum and then in

serum-free medium in the presence or in the absence of various doses of rm KIRREL (A and C) for 48 h, or in presence or absence of 10 ng/ml rm KIRREL, with or without 10^{-8} M FSH, or 10^{-8} M IGF1 (B and D) as described in Materials and Methods. The culture medium was then collected and analyzed for progesterone (A and B) and estradiol (C and D) content by RIA. The results are expressed as the amount of steroid secreted relative to the basal state. The results are means ± SEM of six independent experiments. Bars with different letters are significantly different ($P < 0.05$).

Figure 6: Effect of rm KIRREL on the proliferation of bovine granulosa cells. A, Thymidine incorporation was determined in bovine granulosa cells cultured for 24 h in serum-free medium in the presence of different concentrations of rm KIRREL (1, 5, 10 and 100 ng/ml) as described in Materials and Methods. Results are expressed as thymidine incorporated in cpm (counts per minute). Results are representative of five independent experiments. The results are expressed as means ± SEM. B, Effect of rm KIRREL on the amount of PCNA protein in bovine granulosa cells. Protein extracts from bovine granulosa cells cultured for 48 h in the presence or absence of different concentrations of rm KIRREL (1, 5, 10 and 100 ng/ml) were subjected to SDS-PAGE as described in Materials and Methods. The membranes were incubated with antibodies raised against PCNA. Equal protein loading was verified by reprobing membrane with an anti-tubulin-antibody. A representative blot from three independent experiments is shown. Bars with different letters are significantly different ($P < 0.05$).

Figure 7: Effect of rm KIRREL on phosphorylation of MAPK1/3 (A.) and MAPK14 (B.) in primary bovine granulosa cells. After 18 hours of serum starvation, cells were stimulated during different times (0 to 60 minutes) in enriched McCoy's 5A medium (without FBS)

supplemented with or without rm KIRREL (100 ng/ml). Results are represented as mean ± SEM. The results are representative of 4 independent cultures. Bars with different superscripts are significantly different ($P < 0.05$).

Figure 8: Effects of rm KIRREL treatment on bovine oocyte nuclear maturation. A Bovine oocytes were allowed to mature for 22 h in the presence or absence of various concentrations of rm KIRREL (1, 10 and 100 ng/ml). The percentage of oocytes at the GV stage in the various conditions is shown. Different letters indicate significant differences with $P < 0.05$. The results are presented as mean ± SEM of three independent experiments. Fifty bovine oocytes for each set of conditions in each experiment were used. B. Bovine COCs were cultured for 22 h in maturation medium in the presence or absence of rm KIRREL (1, 10 and 100 ng/ml). COCs were then mechanically separated into oocyte and cumulus cells. Denuded oocytes (50 oocytes per lane) were lysed and subjected to Western blot analysis with antibodies against phospho-MAPK3/1 and MAPK3. Representative blots from three independent experiments are shown. Blots were quantified, and the phosphorylated protein to total protein ratio is shown. Different letters indicate significant differences with $P < 0.05$. The results are presented as means ± SEM. C. Bovine COCs were cultured for 22 h in maturation medium in the presence or absence of various doses of rm KIRREL (1, 10, and 100 ng/ml). The culture medium was then collected, and its progesterone content was analyzed by RIA as described in Materials and Methods. The results are expressed as ng/ml of 50 COC-equivalent cumulus cells. The results are means ± SEM for three independent experiments. Different letters indicate significant differences with $P < 0.05$.

Table 1 Oligonucleotide primer sequences

Primer name	Primer sequence	Accession number
KIRREL		
Sense	5'- GGC AAG GTG GAG TGT TTC AT-3'	XM_003585822
Antisense	5'- GGC AAG GTG GAG TGT TTC AT-3'	
ACTR3		
Sense	5'- ACG GAA CCA CAG TTT ATC ATC -3'	NM_174226
Antisense	5'- GTC CCA GTC TTC AAC TAT ACC -3'	
PPIA		
Sense	5'- GCA TAC AGG TCC TGG CAT CT -3'	NM_178320
Antisense	5'- TGT CCA CAG TCA GCA ATG GT -3'	
RPL19		
Sense	5'- AAT CGC CAA TGC CAA CTC -3'	NM_001040516
Antisense	5'- CCC TTT CGC TTA CCT ATA CC -3'	
EEF1A1		
Sense	5'- ATC CCA GGC TGA CTG TGC TG -3'	NM_174537
Antisense	5'- TGC TAC TGT GTC GGG GTT GT -3'	
COPA		
Sense	5'- ATT GCT TGG GCA CTT AGA CT -3'	NM_001105645
Antisense	5'- GGC ACTC AGA ATC CAA GGG T -3'	
KCNJ10		
Sense	5'- CAG TCG TAG CCG CTC ACA AT -3'	NM_001081601
Antisense	5'- GGT TGA GGC GGA TGT TCT CA -3'	
CDIE		
Sense	5'- GCT GCA GAA GAA TCC CCC TC -3'	NM_001034394
Antisense	5'- TGC TGG CCA AGA CAC TAT CC -3'	
ATP1A2		
Sense	5'- CGA CAT GGA CTG CCC TAT CC -3'	NM_001081524
Antisense	5'- TTG AGG AGA GCT GAC TCG GA -3'	
EF1A1-like		
Sense	5'- TCG TTG TCA TTG GGC ACG TA -3'	XR_083620

Antisense	5’- TCT CTT GTT GAT CCC GCC AC -3’	
PEA15		
Sense	5’- GGA CAT CCC CAG TGA GAA GAG -3’	NM_001075456
Antisense	5’- AGA TCT CAA AGA TGT GCT CGA TA -3’	
CADM3		
Sense	5’- AGC TCC ATG GGG AAT CTA CC -3’	NM_001075946
Antisense	5’- ATG GTT CAC AGA GCA CAC GA -3’	
IFI16		
Sense	5’- AGC CAC CAA ACC TAA GGA CG -3’	XM_863928
Antisense	5’- GTC CTC TGG TCA CTG CTC AC -3’	
SLAMF6		
Sense	5’- GGA CAT TAC CGT GCC CAG AT -3’	NM_001206364
Antisense	5’- CAC GTG GTG TGA TGT GCA AC -3’	

Table 2 Genes differentially expressed between "fertil+" and "fertil-" adipose tissue one week after calving, with adjusted *P*-value<0.05.

Gene Symbol	Biological process	NCBI accession number	Fold change "f+"/ "f-"
Cell development and organization			
VANGL2	Multicellular organismal development	NM_001205875	1.0998
TAGLN2	Muscle organe development	NM_001013599	1.0819
CASQ1	Reticulum endoplasmic organization	NM_001077877	1.0774
IGSF9	Dendrite development	NM_001205532	1.0471
SPTA1	Hemopoiesis	NM_001206588	1.0416
Ion and protein transport			
COPA	**Vesicle-mediated transport**	**NM_001105645**	**1.2486**
KCNJ10	**Potassium ion transport**	**NM_001081601**	**1.1718**
PEX19	Protein targeting to peroxisome	NM_001034540	1.1232
Immune response			
FCER1A	Signal transduction	NM_001100310	1.1468
DARC	Inflammatory response	NM_001015634	1.1439
CD1A	Antigen processing and pre immune response	NM_001102024	1.1261
LOC512286	Antigen processing and pre immune response	XM_003585820	1.1011
CRP	Negative regulation of macrophage	NM_001144097	1.0970
SLAMF1	Lymphocyte activation	NM_174184	1.0469
CD1E	**Antigen processing and presentation**	**NM_001034394**	**0.7694**
Metabolism			
ATP1A2	**ATP biosynthetic process**	**NM_001081524**	**1.2367**

NCSTN	Membrane protein ectodomain proteolysis	NM_001034475	1.1652
PIGM	Glycosylphosphatidylinositol biosynthesis	NM_001015563	1.0579
ATP1A4	ATP biosynthetic process	NM_001144103	1.0557
Olfactory receptors			
LOC519294	Signal transduction	XM_002685904	1.0901
LOC617783	Signal transduction	XM_003581920	0.9664
LOC508806	Signal transduction	XM_002685946	0.9617
OR10T2	Signal transduction	XM_002685925	0.9572
OR10R2	Signal transduction	XM_002685943	0.9506
LOC522554	Signal transduction	XM_002685948	0.9456
OR10K2	Signal transduction	XM_002685942	0.9455
OR6Y1	Signal transduction	XM_002685938	0.9455
OR6P1	Signal transduction	XM_002685937	0.9449
LOC530601	Signal transduction	XM_002685875	0.9442
LOC514540	Signal transduction	XM_002685910	0.9377
Other biological process			
EF1A1-like	**Translation elongation factor activity**	**XR_083620**	**1.6612**
KIRREL	**Excretion**	**XM_003585822**	**1.2695**
PEA15	**Anti-apoptosis**	**NM_001075456**	**1.2301**
CADM3	**Cell adhesion**	**NM_001075946**	**1.2057**
APCS	Response to protein stimulus	NM_001034466	0.9714
Gene ontology unknown in *Bos Taurus*			
IFI16	**Gene ontology unknown in *Bos Taurus***	**XM_863928**	**1.3068**
DCAF8	Gene ontology unknown in *Bos Taurus*	NM_001206419	1.1878
SLAMF6	**Gene ontology unknown in *Bos Taurus***	**NM_001206364**	**1.1857**

VSIG8	Gene ontology unknown in *Bos Taurus*	NM_001205873	1.1660
IGSF8	Gene ontology unknown in *Bos Taurus*	NM_001082439	1.1568
CD84	Gene ontology unknown in *Bos Taurus*	XM_588136	1.1339
SLAMF8	Gene ontology unknown in *Bos Taurus*	NM_001205794	1.0932
CCDC19	Gene ontology unknown in *Bos Taurus*	NM_001038219	1.0345

"f+"/ "f-", "fertil+"/ "fertil-"

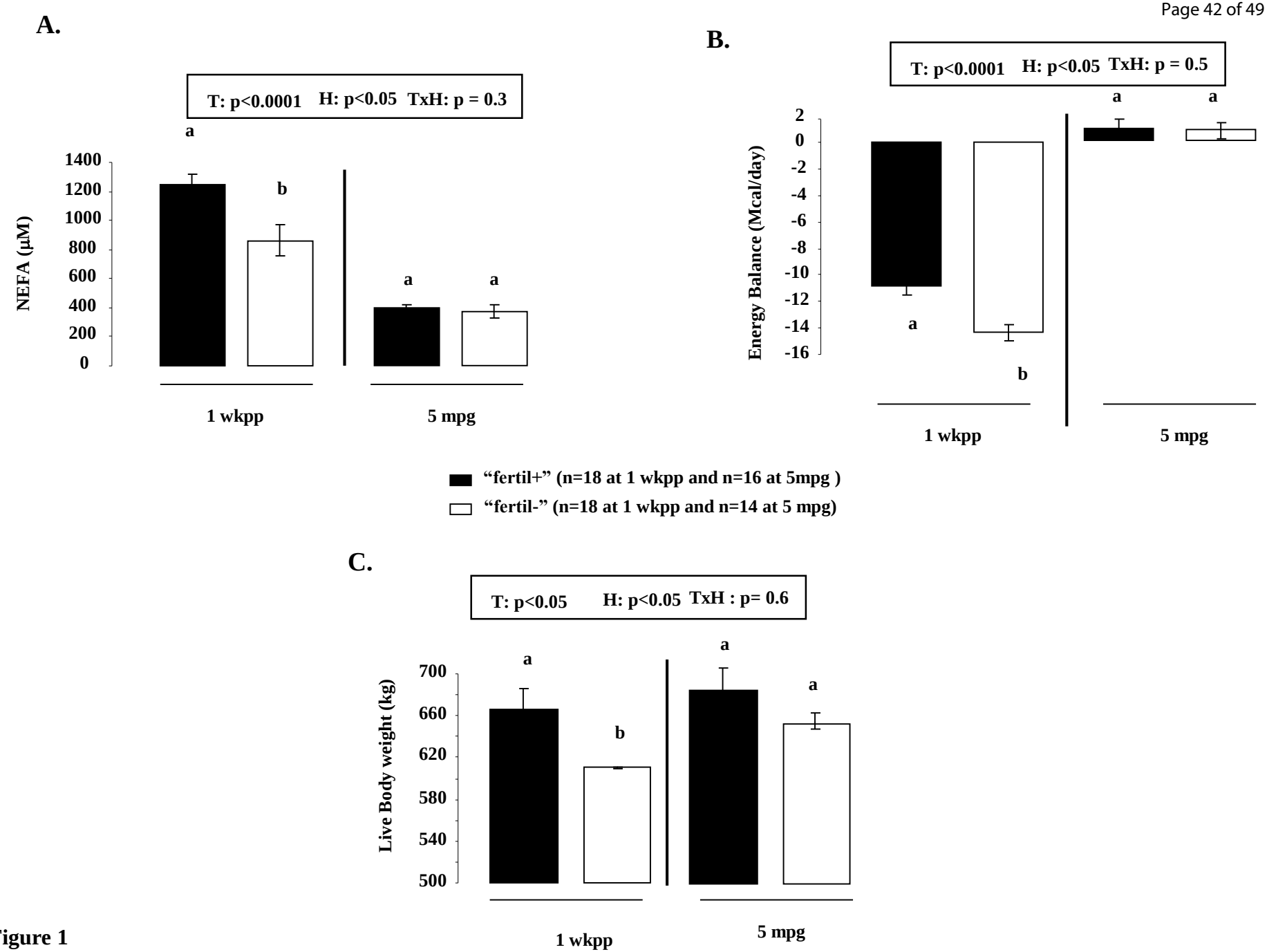
A.
T: p<0.0001 H: p<0.05 TxH: p = 0.3
NEFA (µM)
1400
1200
1000
800
600
400
200
0
a
b
a
a
1 wkpp
5 mpg
B.
T: p<0.0001 H: p<0.05 TxH: p = 0.5
Energy Balance (Mcal/day)
2
0
-2
-4
-6
-8
-10
-12
-14
-16
a
b
a
a
1 wkpp
5 mpg
"fertil+" (n=18 at 1 wkpp and n=16 at 5mpg)
"fertil-" (n=18 at 1 wkpp and n=14 at 5 mpg)
C.
T: p<0.05 H: p<0.05 TxH : p= 0.6
Live Body weight (kg)
700
660
620
580
540
500
a
b
a
a
1 wkpp
5 mpg

Figure 1

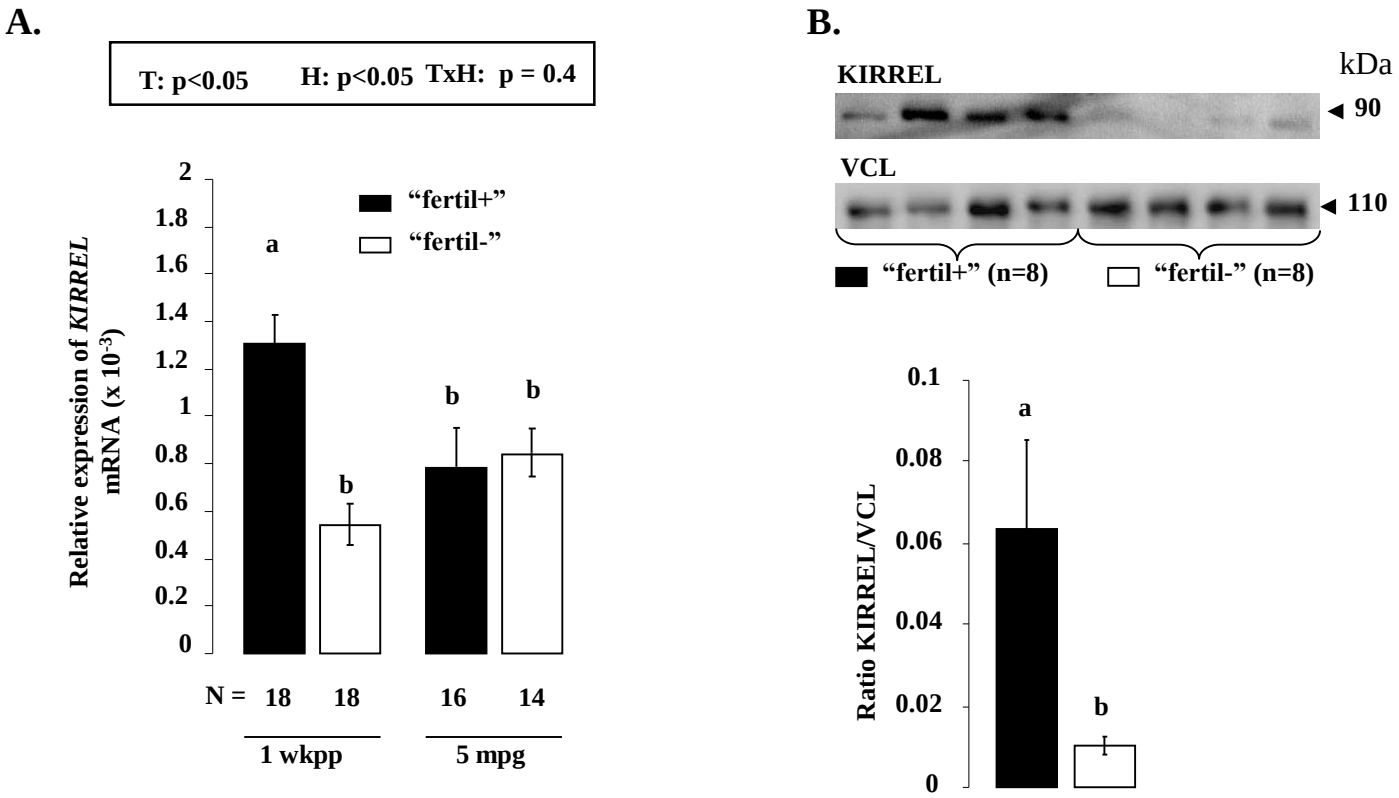

Figure 2

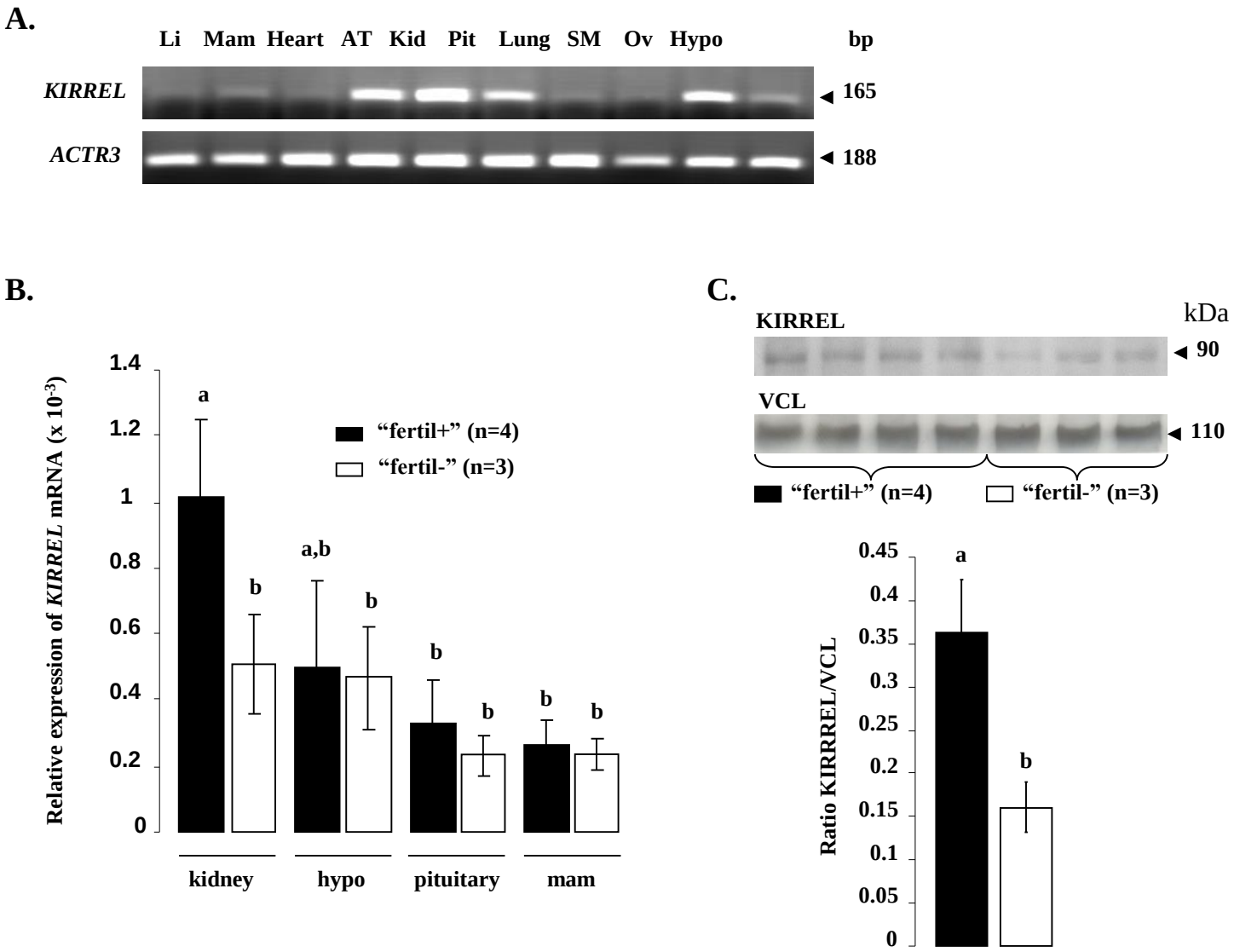
A.
Li Mam Heart AT Kid Pit Lung SM Ov Hypo
bp
KIRREL
165
ACTR3
188
B.
Relative expression of KIRREL mRNA (x 10-3)
1.4
1.2
1
0.8
0.6
0.4
0.2
0
a
b
a,b
b
b
b
b
b
"fertil+" (n=4)
"fertil-" (n=3)
kidney
hypo
pituitary
mam
C.
KIRREL
kDa
90
VCL
110
"fertil+" (n=4)
"fertil-" (n=3)
Ratio KIRREL/VCL
0.45
0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
a
b

Figure 3

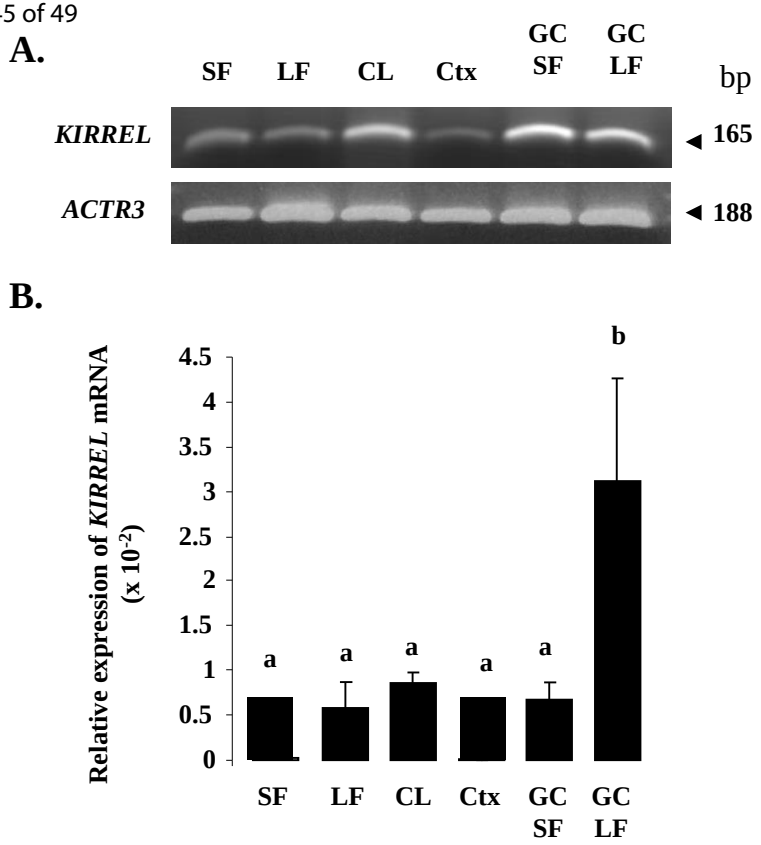
A.
SF
LF
CL
Ctx
GC SF
GC LF
bp
KIRREL
ACTR3
165
188
B.
Relative expression of KIRREL mRNA (x 10-2)
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
a
a
a
a
a
b
SF
LF
CL
Ctx
GC SF
GC LF
C.
Control IgG
KIRREL
x10
GC
TC
FF
x20
Oo
CC
D.
Relative expression of KIRREL mRNA (x 10-3)
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
a
b
"fertil+" (n=3)
"fertil-" (n=3)

Figure 4

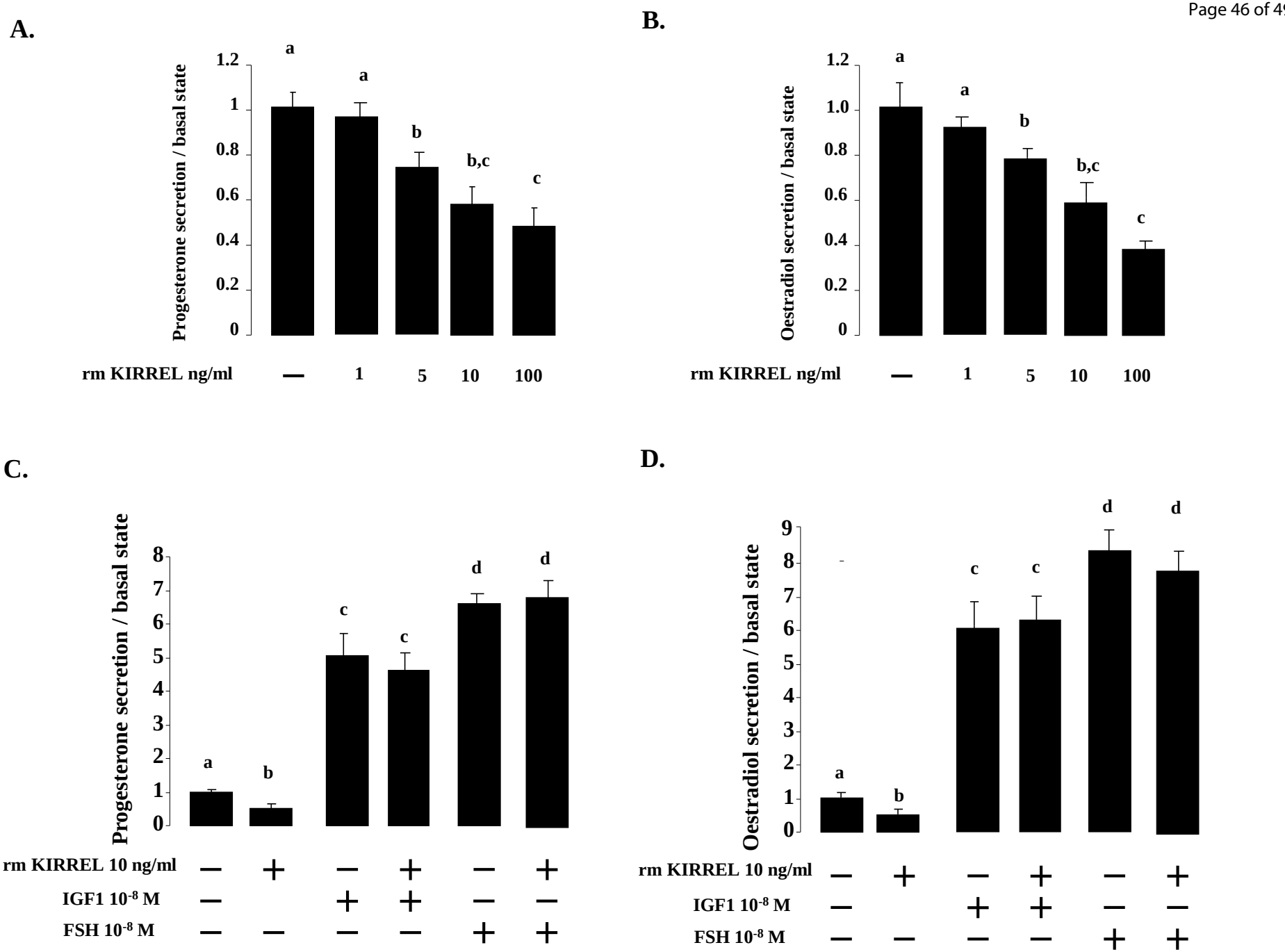
A.
Progesterone secretion / basal state
1.2
1
0.8
0.6
0.4
0.2
0
a
a
b
b,c
c
rm KIRREL ng/ml
—
1
5
10
100
B.
Oestradiol secretion / basal state
1.2
1.0
0.8
0.6
0.4
0.2
0
a
a
b
b,c
c
rm KIRREL ng/ml
—
1
5
10
100
C.
Progesterone secretion / basal state
8
7
6
5
4
3
2
1
0
a
b
c
c
d
d
rm KIRREL 10 ng/ml
IGF1 10-8 M
FSH 10-8 M
D.
Oestradiol secretion / basal state
9
8
7
6
5
4
3
2
1
0
a
b
c
c
d
d
rm KIRREL 10 ng/ml
IGF1 10-8 M
FSH 10-8 M

Figure 5

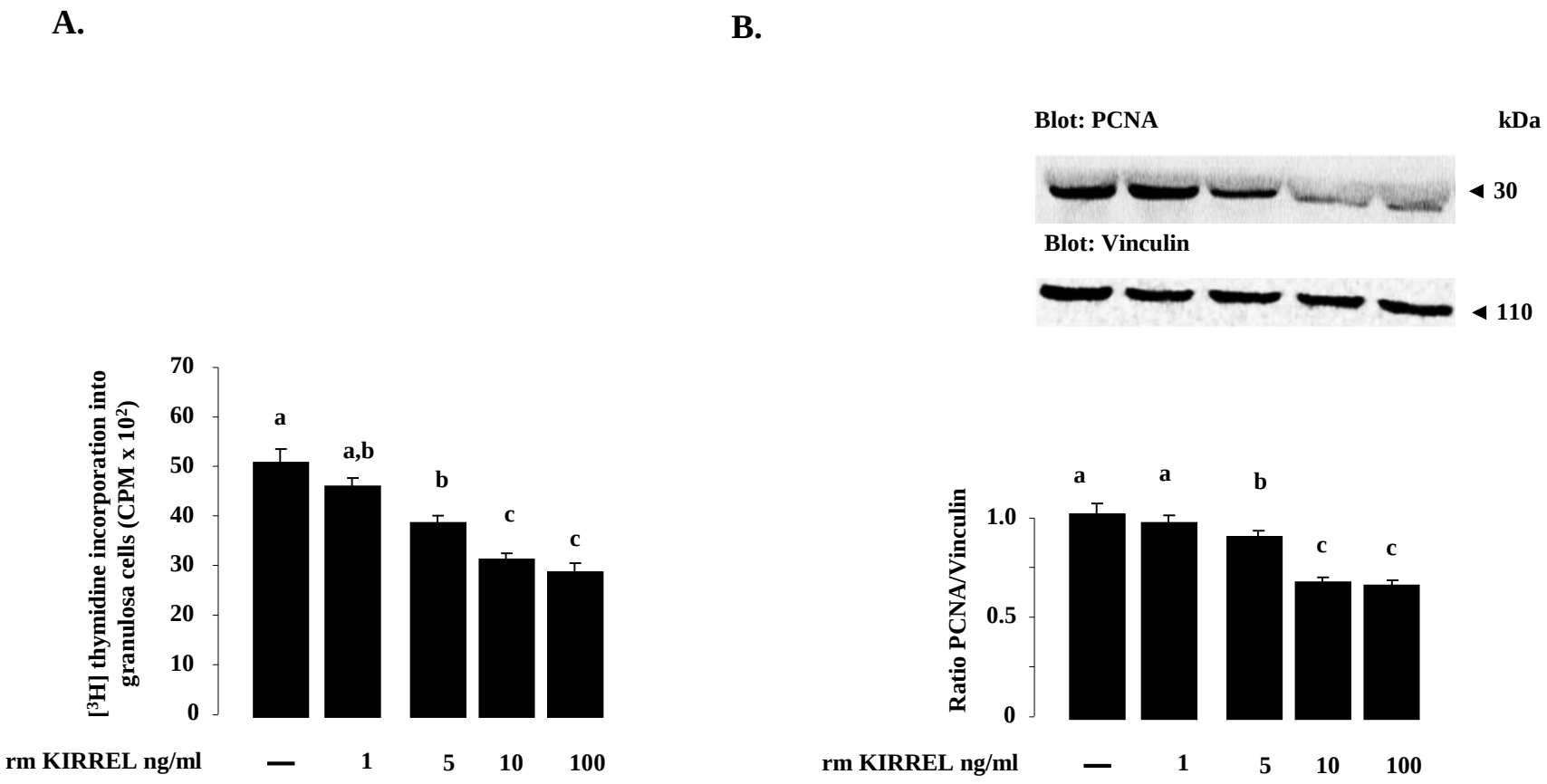
A.
B.
Blot: PCNA
kDa
30
Blot: Vinculin
110
[3H] thymidine incorporation into granulosa cells (CPM x 10^2)
70
60
50
40
30
20
10
0
a
a,b
b
c
c
rm KIRREL ng/ml
—
1
5
10
100
Ratio PCNA/Vinculin
1.0
0.5
0
a
a
b
c
c
rm KIRREL ng/ml
—
1
5
10
100

Figure 6

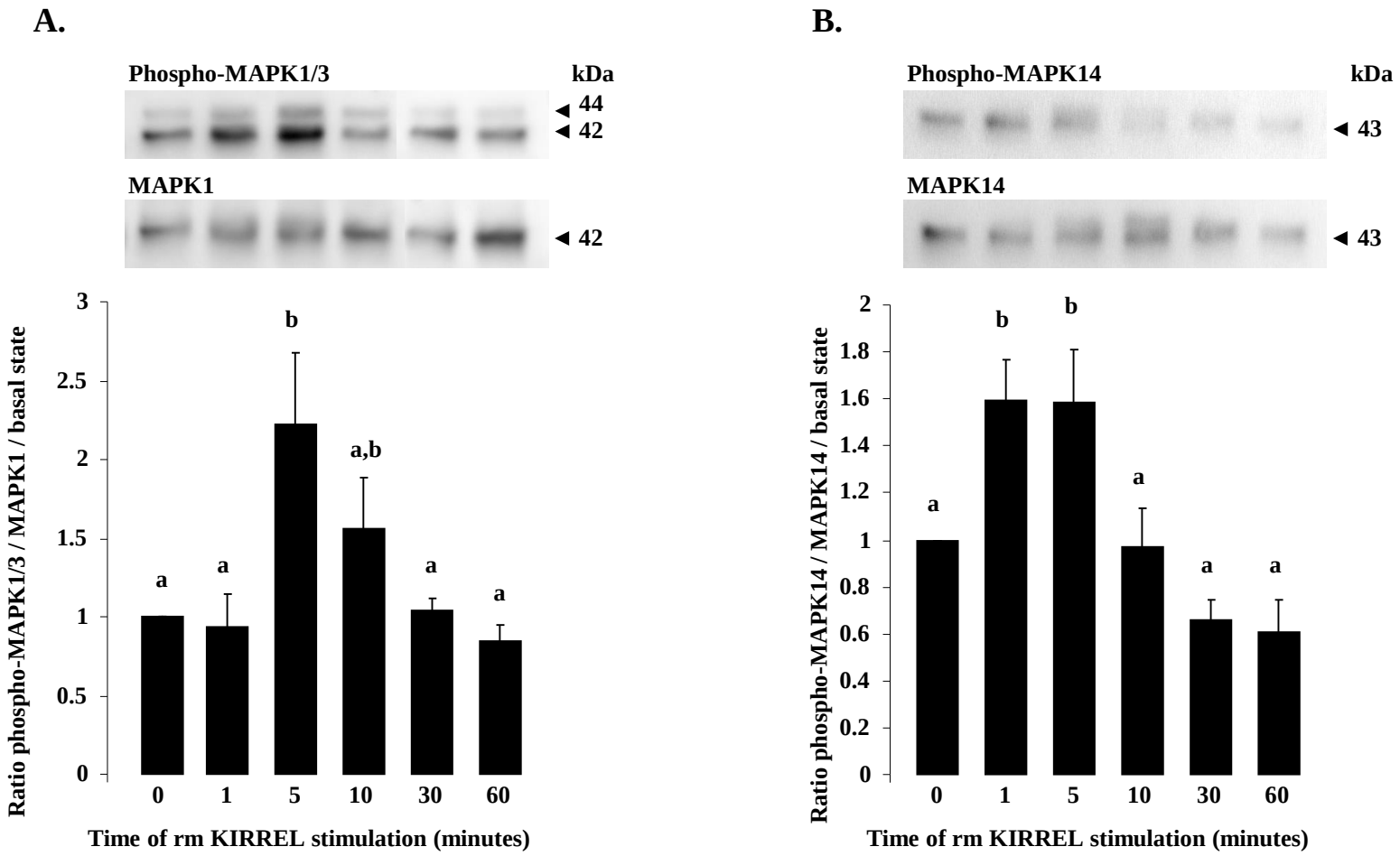
A.
Phospho-MAPK1/3
kDa
44
42
MAPK1
42
Ratio phospho-MAPK1/3 / MAPK1 / basal state
3
2.5
2
1.5
1
0.5
0
a
a
b
a,b
a
a
0
1
5
10
30
60
Time of rm KIRREL stimulation (minutes)
B.
Phospho-MAPK14
kDa
43
MAPK14
43
Ratio phospho-MAPK14 / MAPK14 / basal state
2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
a
b
b
a
a
a
0
1
5
10
30
60
Time of rm KIRREL stimulation (minutes)

Figure 7

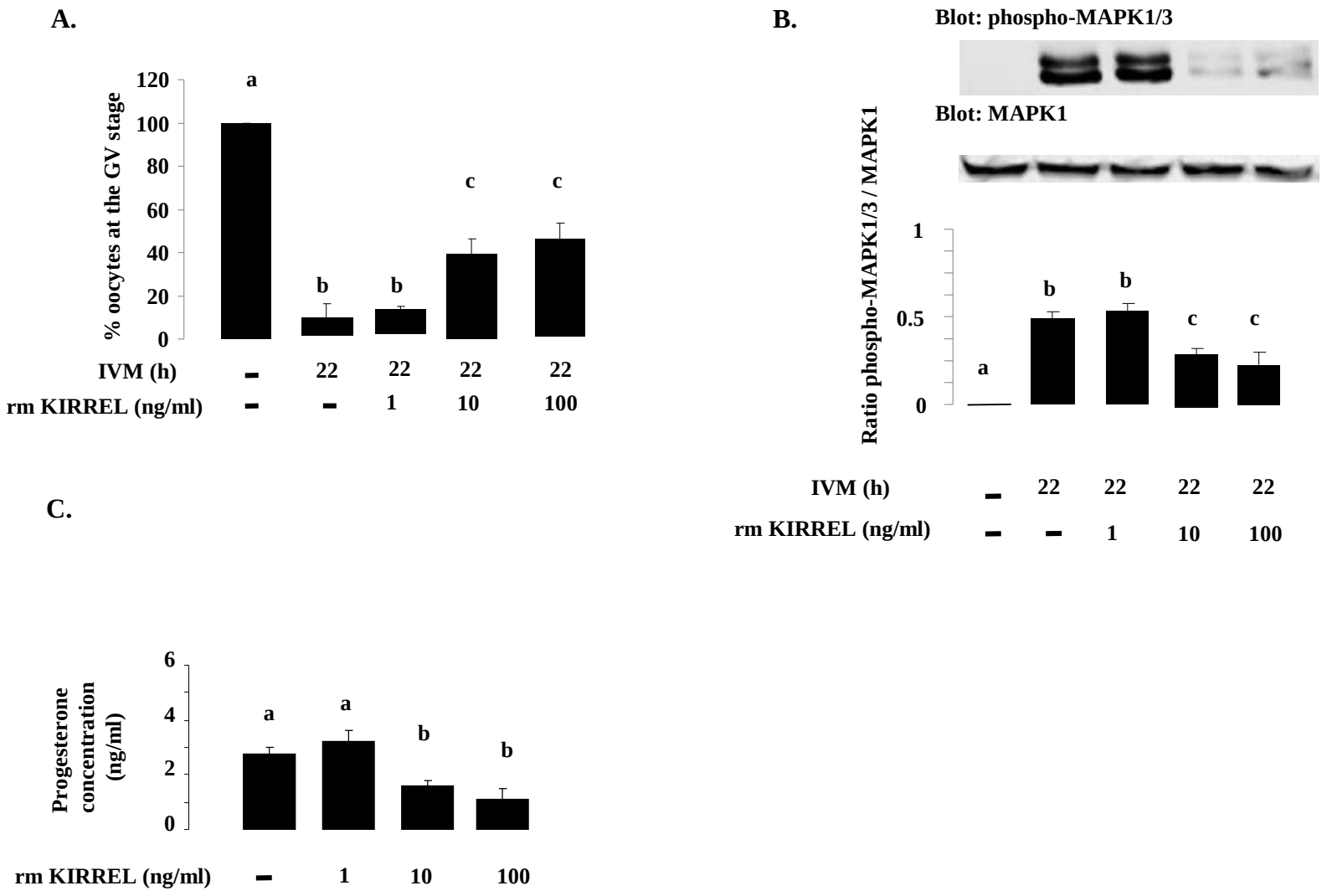

Figure 8

Coyral-Castel, S., Ramé, C., Cognié, J., Lecardonnel, J., Marthey, S., Esquerré, D., Hennequet Antier, C., Elis, S., Fritz, S., Boussaha, M., Jaffrézic, F., Dupont, J. (2018). KIRREL is differentially expressed in adipose tissue from “fertil+” and “fertil-” cows: in vitro role in ovary? . Reproduction, 155 (2), 181-196. . DOI : 10.1530/REP-17-0649