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

3

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24

25 Short title: KIRREL in bovine adipose tissue and ovary

26 **Abstract**

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

46

47

48 **Introduction**

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

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

73 33% of the milk produced in the first month of lactation (Bauman & Bruce Currie 1980).
74 Mobilization of fat results in release of non-esterified fatty acids (NEFA) in blood, which
75 were reviewed as indicators of energy status of ruminants (Bowden 1971). It is now well
76 established that negative energy balance impact reproductive traits at various levels of the
77 hypothalamo-pituitary-gonadal axis (Beam & Butler 1999, Roche *et al.* 2000, Leroy *et al.*
78 2008, Roche *et al.* 2009). Adipose tissue is not only an energy storage organ but it is also able
79 to secrete a number of hormone-like compounds that regulates adipocyte development and
80 metabolic function (Ouchi *et al.*) but also fertility (Campos *et al.* 2008, Tersigni *et al.* 2011).

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

89

90 **Materials and methods**

91

92 ***Ethics***

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

97

98 ***Animals***

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

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

151

152 ***Tiling Array data analysis***

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

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

$$160 \quad Y_{ikrc} = \mu + \alpha_c + \beta_j + (\alpha\beta)_{jc} + \gamma_{jk} + e_{ikrc}$$

161 where α_c corresponds to a condition effect with two levels ($c=1,2$ for “fertil+” and “fertil-”),

162 β_j corresponds to an exon effect j within gene i ($j = 1, \dots, n_i$), and $(\alpha\beta)_{jc}$ is the interaction

163 term between exon and condition. Parameter γ_{jk} corresponds to the probe effect k within

164 exon j ($k = 1, \dots, n_j$). The probe effect is assumed to be a random effect such
 165 that: $Y_{jk} \sim \mathcal{N}(0, \tau_j^2)$, with gene-by-gene variances τ_j^2 . Residuals ϵ_{jkr} are also assumed
 166 independent and normally distributed such that: $\epsilon_{jkr} \sim \mathcal{N}(0, \sigma_j^2)$, with gene-by-gene
 167 variances σ_j^2 . Index r represents the biological replicates ($r = 1, \dots, R$).

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

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

181

182 **Total RNA extraction**

183 Subcutaneous adipose tissue was sampled at the dewlap of 36 second lactation cows
 184 (18 "fertil+" and 18 "fertil-") one week after parturition and at 5 months of pregnancy (16
 185 "fertil+" and 14 "fertil-"), frozen in liquid nitrogen and stored at -80°C until use. Total RNA
 186 was extracted on ice from 250 mg of tissue with an ultraturax homogenizer using 8 ml of
 187 QIAzol lysis reagent (Qiagen, Courtaboeuf, France). Chloroform (0.2 ml) was added to each
 188 sample. Tubes were waved for 15 seconds and left at room temperature for 5 minutes before

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

199

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

209

210 ***cDNA synthesis and labeling, array hybridization, washing and scanning***

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

214 NimbleGen Arrays User's Guide: Gene Expression Analysis v3.2. cDNAs were synthesized
215 using an Invitrogen Superscript Double-Stranded cDNA Synthesis Kit (Invitrogen™ by Life
216 Technologies™). They were then purified using a MinElute Reaction Cleanup Kit (Qiagen).
217 Samples were labelled with Cy3 with a NimbleGen One-Color DNA Labeling Kit (Roche
218 NimbleGen, Inc.). Hybridization solution was prepared from the NimbleGen Hybridization
219 Kit (Roche NimbleGen, Inc.) and Cy3-labeled samples were hybridized on the 385K array at
220 42°C for 18 hours. Finally, arrays were washed with solutions of the NimbleGen Wash Buffer
221 Kit (Roche NimbleGen, Inc.). Arrays were scanned with a GenePix 4000B Scanner at 532
222 nm. Data were extracted with the Roche NimbleScan software (Roche NimbleGen, Inc.).

223

224 ***Reverse Transcription and Polymerase Chain Reaction***

225 Reverse transcription (RT) of total RNA (1 µg) was performed for 1 hour at 37°C in a
226 20 µl mixture as previously described (Coyral-Castel *et al.* 2010). Single-strand cDNAs of
227 *KIRREL* and *ACTR3* were amplified with specific primers (Invitrogen™ by Life
228 technologies™, Table 2). Polymerase chain reaction (PCR) was carried out in a previously
229 described mixture (Coyral-Castel *et al.* 2010) for 30 (*ACTR3*) or 40 (*KIRREL*) PCR cycles (1
230 minute at 94°C, 1 minute at 58°C, 1 minute at 72°C), with a final extension step of 7 minutes
231 at 72°C. PCR products were visualized in a 1.5% (w:v) agarose gel stained with ethidium
232 bromide. *ACTR3* was used as positive control. Finally, DNA was extracted from the agarose
233 gel using the EZNA microelute Gel Extraction kit (VWR, Fontenay-sous-Bois, France)
234 according to the manufacturer's procedure. DNA was sequenced by Beckman Coulter
235 Genomics (Grenoble, France). RT and PCR consumables were purchased from Promega
236 (Charbonnières-les-Bains, France).

237

238 ***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 : $\text{expression} = E^{-Cq}$. 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

264 mg/ml, PAA laboratories), Hepes (20 mM pH = 7.6), bovine apo-transferrin (5 µg/ml, Sigma-
265 Aldrich, Saint-Quentin-Fallavier, France) and androstenedione (0.1 µmol/l, Sigma-Aldrich,
266 Saint-Quentin-Fallavier, France). Cells were centrifuged at 200 g for 5 minutes, washed with
267 fresh enriched McCoy's 5A and the pellet was resuspended in enriched McCoy's 5A
268 supplemented with 10% (v:v) fetal bovine serum (FBS, PAA laboratories, Les Mureaux,
269 France) and amphotericin B (5 µg/ml, PAA laboratories). Approximately 2×10^5 live cells
270 were seeded per well of a 24-well culture plate. After 24 hours of culture, cells were serum
271 starved for 18 hours before treatment with a recombinant mouse (rm) KIRREL (R&D
272 Systems[®], Lille, France), human recombinant IGF1 (Sigma) and/or ovine recombinant FSH
273 (NIDDK, NIH Bethesda, USA). Cultures were performed at 37°C in a humidified air
274 containing 5% CO₂.

275

276 ***Cell viability***

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

279

280 ***Thymidine incorporation into granulosa cells***

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

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^{-8}
293 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 \pm S.E.M of four independent
300 cultures, in which each condition was analyzed in quadruplicate.

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 μ g) 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 Quentin 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

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

323

324 ***Immunohistochemistry***

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

329

330 ***Bovine Oocyte Collection and In Vitro Maturation***

331 Bovine ovaries were collected from a slaughterhouse in sterile NaCl solution and
332 maintained at 37°C until aspiration. The cumulus-oocyte complexes (COCs) were aspirated
333 from follicles 3–8 mm in diameter using an 18-gauge needle connected to a sterile test tube
334 and to a vacuum line (100mmHg) as previously described (Reverchon *et al.* 2014). COCs
335 were then selected under a dissecting microscope. Expanded or nonintact COCs were
336 eliminated: only intact COCs were washed in TCM Hepes 199 (Sigma) supplemented with
337 0.4% BSA and gentamycine (2.5ml/L) under mineral oil (Sigma). The COCs were cultured in
338 TCM 199 (Sigma) with 4 mg/ml BSA supplemented or not with different concentrations of

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

343

344 ***Statistical analysis***

345

346 All statistical analyses were conducted using the SAS software (SAS Institute INC,
347 2009). The MIXED procedure for linear mixed models was used to determine the changes of :
348 i) the live body weight; ii) the energy balance ; iii) the plasma NEFA concentrations ; iv)
349 adipose tissue KIRREL expression. The initial model included time after calving (1 wkpp, 5
350 mpg), haplotype (fertil +, fertil-) and time after calving×haplotype interaction.

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

360

361

362 **Results**

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

364 One week after calving, “fertil+” cows (n=18) had significant lower concentrations of
 365 plasma NEFA than “fertil-” cows (n= 18; $860.6 \pm 105.4 \mu\text{mol/l}$ vs $1247.0 \pm 72.7 \mu\text{mol/l}$,
 366 respectively, $P<0.05$, Fig. 1A.) and a greater energy balance (-10.8 ± 0.7 Mcal/day vs $-14.4 \pm$
 367 0.6 Mcal/day, respectively, $P<0.05$, Fig.1B) and live body weight (666.1 ± 19.6 kg vs $610.2\pm$
 368 0.7 kg, respectively, $P<0.05$, Fig.1C), suggesting a greater adipose tissue mobilization in
 369 “fertil-” than in “fertil+” cows. At 5 months of gestation (mpg) during reconstitution of body
 370 reserves, plasma NEFA, energy balance and live body weight were not significant between
 371 “fertil+” and “fertil-” animals (n=16 “fertil+” and n=14 “fertil-”) (Fig. 1).

372

373 ***Tiling array***

374 To better investigate this difference in mobilization, total adipose tissue RNA from
 375 nine “fertil+” and nine “fertil-” was extracted, reverse transcribed, labelled and hybridized on
 376 a 385K array containing the sequence of the QTL-F-Fert-BTA3. We observed that 43 known
 377 genes were differentially expressed in adipose tissue of “fertil+” and “fertil-” cows ($P<0.05$,
 378 Table 2). Thirty-one genes were over-expressed in “fertil+” adipose tissue as compared to
 379 “fertil-” cows, with fold change (“fertil+”/“fertil-”) ranging from 1.0345 to 1.6612 (Table 2).
 380 Twelve were under-expressed in “fertil+” adipose tissue, with fold change varying from
 381 0.7694 to 0.9714 (Table 2). Genes under-expressed in “fertil+” adipose tissue were mainly
 382 olfactory receptors (10 on 12 genes, Table 2). We then selected about 10 genes represented in
 383 bold in the Table 2 that had the highest fold change to perform expression analysis by
 384 quantitative PCR using specific primers (Table 2). Interestingly, we confirmed the results of
 385 Tiling array by qPCR for only one gene, named *KIRREL* (kin of IRRE like) also known as
 386 *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

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

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

458

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

460 In the literature, KIRREL (NEPH1) has been described to modulate intracellular
461 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.

537 KIRREL is a member of the nephrin-like protein family, which 444 also includes
538 KIRREL2 and KIRREL3. It is a molecule identified in mice by a retrovirus-mediated
539 mutagenesis screen (Donoviel *et al.* 2001). KIRREL contains five extracellular
540 immunoglobulin-like domains and is structurally related to nephrin. In human and rodents,
541 *KIRREL* is abundantly expressed in the kidney but also found in some reproductive organs
542 such as brain, placenta and testis (Donoviel *et al.* 2001, Beall *et al.* 2005). However, the
543 presence of KIRREL in the ovary or pituitary had never been investigated. In bovine tissues,
544 we have detected *KIRREL* mRNA expression in kidney, adipose tissue, ovary and pituitary
545 and less abundantly in hypothalamus and mammary gland. Interestingly, we have shown a
546 greater mRNA and protein expression of KIRREL in “fertil+” kidney compared to “fertil-”
547 cows. In mice, the disruption of the *NEPH1* gene results in effacement of glomerular
548 podocytes, heavy proteinuria, and early postnatal death (Donoviel *et al.* 2001). Thus,
549 KIRREL plays a pivotal role for the development and maintenance of the filtration barrier in
550 the kidney (Donoviel *et al.* 2001, Neumann-Haefelin *et al.* 2010). In a previous work, we
551 have observed that the plasma concentrations of urea were significantly greater in “fertil+”
552 compared to “fertil-” cows (unpublished data) suggesting that the greater KIRREL expression
553 in the kidney of “fertil+” could explain a better renal glomerular filtration in this haplotype. In
554 bovine ovary, we detected *KIRREL* in different cells including granulosa and corpus luteum
555 cells. As in adipose tissue one week after calving and in kidney, we have shown a greater
556 expression of *KIRREL* in granulosa cells from “fertil+” compared to “fertil-” cows. The
557 function of KIRREL in extra renal organ systems is almost unknown. Recent studies revealed
558 that mammalian KIRREL proteins have similar cell-cell recognition functions. Furthermore,
559 KIRREL has been shown to interact with Nephrin and Tight junction protein zona occludens-
560 1 (ZO-1) (Huber *et al.* 2003, Liu *et al.* 2003). ZO-1 is a protein located on a cytoplasmic
561 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 Nephhrin 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

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

598

599

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

607

608

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

626

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- 770
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- 772

1 **Figure legends**

2

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

10

11

12 **Figure 2: Relative expression of *KIRREL* mRNA (A.) and *KIRREL* protein (B.) in**
13 **adipose tissue of “fertil+” and “fertil-” dairy cows.** A. mRNA of *KIRREL* was analysed by
14 RT-qPCR in adipose tissue, sampled 1 week post partum (wkpp) and at 5 months of
15 pregnancy (mpg). The data were normalized to the geometric mean of PPIA and EEF1A1.
16 Results are presented as means \pm SEM and were analyzed using the MIXED procedure for
17 linear mixed models in the SAS software. Information about effects of the time after calving
18 (T, 1 wkpp, 5 mpg), haplotype (H, fertil+, fertil-) and Time x haplotype (TxH) are placed
19 above the graph. B. Protein of *KIRREL* was studied by western blot in adipose tissue
20 collected the first week post partum. VCL was used as a loading control. Results are
21 represented as mean \pm SEM. Bars with different superscripts are significantly different ($P <$
22 0.05).

23

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

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26 skeletal muscle (SM), ovary (Ov) and hypothalamus (Hypo). *ACTR3* was used as positive
 27 control. B. Relative expression of *KIRREL* mRNA in bovine kidney, hypothalamus, pituitary
 28 and mammary gland of “fertil+” and “fertil-” dairy cows. The data were normalized to the
 29 geometric mean of PPIA and *EEF1A1*. C. Protein of *KIRREL* was studied by western blot in
 30 kidney of “fertil+” and “fertil-” dairy cows. VCL was used as a loading control. Results are
 31 represented as mean \pm SEM. Bars with different superscripts are significantly different ($P <$
 32 0.05).

33

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

47

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

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

58

59 **Figure 6: Effect of rm KIRREL on the proliferation of bovine granulosa cells.** A,
60 Thymidine incorporation was determined in bovine granulosa cells cultured for 24 h in
61 serum-free medium in the presence of different concentrations of rm KIRREL (1, 5, 10 and
62 100 ng/ml) as described in Materials and Methods. Results are expressed as thymidine
63 incorporated in cpm (counts per minute). Results are representative of five independent
64 experiments. The results are expressed as means \pm SEM. B, Effect of rm KIRREL on the
65 amount of PCNA protein in bovine granulosa cells. Protein extracts from bovine granulosa
66 cells cultured for 48 h in the presence or absence of different concentrations of rm KIRREL
67 (1, 5, 10 and 100 ng/ml) were subjected to SDS-PAGE as described in Materials and
68 Methods. The membranes were incubated with antibodies raised against PCNA. Equal protein
69 loading was verified by reprobating membrane with an anti-tubulin-antibody. A representative
70 blot from three independent experiments is shown. Bars with different letters are significantly
71 different ($P < 0.05$).

72

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

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

79

80 **Figure 8: Effects of rm KIRREL treatment on bovine oocyte nuclear maturation.** A

81 Bovine oocytes were allowed to mature for 22 h in the presence or absence of various
82 concentrations of rm KIRREL (1, 10 and 100 ng/ml). The percentage of oocytes at the GV
83 stage in the various conditions is shown. Different letters indicate significant differences with
84 $P < 0.05$. The results are presented as mean \pm SEM of three independent experiments. Fifty

85 bovine oocytes for each set of conditions in each experiment were used. B. Bovine COCs
86 were cultured for 22 h in maturation medium in the presence or absence of rm KIRREL (1, 10
87 and 100 ng/ml). COCs were then mechanically separated into oocyte and cumulus cells.

88 Denuded oocytes (50 oocytes per lane) were lysed and subjected to Western blot analysis with
89 antibodies against phospho-MAPK3/1 and MAPK3. Representative blots from three
90 independent experiments are shown. Blots were quantified, and the phosphorylated protein to
91 total protein ratio is shown. Different letters indicate significant differences with $P < 0.05$.

92 The results are presented as means \pm SEM. C. Bovine COCs were cultured for 22 h in
93 maturation medium in the presence or absence of various doses of rm KIRREL (1, 10, and
94 100 ng/ml). The culture medium was then collected, and its progesterone content was
95 analyzed by RIA as described in Materials and Methods. The results are expressed as ng/ml of
96 50 COC-equivalent cumulus cells. The results are means \pm SEM for three independent
97 experiments. Different letters indicate significant differences with $P < 0.05$.

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Table 1 Oligonucleotide primer sequences

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

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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'

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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			
<i>VANGL2</i>	Multicellular organismal development	NM_001205875	1.0998
<i>TAGLN2</i>	Muscle organe development	NM_001013599	1.0819
<i>CASQ1</i>	Reticulum endoplasmic organization	NM_001077877	1.0774
<i>IGSF9</i>	Dendrite development	NM_001205532	1.0471
<i>SPTA1</i>	Hemopoiesis	NM_001206588	1.0416
Ion and protein transport			
<i>COPA</i>	Vesicle-mediated transport	NM_001105645	1.2486
<i>KCNJ10</i>	Potassium ion transport	NM_001081601	1.1718
<i>PEX19</i>	Protein targeting to peroxisome	NM_001034540	1.1232
Immune response			
<i>FCERIA</i>	Signal transduction	NM_001100310	1.1468
<i>DARC</i>	Inflammatory response	NM_001015634	1.1439
<i>CD1A</i>	Antigen processing and pre immune response	NM_001102024	1.1261
<i>LOC512286</i>	Antigen processing and pre immune response	XM_003585820	1.1011
<i>CRP</i>	Negative regulation of macrophage	NM_001144097	1.0970
<i>SLAMF1</i>	Lymphocyte activation	NM_174184	1.0469
<i>CD1E</i>	Antigen processing and presentation	NM_001034394	0.7694
Metabolism			
<i>ATPIA2</i>	ATP biosynthetic process	NM_001081524	1.2367

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<i>NCSTN</i>	Membrane protein ectodomain proteolysis	NM_001034475	1.1652
<i>PIGM</i>	Glycosylphosphatidylinositol biosynthesis	NM_001015563	1.0579
<i>ATPIA4</i>	ATP biosynthetic process	NM_001144103	1.0557
Olfactory receptors			
<i>LOC519294</i>	Signal transduction	XM_002685904	1.0901
<i>LOC617783</i>	Signal transduction	XM_003581920	0.9664
<i>LOC508806</i>	Signal transduction	XM_002685946	0.9617
<i>OR10T2</i>	Signal transduction	XM_002685925	0.9572
<i>OR10R2</i>	Signal transduction	XM_002685943	0.9506
<i>LOC522554</i>	Signal transduction	XM_002685948	0.9456
<i>OR10K2</i>	Signal transduction	XM_002685942	0.9455
<i>OR6Y1</i>	Signal transduction	XM_002685938	0.9455
<i>OR6P1</i>	Signal transduction	XM_002685937	0.9449
<i>LOC530601</i>	Signal transduction	XM_002685875	0.9442
<i>LOC514540</i>	Signal transduction	XM_002685910	0.9377
Other biological process			
<i>EF1A1-like</i>	Translation elongation factor activity	XR_083620	1.6612
<i>KIRREL</i>	Excretion	XM_003585822	1.2695
<i>PEA15</i>	Anti-apoptosis	NM_001075456	1.2301
<i>CADM3</i>	Cell adhesion	NM_001075946	1.2057
<i>APCS</i>	Response to protein stimulus	NM_001034466	0.9714
Gene ontology unknown in <i>Bos Taurus</i>			
<i>IFI16</i>	Gene ontology unknown in <i>Bos Taurus</i>	XM_863928	1.3068
<i>DCAF8</i>	Gene ontology unknown in <i>Bos Taurus</i>	NM_001206419	1.1878
<i>SLAMF6</i>	Gene ontology unknown in <i>Bos Taurus</i>	NM_001206364	1.1857

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

“f+”/ “f-”, “fertil+”/ “fertil-”

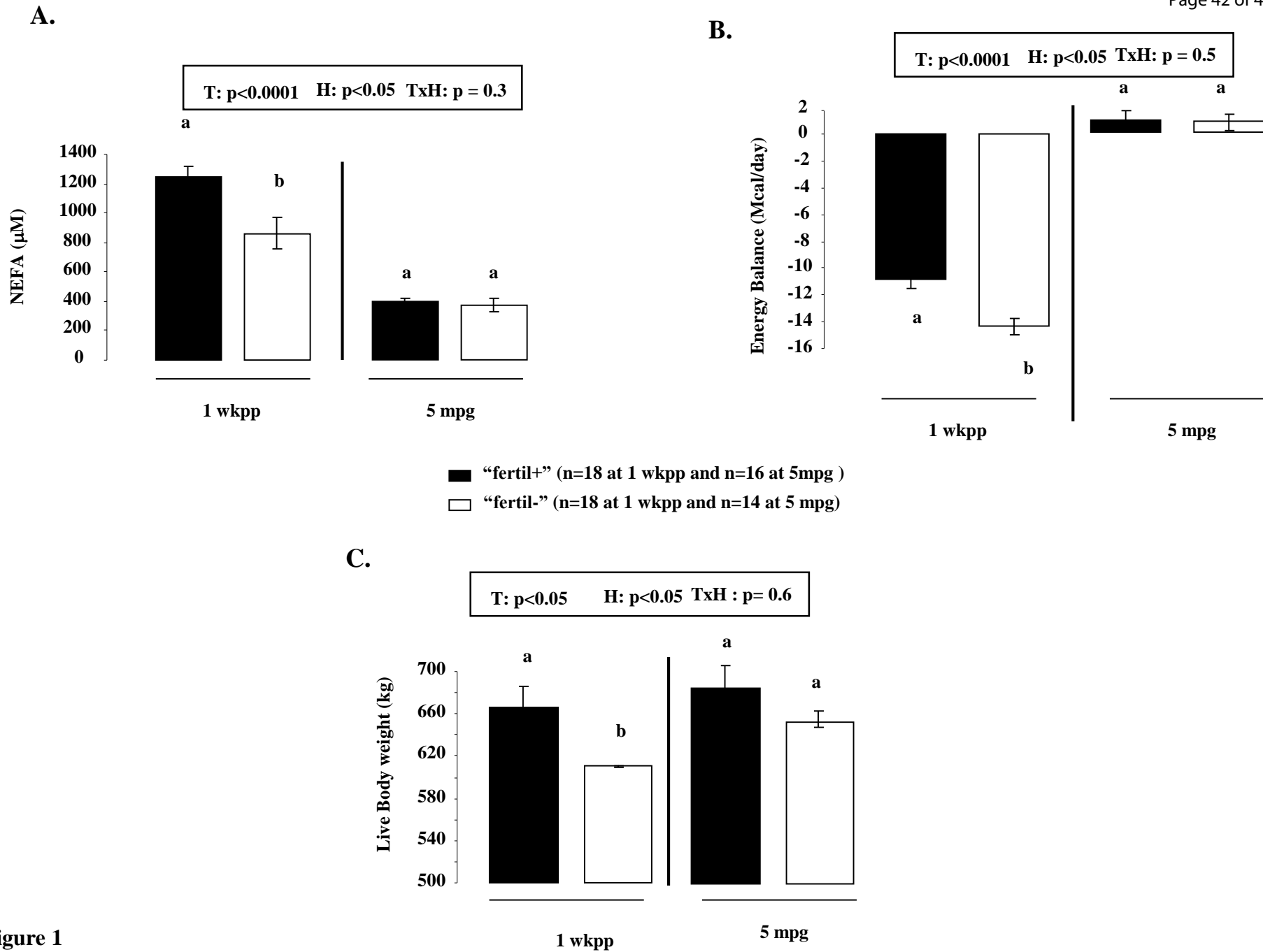
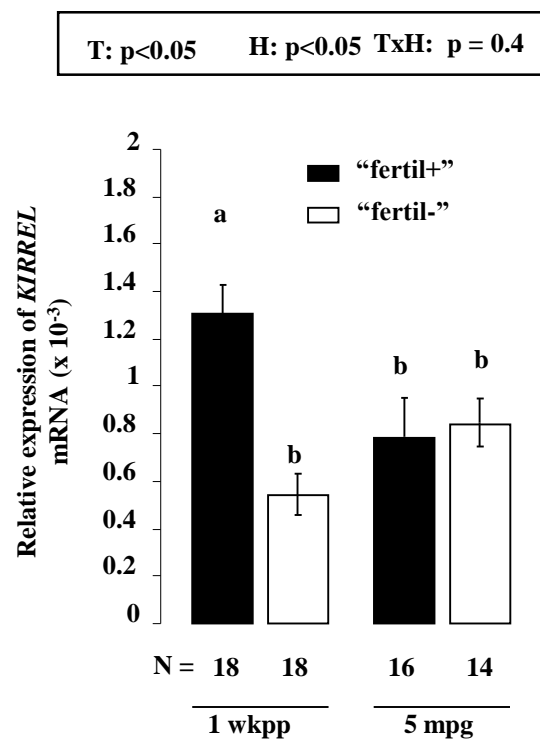


Figure 1

A.



B.

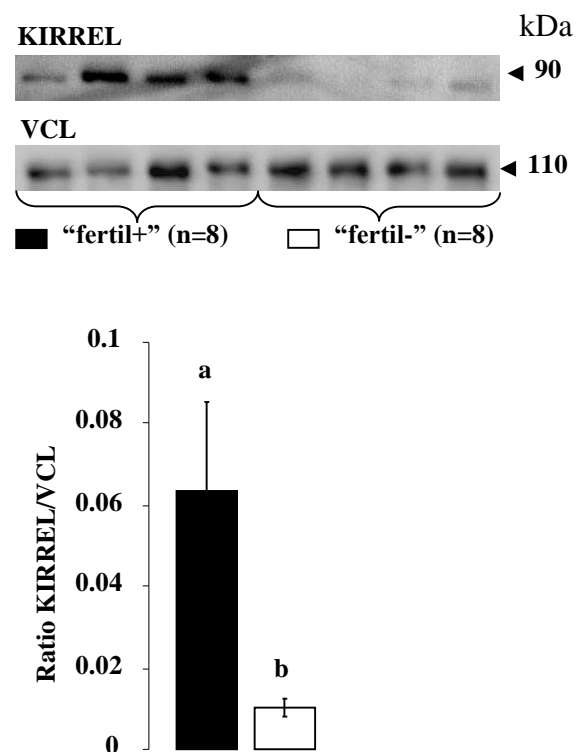


Figure 2

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

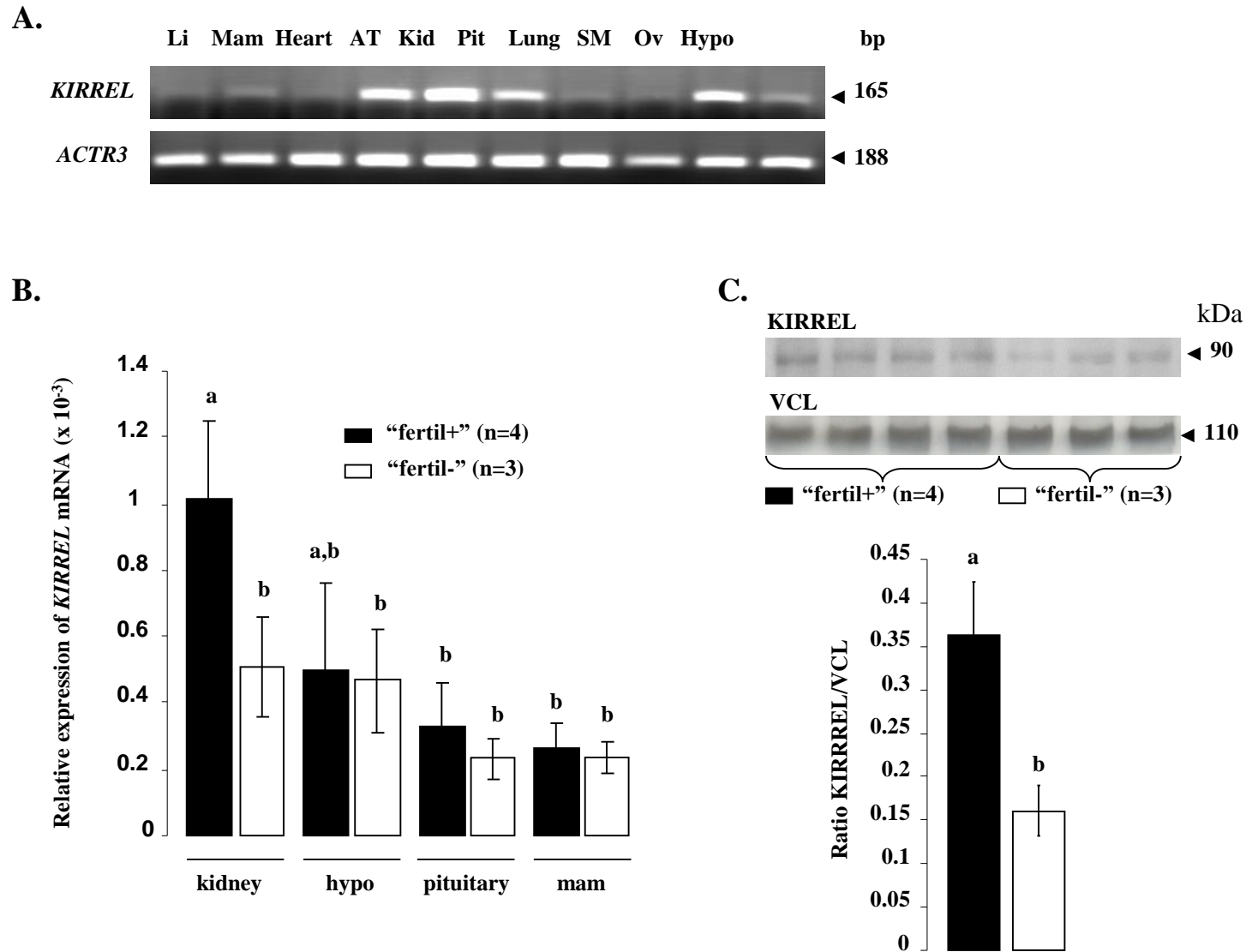


Figure 3

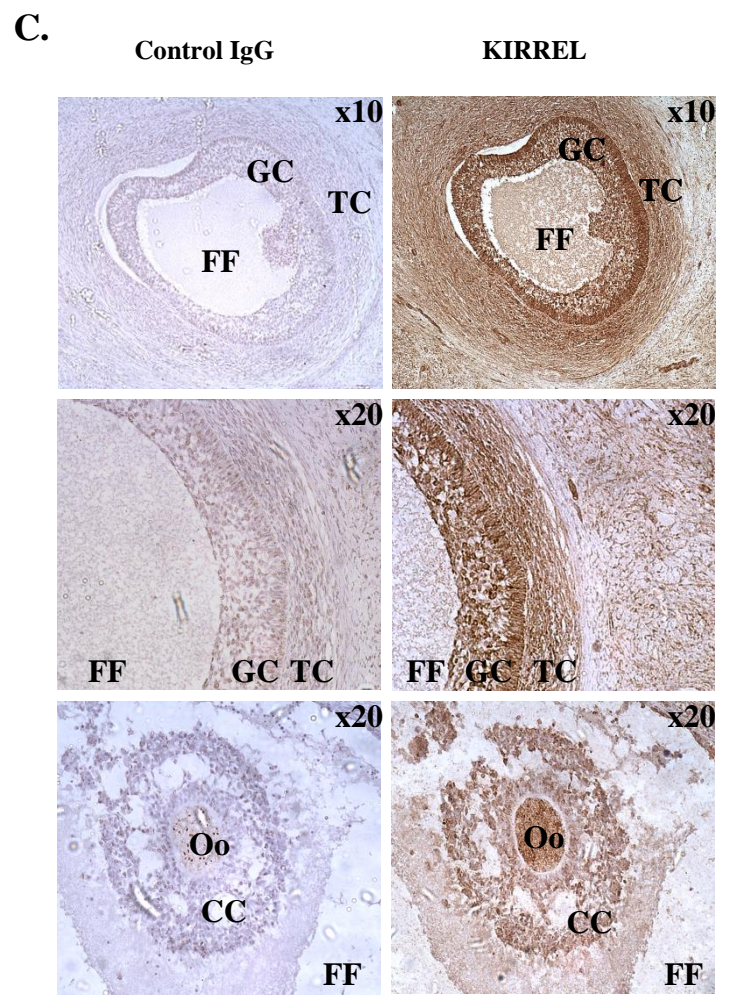
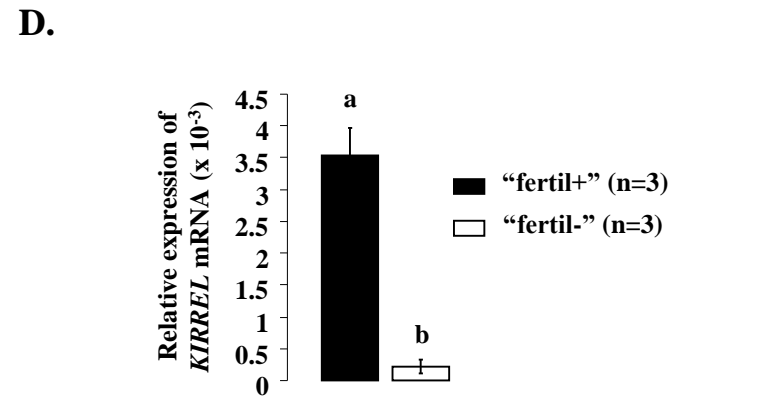
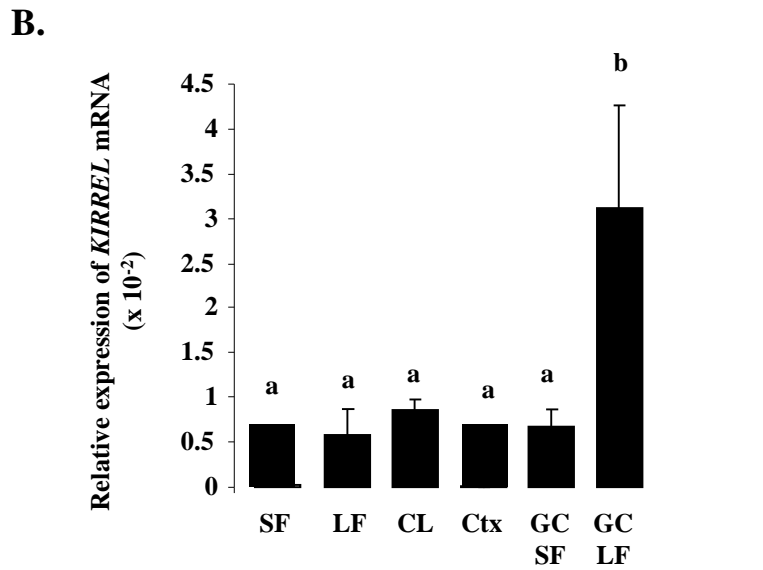
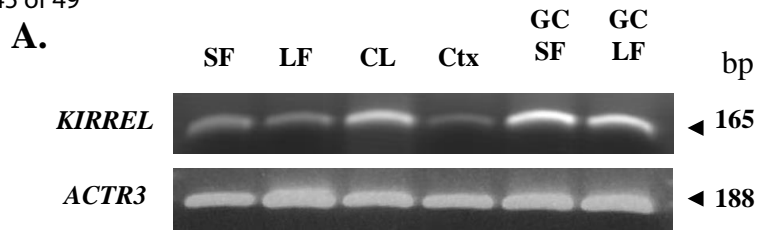
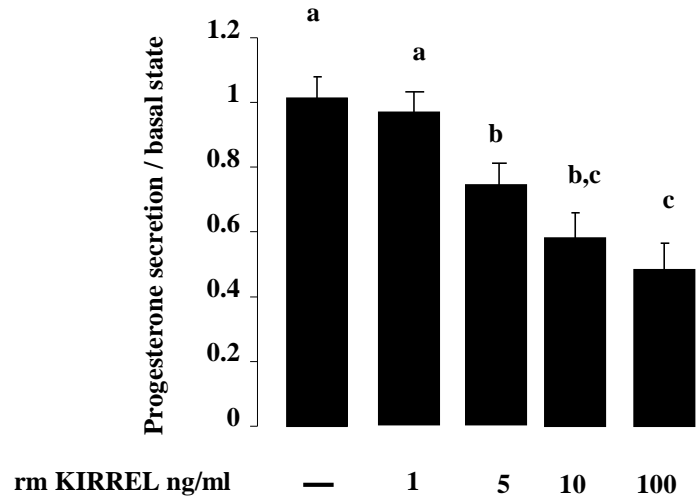
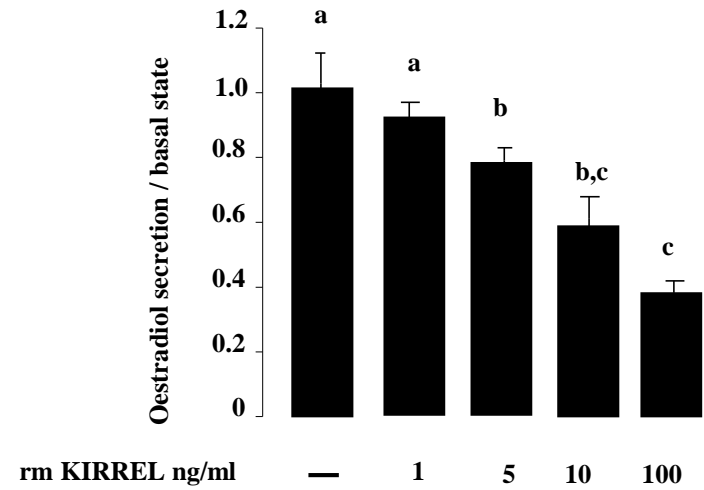


Figure 4

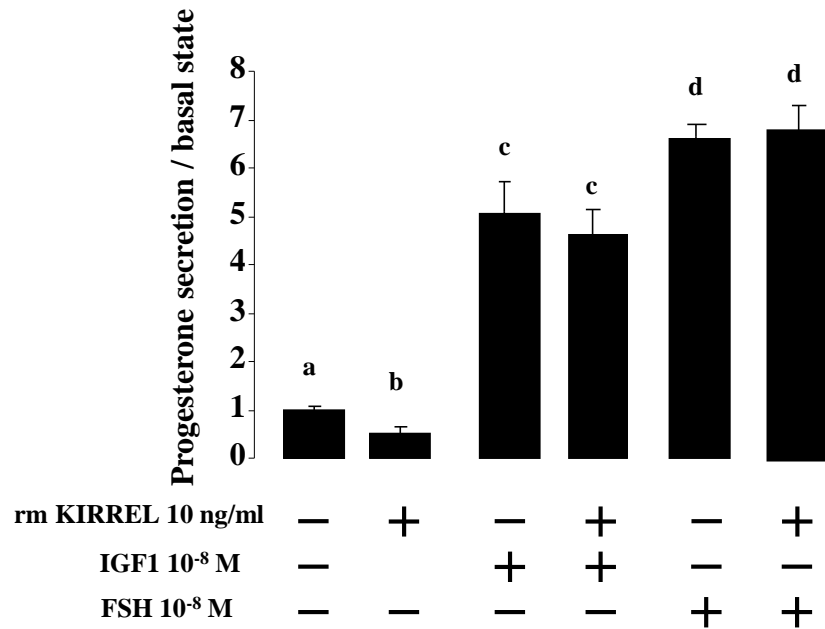
A.



B.



C.



D.

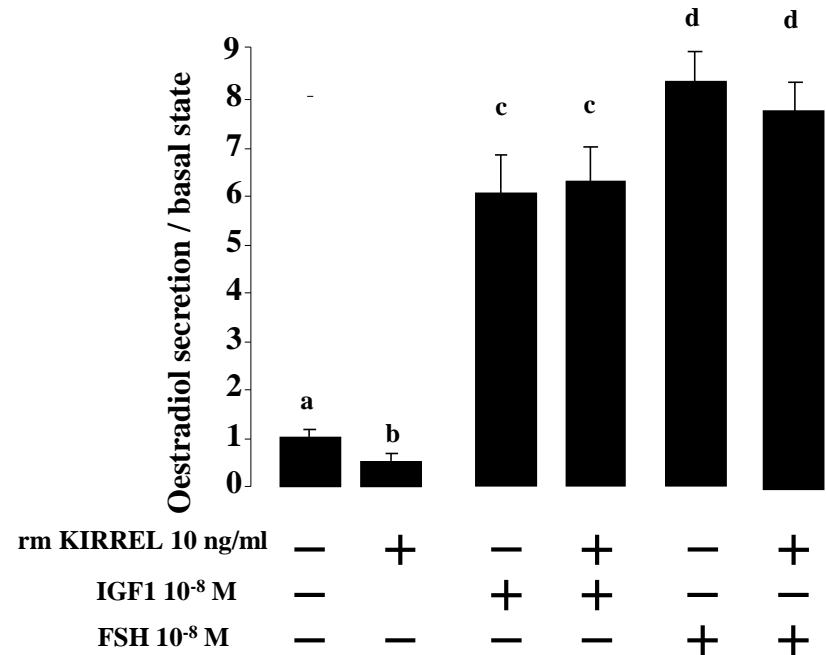
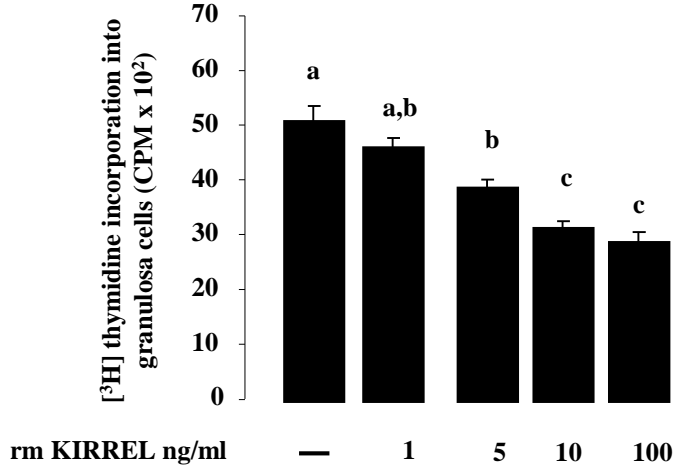


Figure 5

A.



B.

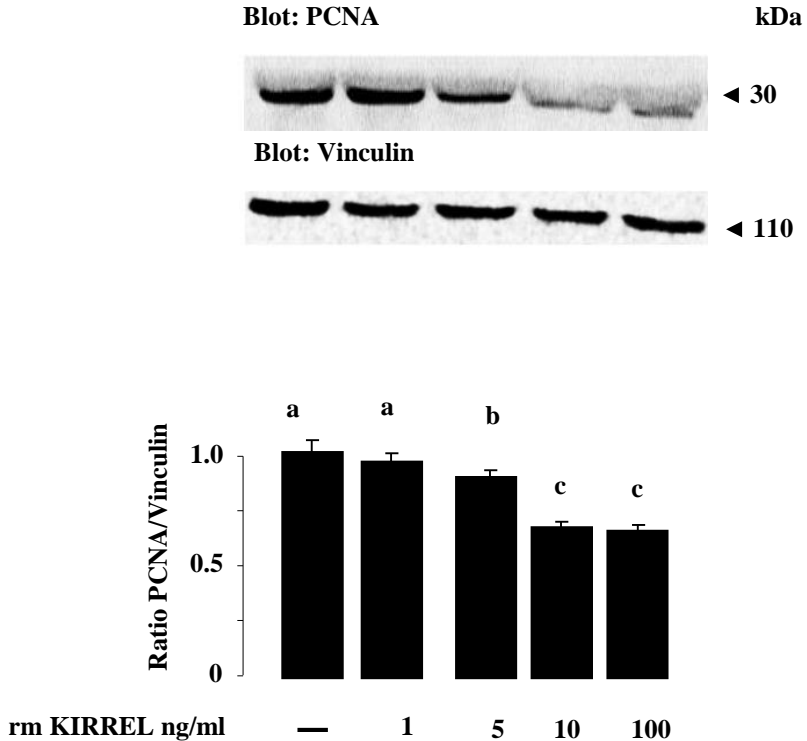


Figure 6

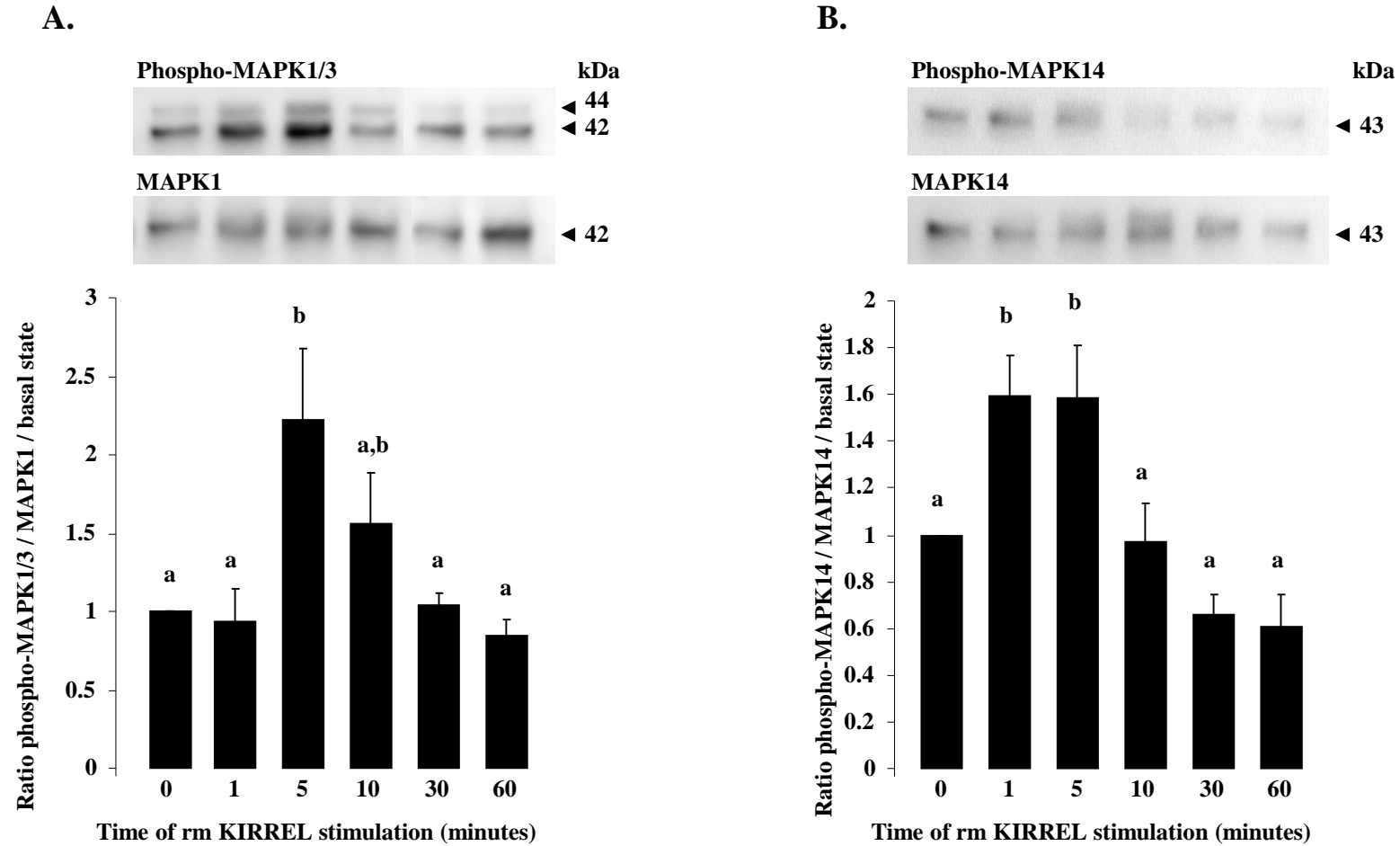


Figure 7

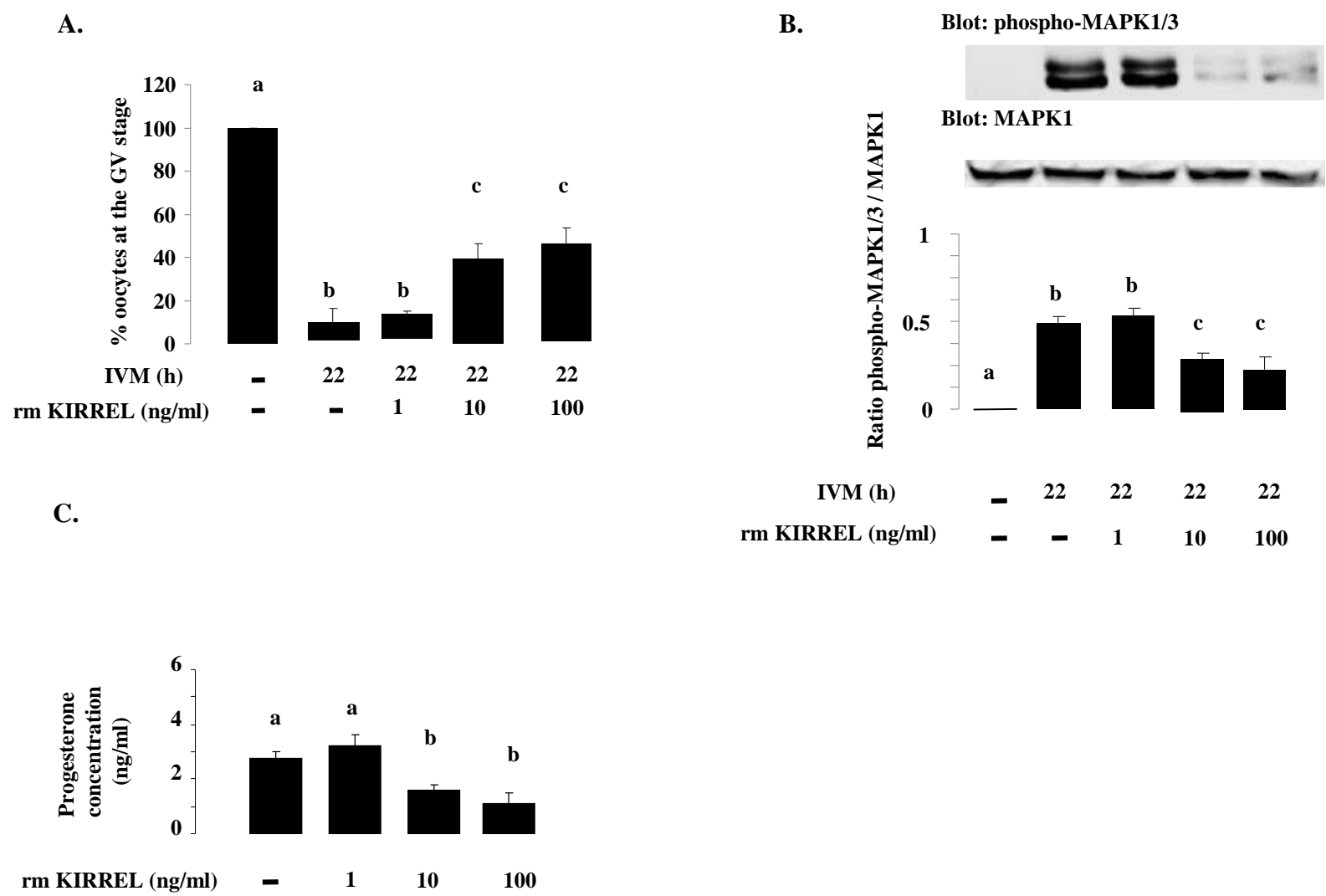


Figure 8

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