Document downloaded from:

http://hdl.handle.net/10251/80318

This paper must be cited as:

Marco Jiménez, F.; Llobat L; Vicente Antón, JS. (2010). Effects of lanosterol on in vitro maturation of porcine oocytes. Animal Reproduction Science. 117(3-4):288-294. doi:10.1016/j.anireprosci.2009.04.008.

The final publication is available at

http://doi.org/10.1016/j.anireprosci.2009.04.008

Copyright Elsevier Masson

Additional Information

Elsevier Editorial System(tm) for Animal Reproduction Science
Manuscript Draft

Manuscript Number: ANIREP-D-08-1643R1

Title: Effects of Lanosterol on In vitro Maturation of Porcine Oocytes

Article Type: Research Paper

Keywords: Lanosterol; Sterols; Porcine; Δ7-Reductase; Real-Time PCR; Nile Red.

Corresponding Author: Mr Francisco Marco-Jimenez, PhD

Corresponding Author's Institution: Polytechnic University of Valencia

First Author: Francisco Marco-Jimenez, PhD

Order of Authors: Francisco Marco-Jimenez, PhD; Lola Llobat, PhD student; Jose-Salvador Vicente, M.D., PhD

1 Effects of Lanosterol on *In vitro* Maturation of Porcine Oocytes

2

3

4 Francisco Marco-Jiménez*, Lola Llobat, José-Salvador Vicente

5

6

7 **Institution address:** Laboratorio de Biotecnología de la Reproducción. Grupo de
8 Mejora Animal. Instituto de Ciencia y Tecnología Animal. Universidad Politécnica de
9 Valencia. Camino de Vera s/n 46022 Valencia, Spain.

10

11 **Correspondence to:** PhD Francisco Marco Jiménez, Reproduction Biotechnology
12 Laboratory. Institute of Science and Animal Technology (ICTA) at the Polytechnic
13 University of Valencia, C/Camino de Vera s/n, 46071 Valencia, Spain (e-mail:
14 fmarco@dca.upv.es).

15

16

17

18 **Abstract**

19

20 FF-MAS and T-MAS sterols, intermediaries in the cholesterol biosynthetic pathway
21 present in all cells, may represent the physiological signal that instructs the oocyte to
22 reinitiate meiosis. The purpose of this study was to examine the hypothesis that
23 exogenous lanosterol could be included in the sterol biosynthetic pathway from
24 acetate to cholesterol and induce resumption of meiosis in oocytes cultured in vitro.
25 Porcine oocytes were in vitro matured in medium supplemented with different
26 concentrations of lanosterol. First, after 22 h of in vitro maturation, cumulus cells
27 were recovery and Δ7-Reductase gene expression was quantified using Real-Time
28 PCR. Second, after 44 h of in vitro maturation, chromatin configuration was
29 evaluated using Hoechst 33342. Lipid content was also evaluated at 22 and 44 h of in
30 vitro maturation using Nile red staining. The results showed that the addition of
31 lanosterol increased the Δ7-Reductase gene expression and influenced resumption of
32 meiosis using 50 and 100 μM, as well as enhancing higher cytoplasmic lipid
33 accumulation after in vitro maturation. The results demonstrate that exogenous
34 lanosterol acts in the sterol biosynthetic pathway from acetate to cholesterol and it
35 was responsible for a higher resumption of meiosis in porcine oocytes cultured in
36 vitro.

37

38

39 **Keywords:** Lanosterol, Sterols, Porcine, Δ7-Reductase, Real-Time PCR, Nile Red.

40

41

42 **1. Introduction**

43

44 Despite the progress of *in vitro* maturation (IVM) research, the quality of oocytes
45 matured *in vitro*, defined as their potential to develop into viable offspring, is still not
46 satisfactory (Lee et al., 2005). Oocyte maturation comprises cytoplasmic and nuclear
47 development: cytoplasmic refers to the processes modifying the oocyte cytoplasm
48 essential for activation, pronuclear formation, and preimplantation development, while
49 nuclear maturation refers to the ability of the oocyte to progress through meiosis (Ptak
50 et al., 1999). The mechanism of meiotic arrest and subsequent resumption received
51 increasing attention in the last decade, although the mechanism by which the
52 stimulation operates remains largely unknown (Grøndahl et al., 1998). It is generally
53 accepted that gonadotrophins induce the resumption of oocyte maturation by
54 triggering the production of paracrine hormones in the somatic cell compartment
55 surrounding the oocyte (Faerge et al., 2006).

56

57 The follicle and the cumulus cells play a very important role during oocyte growth
58 and maturation. Follicular fluids contain a variety of hormones, cytokines and other
59 substances, creating a microenvironment that contributes to the physiological
60 maturation of the oocyte. The cumulus cells are known to supply nutrients (Eppig,
61 1982; Laurincík et al., 1992) and/or messenger molecules for the development of the
62 oocyte (Lawrence et al., 1978; Buccione et al., 1990). Many of the factors secreted by
63 gonadotrophin-stimulated cumulus cells can affect the meiotic maturation of oocytes
64 (Xie et al., 2004). Although still not proven, growth factors may also stimulate
65 cumulus granulosa cells to produce steroids, follicular fluid-derived meiosis-
66 activating sterol (FF-MAS), which play a role in the control of oocyte maturation

67 (Byskov et al., 1995; Ruan et al., 1998; Jamnongjit and Hammes, 2005). These
68 sterols are intermediaries in cholesterol biosynthesis from lanosterol (Schroepfer,
69 1982), a synthesis occurring in most tissue, though without accumulation due to rapid
70 conversion of the intermediaries to cholesterol. Yamashita et al. (2003) showed that
71 FF-MAS is synthesised in cumulus cells and does not accumulate, but is metabolised
72 to cholesterol, which is then converted into progesterone, accelerating ongoing
73 germinal vesicle breakdown in porcine oocytes. The findings of Grøndahl et al.
74 (1998) strengthened the evidence that FF-MAS could be an endogenous signalling
75 molecule involved in the resumption of meiosis. Faerge et al. (2006) found that FF-
76 MAS was effective in influencing the quality of porcine oocyte maturation and thus
77 the developmental potential of porcine oocytes exposed during maturation. However,
78 little is known about the mechanisms that underlie the activity of FF-MAS or its
79 analogues on either meiotic maturation or competence to complete preimplantation
80 development (Marín Bivens et al., 2004). Numerous enzymes, expressed in cumulus
81 cells, mediate in the conversion of lanosterol to cholesterol (Stressed et al., 1996;
82 Yamashita et al., 2005). Lanosterol 14∝-demethylase converts lanosterol to FF-MAS,
83 which is further reduced to T-MAS by sterol Δ14-Reductase, and T-MAS is reduced
84 to cholesterol by sterol Δ7-Reductase (Yamashita et al., 2005; Bokal et al., 2006).
85

86 Usually, only nuclear maturation has been taken into account for the assessment of *in*
87 *vitro* maturation, and cytoplasmic maturation was mostly overlooked. However,
88 nuclear and cytoplasmic maturation are normally coordinated processes, and
89 cytoplasmic maturation may influence the nuclear maturation (Eppig et al., 1996).
90 Some elements of cytoplasmic maturation can be visualised (e.g. the line-up of
91 cortical granules) whereas many other elements of cytoplasmic maturation are

92 molecular and very challenging to visualise or monitor (Grøndahl et al., 2008).
93 Previous studies have shown that pig oocytes are rich in lipid, triglycerides being the
94 major component of intracellular lipid in the oocytes (Homa et al., 1986; McEvoy et
95 al., 2000; Sturmey and Leese, 2003). Lipids provide a great potential in terms of
96 energy reserves, cell structure and modifying the physical properties and metabolic
97 function of biological membrane (Kim et al., 2001; Sturmey and Leese, 2003).
98
99 The aim of this study was to determine the effect of lanosterol added to the maturation
100 media on the resumed meiosis and cytoplasmic maturation of porcine oocytes in vitro,
101 analysing the Δ7-Reductase gene expression in cumulus cells as indicative of
102 incorporate endogenous lanosterol in the naturally biosynthetic pathway between
103 lanosterol to cholesterol.
104
105
106 **2. Material and methods**
107
108 All chemicals, unless otherwise stated, were reagent grade and purchased from
109 Sigma-Aldrich Química S.A. (Alcobendas, Madrid, Spain).
110
111 *2.1. Isolation and Culture of Porcine COCs*

112 Porcine ovaries were collected from prepubertal gilts at a local slaughterhouse and
113 transported within 1.5 h to the laboratory in Dulbecco's phosphate buffered saline
114 (DPBS) supplemented with antibiotics at about 30°C. Oocyte-cumulus cell
115 complexes were collected from non-atretic follicles (3-6 mm diameter), washed twice
116 in 35 mm plastic Petri dishes containing DPBS supplemented with 4 mg/ml polyvinyl

117 alcohol (PVA), and twice more in maturation medium previously equilibrated for at
118 least 3 h at 38.5 °C under an atmosphere of 5% CO_2 and 100% humidity. Oocytes
119 having evenly granulated cytoplasm with at least four layers of unexpanded cumulus
120 oophorus cells and harvested within 2 h of slaughter were selected. The basic
121 maturation medium was NSCU37 (Petters and Wells, 1993), supplemented with
122 10 IU/ml eCG (Folligon, Intervet International B.V., Boxmeer, Holland), 10 IU/ml
123 hCG (Chorulon, Intervet International B.V., Boxmeer, Holland), 10% (v/v) foetal calf
124 serum (FCS), 10 ng/ml epidermal growth factor, 0.02 mg/ml penicillin and 0.04
125 mg/ml streptomycin. The COCs were cultured in the maturation medium
126 supplemented with 10, 50 and 100 µM of lanosterol. Dilutions of lanosterol were
127 prepared from a stock solution containing 5 mg lanosterol dissolved in 1 ml ethanol.
128 In order to get a 0 µM (control group), 10, 50 and 100 µM lanosterol use
129 concentration we added a 0 µl, 0.84 µl, 4.2 µl or 8.4 µl of lanosterol stock solution,
130 respectively, together with 8.4 µl, 4.2 µl, 7.56 µl, or 0 µl ethanol, respectively, to give
131 a final volume of 500 µl maturation medium. The final ethanol concentration in all
132 culture media, including the control group, was 1.68%. The COCs were cultured
133 covered with mineral oil at 38.5°C in a humidified atmosphere of 5% CO_2.
134
135 *2.2. Effect of lanosterol on Δ7-Reductase gene expression quantification*
136
137 *Isolation of Total RNA*

138 After 22 hours of *in vitro* maturation, cumulus cells from 35 COCs were removed
139 from oocytes using 3 mg/ml hyaluronidase in DPBS and repeated pipetting.
140 Yamashita et al. (2005) detected a higher level of Δ7-Reductase expression at 20 h in

141 cumulus cells cultured in vitro. A total of 420 oocytes were used in three batches per
142 lanosterol level.
143
144 Total RNA was isolated from cumulus cells using the Trizol® method (Invitrogen,
145 Carlsbad, CA) according to the manufacturer's instruction. Samples were treated
146 with DNAse as described by the manufacturer. Total RNA was collected in elution
147 tubes in a volume of 10 microlitres. RNA quality and quantity were determined
148 electrophoretically by OD_{260}/OD_{280} nm absorption ratio > 1.9.
149
150 Reverse Transcription

151 Reverse transcription was performed on 8 ng total RNA/sample using Quantitec Kit
152 (Qiagen). Before addition of RT enzyme and random primers, samples and buffer
153 were incubated at 42ºC for 2 min. RT and random primers were added and reactions
154 were incubated at 42ºC for 15 min. The reaction was inactivated by incubation at
155 95ºC for 3 min. Cumulus cells cDNA were stored at -20ºC.
156
157 Real-Time PCR Analysis

158 Quantitative real-time PCR analysis was performed, in duplicate, in a total volume of
159 25 microlitres which consisted of SYBR Green Master Mix (Applied Biosystems,
160 Foster City, CA), 25 µM each of sequence-specific primers (Table 1) and 1 ng of
161 cDNA. The PCR protocol included an initial step at 50ºC (2 min) and a second step
162 for denaturalisation at 94ºC (5 min), followed by 50 cycles at 94ºC (30 sec), annealing
163 temperature for each gene (60 sec) and 72ºC (60 sec). At the end of the amplification
164 cycles, a melting curve analysis was performed to verify specific amplification.

165 Melting curve data were collected between 60º and 95ºC with a ramp time of 20
166 minutes. Relative quantification was based on the comparison of Ct at a constant
167 level of fluorescence. The amount of transcript present was inversely related to the
168 observed Ct. The relative expression ratio was calculated with the $2^{-\Delta\Delta Ct}$ method
169 (Pfaffl, 2001; Livak and Schmittgen, 2001). That is, to determine a normalised
170 arbitrary value for each gene, every data point was normalised to the reference gene
171 GADPH (housekeeping), as well as to their respective control. The real time
172 quantitative PCR was evaluated according to efficiency by melting curve (Pfaffl,
173 2001) and a coefficient of determination with serial dilutions for the cDNA of
174 recovered sample pools.
175
176 *2.3. Nuclear stage evaluation*
177
178 After 44 hours of *in vitro* maturation, 546 oocytes were denuded using 3 mg/ml
179 hyaluronidase in DPBS and repeated pipetting. The oocytes were fixed for 22 hours
180 with 2% (v/v) glutaraldehyde and 2% (v/v) formaldehyde in DPBS. Oocytes were
181 subsequently washed twice in DPBS and then incubated in 1% (w/v) of Hoechst
182 33342 in DPBS for 15 min to identify chromatin configuration. The oocytes were
183 given three final washes in DPBS containing 1 mg/ml polyvinylpyrrolidone and
184 mounted on glass slides. Nuclear maturation rate was defined as percentage of MII
185 oocytes relate to total cultured oocytes in each group.
186
187 *2.4. Lipid content*
188 A total of 1040 oocytes were evaluated, 146 of them immature oocytes without in
189 vitro maturation. After 22 (444 oocytes) and 44 hours (450 oocytes) of *in vitro*

190 maturation, oocytes were denuded and fixed as previously. Oocytes were then
191 incubated in 10 μg/ml Nile red solution (Molecular Probes, Inc., Eugene, OR, USA)
192 dissolved in physiological saline (0.9% NaCl) with 1 mg/ml polyvinylpyrrolidone (in
193 accordance with Genicot et al. 2005). The Nile red stock solution (1 mg/ml) was
194 prepared by dilution in DMSO and stored at room temperature in the dark. Oocytes
195 were stained overnight in the dark and at room temperature unless otherwise
196 indicated. The oocytes had three final washes in DPBS containing 1 mg/ml
197 polyvinylpyrrolidone and were mounted on glass slides. Lipid droplets were
198 visualised using a fluorescence stereomicroscope (Figure 1). The amount of emitted
199 fluorescent light of the whole oocyte was evaluated with an inverted fluorescence
200 microscope (excitation: 400–500 nm and emission: 515LP) using a 100× lens
201 equipped with a digital camera (DMX1200F, Nikon). Individual photographs were
202 taken of stained oocytes and arbitrary units of fluorescence measurement were
203 quantified with Gene Tools software (Syngene, IZASA, Spain). Results were
204 expressed in arbitrary units of fluorescence, a high fluorescence being associated to a
205 high lipid content. Unless otherwise indicated, one measurement was performed per
206 oocyte.

207 *2.5. Statistical analysis*

208 The effects of lanosterol on the percentage of oocytes that reached metaphase II stage
209 after IVM were analysed by Logistic regression, while the effects of lanosterol on the
210 quantification of Δ7-Reductase gene expression and lipid content were analysed by
211 ANOVA using the general linear models (GML). All procedures were performed
212 with the Statgraphics®Plus 5.1 (Statistical Graphics Corp., Rockville, MO, USA).
213 Significance level was set at $P < 0.01$.

214

215 **3. Results**

216

217 *3.1. Effect of lanosterol concentrations on Δ7-Reductase gene expression*

218

219 The expression levels of Δ7-Reductase gene in granulose cell showed significant
220 differences between cumulus cultured with 10, 50 and 100 μM of lanosterol and
221 control group ($P<0.01$, Fig. 2).

222

223 *3.2. Effect of lanosterol concentrations on nuclear stage*

224

225 As shown in Fig. 3, the presence of 50 and 100 μM of lanosterol promote the oocyte
226 meiosis resumption significantly compared with control (85.3 ± 0.4 and 87.4 ± 0.4 %
227 vs 73.7 ± 0.4 %, for 50, 100 μM of lanosterol vs control, respectively. $P < 0.01$).
228 Compared with control, 10 μM increased compared with control (79.5 ± 0.4 vs 73.7 ±
229 0.4%) but had no significant differences.

230 *3.3. Effect of lanosterol concentrations on lipid content*

231

232 Immature oocytes showed higher arbitrary units of fluorescence than oocytes IVM
233 without lanosterol ($1.31 \pm 0.02 \times 10^6$ *vs* $1.20 \pm 0.02 \times 10^6$ and $0.89 \pm 0.02 \times 10^6$ arbitrary
234 units of fluorescence, for immature *vs* IVM during 22 and 44 h respectively, $P < 0.01$.
235 Figure 4). Culture in the presence of lanosterol, for each of the 3 concentrations,
236 during 22 hours causes reduction of emitted fluorescence light respect to the control
237 group ($1.13 \pm 0.02 \times 10^6$, $1.24 \pm 0.02 \times 10^6$, $1.16 \pm 0.02 \times 10^6$ vs $1.27 \pm 0.02 \times 10^6$

238 arbitrary units of fluorescence, for 10, 50 and 100 µM vs control group, respectively,
239 $P < 0.01$. Fig 4). However, after 44 of IVM the oocytes culture in the presence of
240 lanosterol for each of the 3 concentrations, showed a higher emitted fluorescence light
241 respect to the control group ($0.89 \pm 0.03 \times 10^6$ vs $1.04 \pm 0.03 \times 10^6$, $1.07 \pm 0.02 \times 10^6$
242 and $1.10 \pm 0.02 \times 10^6$ arbitrary units of fluorescence, for 10, 50 and 100 µM,
243 respectively, $P < 0.01$, Fig 4).
244
245 **4. Discussion**
246
247 Meiosis activating sterols (T-MAS and FF-MAS, C_{29} sterols) are intermediaries in the
248 cholesterol biosynthetic pathway from lanosterol (Rozman et al., 2002; Faerge et al.,
249 2006). FF-MAS has shown an effect on meiosis-promoting and the activity seems to
250 be non-specific across species (Byskov et al., 1995; Ruan et al., 1997; Grøndahl et al.,
251 1998:2000; Leemuis et al., 1998; Hegele-Hartong et al., 1999:2001). However, the
252 vast majority of sterols were not effective (Byskov et al., 2002). In porcine, 10 µM of
253 FF-MAS increased the number of zygotes with advanced maternal pronuclear stage
254 and reduced the polyspermic penetration rate (Faerge et al., 2006). Nevertheless, the
255 mechanism whereby meiosis activating sterols mediate in the resumption of meiosis
256 in the oocyte is largely unknown (Grøndahl et al., 1998). Lanosterol metabolic
257 products are requisite for primordial follicle formation (Zhang et al., 2008).
258 Lanosterol, first intermediary with a sterol ring, is structurally very homologous to T-
259 MAS (Bokal et al., 2006). Previously, lanosterol has tested inactive on meiosis
260 promoting activity (Byskov et al., 1995; Grøndahl et al., 1998). In our study,
261 lanosterol added at 10, 50 and 100 µM stimulated Δ7-Reductase gene expression in
262 cumulus cells. These data suggest that exogenous lanosterol could be used by the

263 cumulus cells to incorporate in the cholesterol biosynthetic pathway and must
264 enhance the production of FF-MAS and T-MAS. It has been proposed that FF-MAS
265 is secreted from cumulus cells after gonadotrophin stimulation and promotes meiotic
266 maturation of mouse oocytes using the cholesterol biosynthetic pathway from
267 lanosterol (Xia et al., 1994; Byskov et al., 1997; Hegele-Hartung et al., 1999; Xie et
268 al., 2004). Previous reports showed that lanosterol was not effective in naked oocytes
269 (Byskov et al., 1995; Grøndahl et al., 1998). Faerge et al. (2006) observed that FF-
270 MAS effect is highly dose dependent and positive effect was only seen in the dose
271 range 1-10 μM and not in 30-100 μM concentrations. FF-MAS and lanosterol were
272 shown to be present in the follicle at high concentrations (~1.2 μM and 0.13 μM,
273 respectively). It is therefore proposed that μM doses of FF-MAS do not necessarily
274 represent pharmacological active but more likely physiologically active
275 concentrations (Hegele-Hartung et al., 2001). Sterols are highly sticky and lipophilic
276 molecules exhibiting extremely poor water solubility (Hall, 1985 en Hegele-Hartung
277 et al., 2001). However, more detailed studies of sterol concentrations in culture
278 solutions are required (Hegele-Hartung et al., 2001). Grøndahl et al. (1998) evaluated
279 different concentrations of lanosterol but without results, possibly due to the low
280 concentrations assayed (0.07-7 μM). Addition of lanosterol was shown to enhance
281 the expression of Δ7-Reductase in cumulus cells for all concentrations evaluated
282 compared with the control group, although treatment with 10 μM showed no
283 significant differences in nuclear maturation *versus* control group.
284
285 Maturation of oocytes includes two aspects: nuclear and cytoplasmic maturation.
286 Generally, an oocyte is considered to be morphologically mature when the first polar
287 body is extruded (nuclear maturation) and the oocyte is arrested at metaphase of the

288 second meiotic division stage (Lee et al., 2005). Although oocytes exhibiting nuclear
289 maturation can be fertilised, they may be developmentally incompetent because of a
290 deficiency in the cytoplasmic factors needed for full development (cytoplasmic
291 maturation, Sun and Nagai, 2003). Niimura et al. (2002) considered that the
292 transformation of lipids in the cytoplasm is closely related to the resumption of
293 meiotic maturation, regardless of *in vitro* maturation. The role of lipids in oocyte
294 maturation and embryo development is unclear, although they may have a potential
295 role as reserve fuels, providing ATP for the protein synthesis that is necessary for
296 continuation of cytoplasmic maturation and meiosis (Sturmey and Leese, 2003).
297 Changes in the cytoplasmic lipid droplets during meiotic maturation have previously
298 been examined in porcine oocytes (Niimura et al., 2002). In porcine oocytes,
299 triglycerides are the main component of intracellular lipids (Homa et al., 1986). Nile
300 red technique is highly sensitive and repeatable and contrasts with previously
301 described techniques to detect differences in lipid content (Genicot et al., 2005; Leroy
302 et al., 2005; Ferguson and Lee, 1999; McEvoy et al., 2000; Kim et al., 2001). Nile
303 Red has been used to specifically evaluate, not absolutely but relatively, lipid droplets
304 and lipid content in murine, porcine and bovine oocytes and embryos (Leroy et al.,
305 2005). Triglyceride content in in vitro matured oocytes is lower than that of immature
306 oocytes, in line with previous results (Kim et al., 2001). In the present study, the
307 addition of lanosterol to the maturation medium suggested lipid accumulation after in
308 vitro maturation and it may be used as an energy source (McEvoy et al., 2000;
309 Sturmey and Leese, 2003). The availability of lipids reserves and of lipase activity
310 would enable the oocyte to use lipids as oxidative substrates after it is separated from
311 cumulus cells during maturation (Cetica et al., 2002). However, further study is
312 needed to determine the functional role of cytoplasmic lipid in immature pig oocytes

during maturation.

In conclusion, we have demonstrated that lanosterol enhanced the Δ7-reductase gene expression in the cumulus cells and increased the resumption of meiosis and showed higher lipid accumulation after *in vitro* maturation. However, it is clear from the data presented here that determination of the correct dose of lanosterol is crucial in order to draw proper conclusions concerning this compound. Additional experiments will be needed to understand the involvement of lanosterol in the cholesterogenic pathway.

References

Bokal, E.V., Tacer, K.F., Vrbnjak, M., Leposa, S., Virant Klun, I., Verdenik, I., Rozman, D., 2006. Follicular sterol composition in gonadotrophin stimulated women with polycystic ovarian syndrome. Mol. Cell. Endocrinol. 249,92-98.

Buccione, R., Schroeder, A.C., Eppig, J.J., 1990. Interactions between somatic cells and germ cells throughout mammalian oogenesis. Biol. Reprod. 43, 543-547.

Byskov, A.G., Andersen, C.Y., Hossaini, A., Xia, G.L., 1997. Cumulus cells of oocyte-cumulus complexes secrete a meiosis-activating substance when stimulated with FSH. Mol. Reprod. Dev. 46, 296-305.

Byskov, A.G., Andersen, C.Y., Leonardsen, L., 2002. Role of meiosis activating

339 sterols, MAS, in induced oocyte maturation. Mol. Cell. Endocrinol. 22, 189-96.

340

341 Byskov, A.G., Andersen, C.Y., Nordholm, L., Thogersen, H., Xia, G., Wassmann, O.,
342 Andersen, J.V., Guddal, E., Roed, T., 1995. Chemical structure of sterols that activate
343 oocyte meiosis. Nature 374, 559-62.

344

345 Cetica, P., Pintos, L., Dalvit, G., Beconi, M., 2002. Activity of key enzymes involved
346 in glucose and triglyceride catabolism during bovine oocyte maturation in vitro.
347 Reproduction. 124:675-681.

348

349 Eppig, J.J., 1982. The relationship between cumulus cell-oocyte coupling, oocyte
350 meiotic maturation, and cumulus expansion. Dev. Biol. 89, 268-272.

351

352 Eppig, J.J., 1996. Coordination of nuclear and cytoplasmic oocyte maturation in
353 eutherian mammals. Reprod. Fertil. Dev. 8, 485-489.

354

355 Faerge, I., Strejcek, F., Laurincik, J., Rath, D., Niemann, H., Schellander, K.,
356 Rosenkranz, C., Hyttel, P.M., Grøndahl, C., 2006. The effect of FF-MAS on porcine
357 cumulus-oocyte complex maturation, fertilization and pronucleus formation in vitro.
358 Zygote 14, 189-99.

359

360 Ferguson, E.M., Leese, H.J., 1999. Triglyceride content of bovine oocytes and early
361 embryos. J. Reprod. Fert. 116,373-378.

362

363 Genicot, G., Leroy, J.L., Soom, A.V., Donnay, I., 2005. The use of a fluorescent dye,

364 Nile red, to evaluate the lipid content of single mammalian oocytes. Theriogenology
365 63, 1181-1194.
366
367 Grøndahl, C., 2008. Oocyte maturation. Basic and clinical aspects of in vitro
368 maturation (IVM) with special emphasis of the role of FF-MAS. Dan. Med. Bul. 55,1-
369 16
370
371 Grøndahl, C., Lessl, M., Faerge, I., Hegele-Hartung, C., Wassermann, K., Ottesen,
372 J.L., 2000. Meiosis-activating sterol-mediated resumption of meiosis in mouse
373 oocytes in vitro is influenced by protein synthesis inhibition and cholera toxin. Biol.
374 Reprod. 62,775-780.
375
376 Grøndahl, C., Ottesen, J.L., Lessl, M., Faarup, P., Murray, A., Gronvald, F.C.,
377 Hegele-Hartung, C., Ahnfelt-Ronne, I., 1998. Meiosis-activating sterol promotes
378 resumption of meiosis in mouse oocytes cultured *in vitro* in contrast to related
379 oxysterols. Biol. Reprod. 58, 1297-1302.
380
381 Hall, P.F.,1985. Role of cytochromes P-450 in the biosynthesis of steroid hormones.
382 Vitam. Horm. 42,315-368.
383
384 Hegele-Hartung, C., Grützner, M., Lessl, M., Grøndahl, C., Ottesen, J., Brännström,
385 M., 2001. Activation of meiotic maturation in rat oocytes after treatment with
386 follicular fluid meiosis-activating sterol in vitro and ex vivo. Biol. Reprod. 64,418-
387 424.
388

389 Hegele-Hartung, C., Kuhnke, J., Lessl, M., Grøndahl, C., Ottesen, J., Beier, H.M.,
390 1999. Nuclear and cytoplasmic maturation of mouse oocytes after treatment with
391 synthetic meiosis-activating sterol in vitro. Biol. Reprod. 61, 1362-1372.
392
393 Homa, S.T., Racowsky, C., McGaughey, R.W., 1986. Lipid analysis of immature pig
394 oocytes. J. Reprod. Fertil. 77, 425-434.
395
396 Jamnongjit, M., Hammes, S.R., 2005. Oocyte maturation: the coming of age of a
397 germ cell. Semin. Reprod. Med. 23, 234-241.
398
399 Kim, J.Y., Kinoshita, M., Ohnishi, M., Fukui, Y., 2001. Lipid and fatty acid analysis
400 of fresh and frozen-thawed immature and *in vitro* matured bovine oocytes.
401 Reproduction 122:131-138.
402
403 Laurincík, J., Kroslak, P., Hyttel, P., Pivko, J., Sirotkin, A.V., 1992. Bovine cumulus
404 expansion and corona-oocyte disconnection during culture *in vitro*. Reprod. Nutr.
405 Dev. 32, 151-161.
406
407 Lawrence, T.H., Beers, W.H., Guila, N.B., 1978. Transmission of hormonal
408 stimulation by cell-to-cell communication. Nature 272, 501-506.
409
410 Lee, M.S., Kang, S.K., Lee, B.C., Hwang, W.S., 2005. The Beneficial Effects of
411 Insulin and Metformin on *In vitro* Developmental Potential of Porcine Oocytes and
412 Embryos. Biol. Reprod. 73, 1264-1268.
413

414 Leemuis, J.A.J., Van der Louw, J., Groen, M.B., 1998. 17-
415 allyloxy(thio)alkylandrostane derivatives for the modulation of meiosis. The Patent
416 Cooperation Treaty, WO 98/55498.
417
418 Leroy, J.L., Genicot, G., Donnay, I., Van Soom, A., 2005. Evaluation of the lipid
419 content in bovine oocytes and embryos with Nile Red: A practical approach. Reprod.
420 Domest. Anim. 40, 76-78.
421
422 Livak, K.J., Schmittgen, T.D., 2001. Analysis of relative gene expression data using
423 real-time quantitative PCR and the 2-ΔΔCt method. Methods 25, 402-408.
424
425 Marín-Bivens, C.L., Lindenthal, B., O'Brien, M.J., Wigglesworth, K., Blume, T.,
426 Grøndahl, C., Eppig, J.J., 2004. A synthetic analogue of meiosis-activating sterol (FF-
427 MAS) is a potent agonist promoting meiotic maturation and preimplantation
428 development of mouse oocytes maturing in vitro. Hum. Reprod. 19,2340-2344.
429
430 McEvoy, T.G., Coull, G.D., Broadbent, P.J., Hutchinson, J.S., Speake, B.K., 2000.
431 Fatty acid composition of lipids in immature cattle, pig and sheep oocytes with intact
432 zona pellucida. J. Reprod. Fertil. 118, 163-170.
433
434 Niimura, S., Takano, H., Onishi, A., Hosoe, M., 2002. Changes in the amount of
435 proteins, glycogen and lipids in porcine oocytes during *in vitro* meiotic maturation.
436 Anim. Sci. J. 73, 327-332.
437
438 Petters, R.M., Wells, K.D., 1993. Culture of pig embryos. J. Reprod. Fertil. 48, 61-73.

439

440 Pfaffl, M.W., 2001. A new mathematical model for relative quantification in real-time
441 RT-PCR. Nucleic. Acids. Res. 29, e45.
442

443 Ptak, G., Loi, P., Dattena, M., Tischner, M., Cappai, P., 1999. Offspring from one-
444 month-old lambs: studies on the developmental capability of prepubertal oocytes.
445 Biol. Reprod. 61, 1568-1574.
446

447 Rozman, D., Cotman, M., Frangez, R., 2002. Lanosterol 14alpha-demethylase and
448 MAS sterols in mammalian gametogenesis. Mol. Cell. Endocrinol. 187, 179-87.
449

450 Ruan, B., Gerst, N., Emmons, G.T., Shey, J., Schroepfer, G.J.J., 1997. Sterol
451 synthesis. A timely look at the capabilities of conventional and silver ion high
452 performance liquid chromatography for the separation of C27 sterols related to
453 cholesterol biosynthesis. J. Lipid. Res. 38, 2615-2626.
454

455 Ruan, B., Watanabe, S., Eppig, J.J., Kwoh, C., Dzidic, N., Pang, J., Wilson, W.K.,
456 Schroepfer, G.J.Jr., 1998. Sterols affecting meiosis: novel chemical syntheses and the
457 biological activity and spectral properties of the synthetic sterols. J. Lipid. Res. 39,
458 2005-2020.
459

460 Schroepfer, G.J., 1982. Sterol biosynthesis. Annu. Rev. Biochem. 51,555-585.
461 Review.
462

463 Strömstedt, M., Rozman, D., Waterman, M.R., 1996. The ubiquitously expressed

464 human CYP51 encodes lanosterol 14-demethylase, a cytochrome P450 whose
465 expression is regulated by oxysterols. Arch. Biochem. Biophys. 329,73-81.
466
467 Sturmey, R.G., Leese, H.J., 2003. Energy metabolism in pig oocytes and early
468 embryos. Reproduction 126, 197-204.
469
470 Sun QY, Nagai T., 2003. Molecular mechanisms underlying pig oocyte maturation
471 and fertilization. J. Reprod. Dev. 49, 347-359
472
473 Xia, G.L., Byskov, A.G., Andersen, C.Y., 1994. Cumulus cells secrete a meiosis-
474 inducing substance by stimulation with forskolin and dibutyric cyclic adenosine
475 monophosphate. Mol. Reprod. Dev. 39, 17-24.
476
477 Xie, H.R., Xia, G.L., Byskov, A.G., Andersen, C.Y., Bo, S.M., Tao, Y., 2004. Role of
478 gonadotropins and meiosis-activating sterols in meiotic resumption of cultured
479 follicle-enclosed mouse oocytes. Mol. Cell. Endocrinol. 218, 155-163.
480
481 Yamashita, Y., Nishibori, M., Terada, T., Isobe, N., Shimada, M., 2005.
482 Gonadotropin-induced delta14-reductase and delta7-reductase gene expression in
483 cumulus cells during meiotic resumption of porcine oocytes. Endocrinology 146, 186-
484 94.
485
486 Yamashita, Y., Shimada, M., Okazaki, T., Maeda, T., Terada, T., 2003. The
487 production of progesterone from de novo-synthesised cholesterol in cumulus cells,
488 and its physiological role during meiotic resumption of porcine oocytes. Biol. Reprod.

489 68, 1193-1198.

490

491 Zhang, H., Xu, B., Xie, H., Zhou, B., Ouyang, H., Ning, G., Li, G., Zhang, M., Xia,
492 G., 2008. Lanosterol metabolic product(s) is involved in primordial folliculogenesis
493 and establishment of primordial folliclepool in mouse fetal ovary. Mol. Reprod. Dev.
494 in press.

495

496 Zhang, X., Armstrong, D.T., 1989. Effects of follicle-stimulating hormone and
497 ovarian steroids during *in vitro* meiotic maturation on fertilization of rat oocytes.
498 Gamete. Res. 23, 267-277.

499

500 FIGURE 1.

501 Stained mature porcine oocytes (after Nile red staining) with a granulated cytoplasm
502 under visible light (A), after UV excitation (C) and mixed visible light and after UV
503 excitation (B) (200× magnification).

504

505 FIGURE 2.

506 Quantification of relative mRNA expression of Δ7-sterol Reductase gene in cumulus

507 cells during *in vitro* maturation of porcine oocytes. Data represent the least square

508 mean ± standar error mean. [abcd] Groups with different superscripts are significantly

509 different ($P < 0.01$).

510

511 FIGURE 3.

512 Effect of lanosterol on nuclear maturation in porcine oocytes after 44h IVM. Data
513 represent the least square mean ± standar error mean. [abc] Groups with different
514 superscripts are significantly different ($P < 0.01$). Numbers inside bars indicate
515 numbers of oocytes examined for each lanosterol concentration.

516

517 FIGURE 4.

518 Amount of emitted fluorescence of oocytes cultured during IVM process for 22 and
519 44 h in different lanosterol concentration media and stained with Nile Red. Data
520 represent the least square means ± standard error means. [ab] Groups with different
521 superscripts are significantly different ($P < 0.01$). Numbers inside bars indicate
522 numbers of oocytes examined.

523

524

525

526

527

Figure 1
Click here to download high resolution image

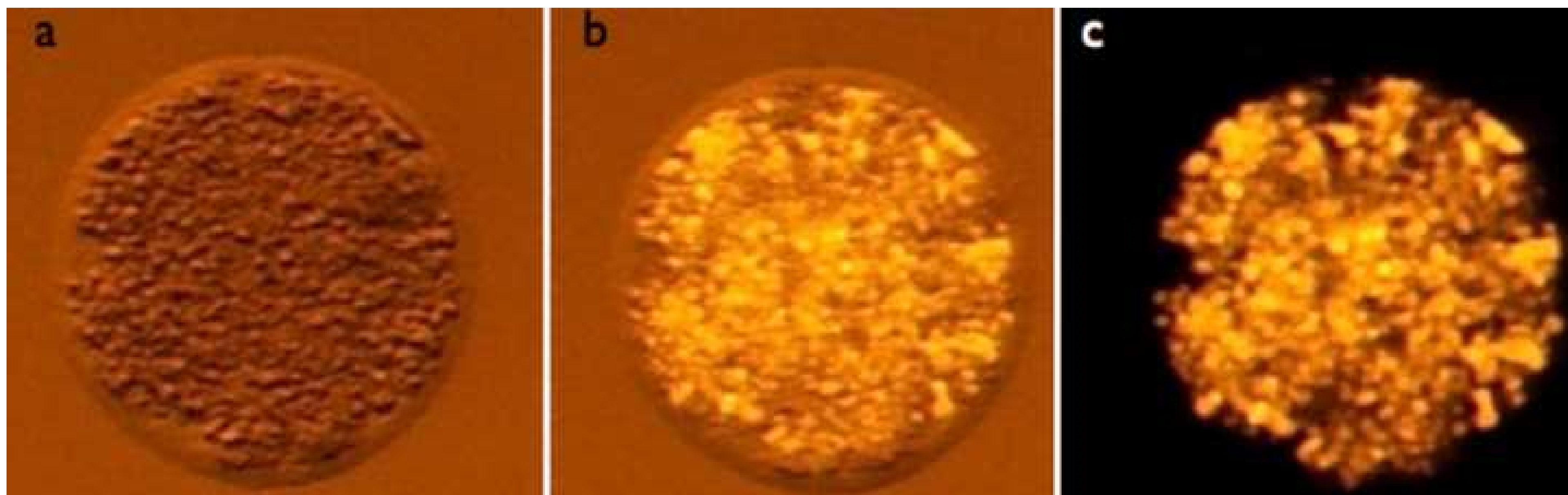

Figure 2
Click here to download high resolution image

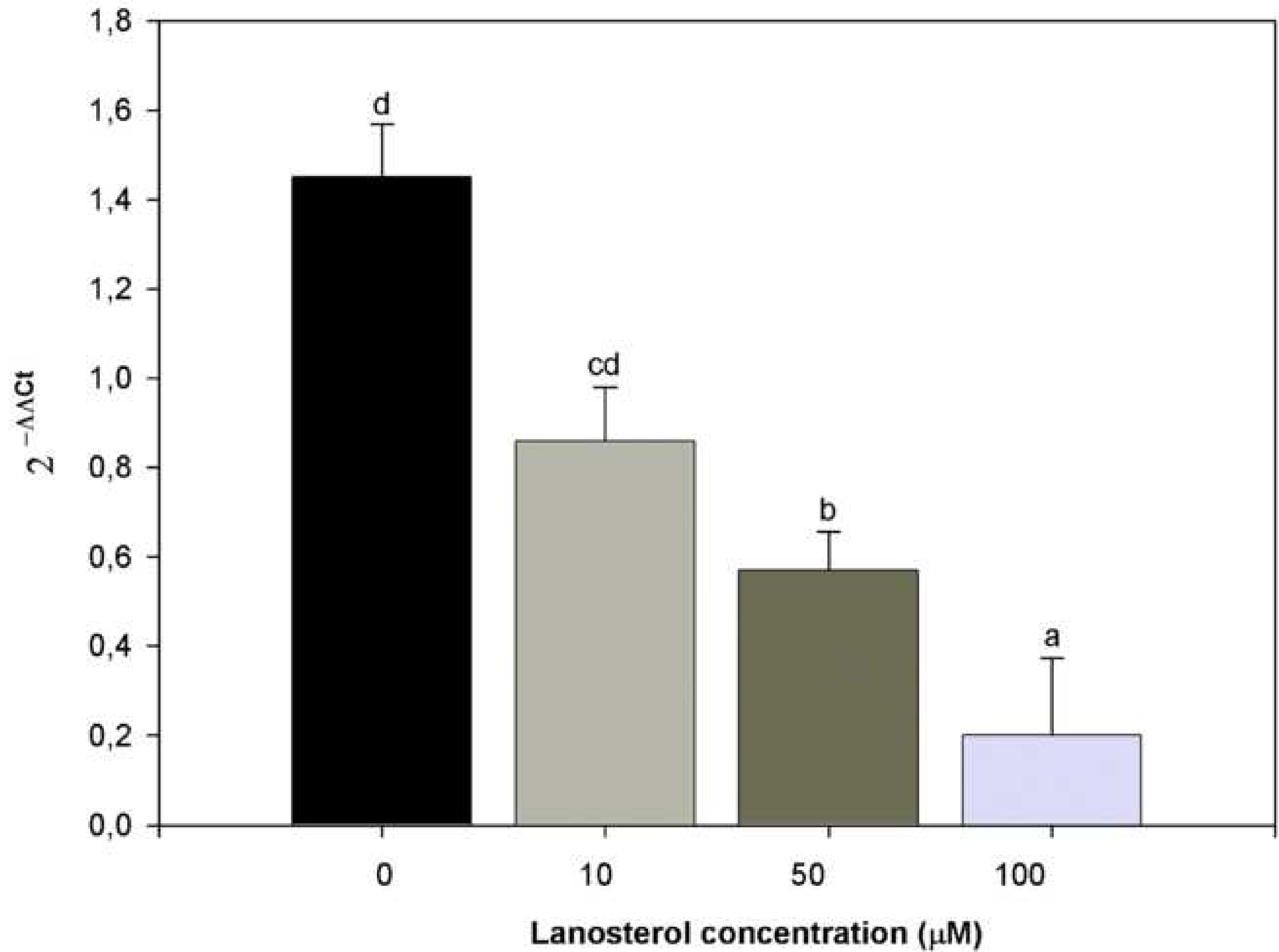

Figure 3
Click here to download high resolution image

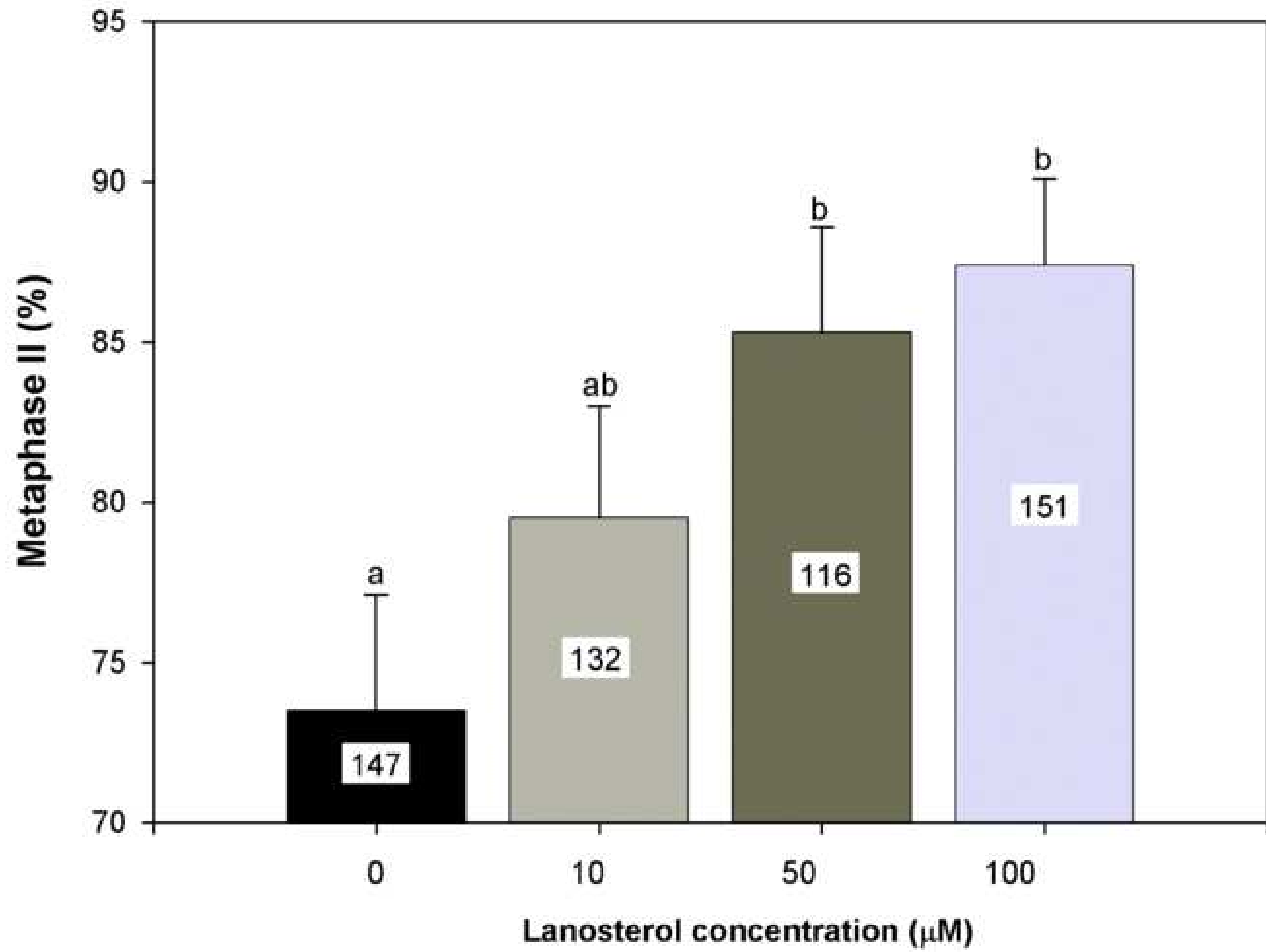

Figure 4
Click here to download high resolution image

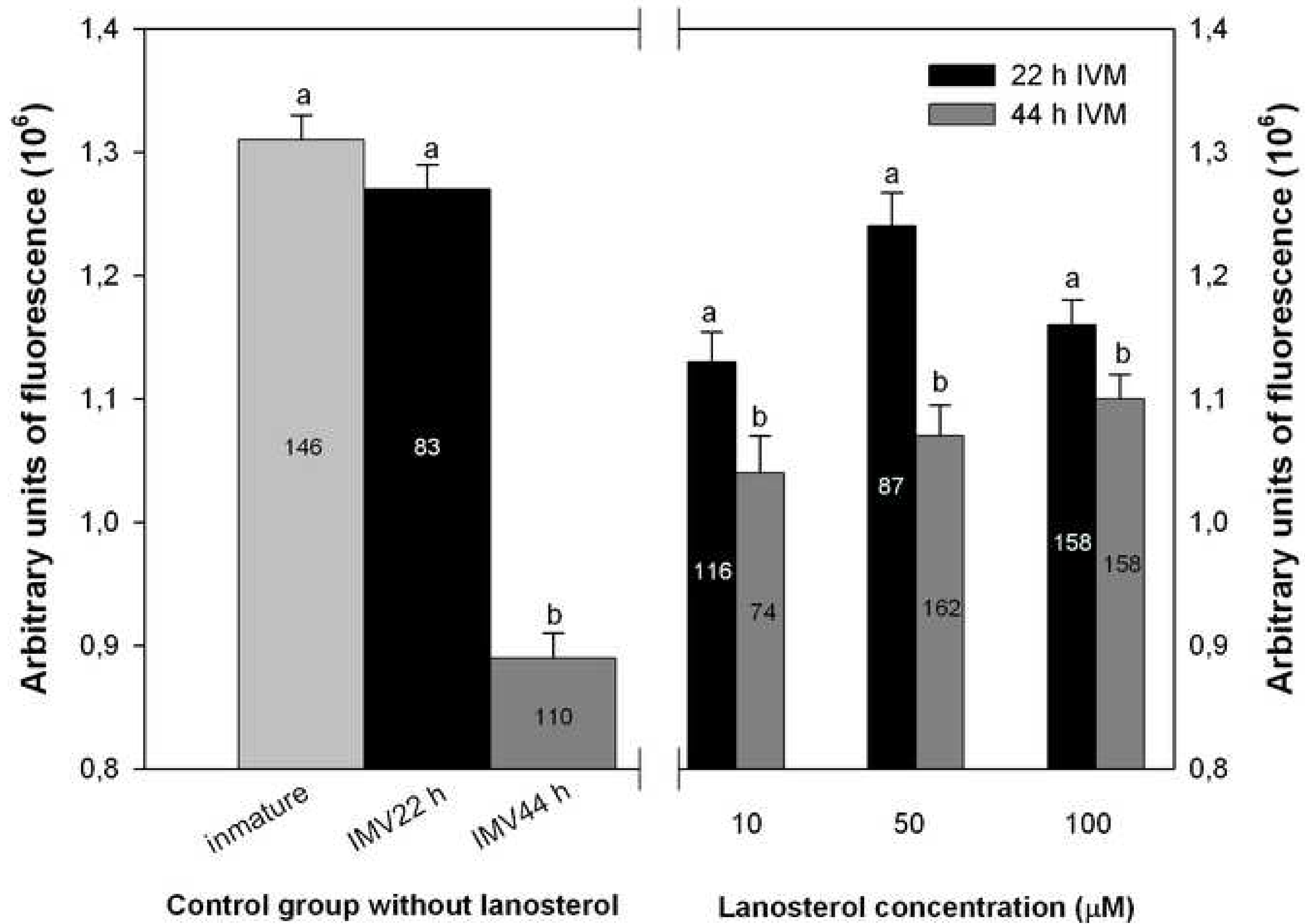

Table 1

Table 1. Oligonucleotide primers used for gene expression analysis

Gene	Primer	Sequence (5'-3')	FS (bp)	AT (ºC)	Gba
GADPH	Forward	CAAGGTCATCCATGACAACT		60	AF069649
	Reverse	CTGTTGCTGTAGCCAAATTC			
Δ7-sterol	Forward	TTGACTTCAAGCTGTTCTTCAATG	511	56	
Reductase	Reverse	CAGTAGGCCAGGCTGCCCATCAGG			AF034544

FS: Fragment size. AT: annealing Temperature. Gba: Gene bank accession number.