Glycolytic pathway activity: effect on *in vitro* maturation and oxidative metabolism of bovine oocyte

Running head: Glycolysis activity in COCs.

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Abstract

The aim of this study was to determine the influence of altering glycolytic pathway activity during bovine IVM on the meiotic maturation rate, oxidative activity, mitochondrial activity, and the mitochondrial distribution within oocytes. Glycolytic activity was manipulated using two inhibitors (ATP, NaF) and a stimulator (AMP) of key enzymes of the pathway. Inhibition of glucose uptake, lactate production and meiotic maturation rates was observed when media was supplemented with ATP or NaF. The addition of AMP in the maturation medium had no effect on glucose uptake, lactate production and meiotic maturation. However, in the absence of gonadotrophin supplementation, AMP stimulated both glucose uptake and lactate production. However, AMP also decreased cytoplasmic maturation, as determined by early cleavage. During IVM, oocyte oxidative and mitochondrial activity was observed to increase at 15 and 22 h of maturation. Inhibiting glycolysis with ATP or NaF led to a reduced oxidative and mitochondrial pattern compared with their respective control groups. Stimulation of the pathway with AMP increased oxidative and mitochondrial activity. A progressive mitochondrial migration to the central area was observed during maturation; oocytes treated with ATP, NaF or AMP showed limited migration. This study reveals the impact of altering the glycolytic pathway activity in COCs, revealing the link between glycolysis of the COC and oxidative and mitochondrial activity of the oocyte.

Key words: COC, oocyte, maturation, glycolysis, metabolism

Introduction

Once the germinal vesicle (GV) oocyte is released from the ovarian antral follicle, it will mature spontaneously in vitro under appropriate conditions. The maturation process that is manifested initially by the germinal vesicle breakdown (GVBD) also involves cumulus expansion and cytoplasmic maturation. In cattle oocytes, an adequate concentration of glucose in the maturation media is necessary for this maturation process and also for subsequent embryo development (Rose-Hellekant et al. 1998; Lim et al. 1999; Khurana and Niemann 2000). Indeed, manipulation of glucose concentration during maturation can affect the kinetics of bovine oocyte meiosis (Sutton-McDowall et al. 2005).

As in other cell types, cumulus oocyte-complexes (COCs) metabolize glucose via glycolysis, pentose phosphate pathway and the hexosamine biosynthesis pathway (Downs and Utech 1999; Sutton et al. 2003; Gutnisky et al. 2007), as well as the polyol pathway (reviewed by Sutton-McDowall et al. 2010). However, cumulus cells have a great capacity for glycolysis, and this represents the predominant pathway in COCs. It has been suggested that cumulus cell glycolytic activity is high in order to generate ATP and produce pyruvate, lactate, malate and/or oxalacetate, which are readily used as oxidative substrates by the oocyte (Bracket and Zuelke 1993; Cetica et al. 1999; 2002; 2003). In contrast, oocytes appear~~s~~ to lack capacity to undertake significant glucose metabolism (Dumollard et al. 2006; Sutton et al. 2003; Zuelke and Bracket 1992). In agreement with these observations, cumulus cells express a high affinity glucose transporter, Glut 4 (Roberts et al. 2004), whereas the oocyte does not.

In mice, a high glucose concentration in the maturation media inhibits maturation by increasing intracellular ATP levels (Downs and Mastropolo 1994). It has also been proposed that the metabolic activity of the oocyte increases in the latter half of the meiotic

maturation process, coinciding with a rise in ~~the~~ lactate production, which has been proposed as an indicator of oocyte quality (Preis et al. 2005).

In somatic cells, the major regulatory point of the glycolytic pathway is the enzyme phosphofructokinase 1 (E.C.2.7.1.11), with AMP and ATP having important positive and negative allosteric regulating roles, respectively (Schrimer and Evans 1990; Clarenburg 1992). Sodium fluoride (NaF) is also a well known inhibitor of the pathway, inactivating the glycolytic enzyme enolase (E.C. 4.2.1.11) (Mayes and Bender 2004). The intermediary metabolism of glucose also produces the reducing equivalent, NADH. This metabolite is mainly synthesized by cumulus cells in the glycolytic pathway by ~~the~~ glyceraldehyde 3-P dehydrogenase and by the oocyte through the reaction catalyzed by the α-ketoglutarate dehydrogenase and malate dehydrogenase. NADH, besides being used for ~~oxidative phosphorylation or~~as a co-factor for anabolic pathways, is a key REDOX ~~regulator~~ molecule (Dumollard et al. 2007), important in both cystolic and mitochondrial REDOX regulation. The REDOX state describes the sum of interactions between oxidized and reduced forms of a variety of molecules that includes NAD(P):NAD(P)H; FAD:$FADH_2$, GSH:GSSG (reviewed by Harvey et al. 2002; Herrick et al. 2006).

Oocyte maturation also includes cytoplasm changes such as synthesis and accumulation of mRNA, proteins, transcription factors and the redistribution of organelles, especially mitochondria. Mitochondrial activity is essential for oocyte competence, and ATP content of oocytes generated from the reducing equivalents derived from carboxylic acid metabolism through the TCA cycle is highly correlated to oocyte competence (Stojkovic *et al.* 2001; Dumollard *et al.* 2007).

Mitochondrial distribution differs within in vitro maturing oocytes between different species. In mouse oocytes, mitochondria aggregate around the nucleus from the GV-stage

through to GVBD. With the movement of the nucleus throughout maturation, mitochondria concentrate around the nucleus (Nishi et al. 2003). In pig oocytes, mitochondria accumulate in the perinuclear area during meiotic progression from GVBD to anaphase I. In matured oocytes, mitochondrial foci are formed and moved to the inner cytoplasm (Sun *et al*. 2001). On the other hand, in bovine oocytes after 12 - 18 h of in vitro maturation, the mitochondria move from a peripcorticalheral location to an evenly distributed pattern (Hyttel *et al*. 1986). Furthermore, there appears to be an association between the oocyte ATP content and mitochondria redistribution; specifically for oocytes with low ATP content, mitochondria migration does not happen occur (Stojkovic *et al*. 2001).

Although it was proposed that glycolytic end products produced by cumulus cells are metabolized by the bovine oocyte during IVM, there are not studies that evaluate the influence of glycolytic activity in COCs on oocyte oxidative metabolism and maturational capacity. Thus, the main aim of this work was to study the glycolytic pathway activity during cattle oocyte IVM, analyzing the effects of two inhibitors (ATP, NaF) and a stimulator (AMP) of the key enzymes of glycolysis on the maturation rate, the oxidative and mitochondrial activity, and the mitochondrial distribution in the oocytes.

Matherials and Methods

Materials

Unless specified, all chemicals and reagents were purchased from Sigma Chemical (St. Louis, MO, USA).

Recovery of cumulus-oocyte complexes

Bovine ovaries were collected at an abattoir within 30 min of slaughter and kept warm (30˚C) during the 2 h journey to the laboratory. Ovaries were washed with physiological saline containing 100000 IU L^{-1} penicillin and 100 mg L^{-1} streptomycin. COCs were recovered by aspiration of antral follicles (2-5 mm in diameter) and only oocytes completely surrounded by a compact and multilayered cumulus oophorus were used.

In vitro maturation of cumulus-oocyte complexes

COCs were cultured in Medium 199 (Earle´s salts, L-glutamine, sodium bicarbonate 2.2 mg L^{-1} GIBCO, Grand Island, NY, USA) supplemented with 5% (v/v) fetal bovine serum (FBS; GIBCO), 0.2 mg porcine L^{-1} follicle-stimulating hormone (FSH; Folltropin-V; Bioniche, Belleville, Ontario, Canada), 2 mg L^{-1} porcine luteinizing hormone (LH; Lutropin-V; Bioniche) and 50 mg L^{-1} gentamicin~~e~~ sulfate under mineral oil at 39˚C for 22 h in an atmosphere of humidified 5% CO_2 in air.

To study the effect of different enzyme modulators of glycolysis on COC glycolytic pathway activity and **oocyte meiotic maturation**, COCs were individually cultured in 20 µl drops of maturation media supplemented with increasing concentrations of ATP (1, 10, 20 and 40 mM), NaF (2, 3 ,4 and 5 mM) or AMP (1, 10, 20 and 40 mM) under the conditions described above.

To study the effect of manipulating COC glycolytic pathway activity on subsequent oxidative activity, mitochondrial activity**, mitochondrial distribution and oocyte nuclear morphology in the oocytes**, COCs were cultured in groups of 50 in 500 µl drops of maturation media supplemented with 10 mM of ATP or 3mM of NaF (determined as inhibitory concentrations in the previous experiments) during 9, 15 and 22 ~~hs~~ hr and AMP

10 mM (determined as stimulatory concentration in the previous experiment) without gonadotrophins supplementation. These maturations time points were chosen for being consecutives to key events of the maturation process: GVBD, metaphase I and extrusion of the first polar body, respectively (Fleming and Saacke 1972; Kruip et al. 1983; Gordon 1994).

Determination of glycolytic activity of COCs

COCs were removed from each 20 µl drop and the glucose content was determined from the spent maturation medium. Glucose concentration was measured using a spectrophotometric assay based on the oxidation of glucose by glucose oxidase and subsequent ~~revealed~~ production of hydrogen peroxide involved (Trinder 1969). Positive **controls** comprising 20 µl drops of maturation media were included in each experiment.

~~The determination of l~~Lactate production in the culture medium was ~~conducted~~ determined in the same droplets ~~that~~ as glucose uptake ~~was determined~~. Lactate production was measured using a spectrophotometric assay based on the oxidation of lactate and subsequent ~~revealed~~ production of hydrogen peroxide (Trinder 1969; Barhan and Trinder 1972).

Evaluation of oocyte meiotic maturation

The removed COCs from each 20 µl drop were used to evaluate meiotic maturation rates. Oocytes were denuded mechanically by repeated pipetting in PBS with 1 g L^{-1} hyaluronidase. Denuded oocytes were placed in a hypotonic medium of 2.9 mmol L^{-1} sodium citrate at 37˚C for 15 min, fixed on a slide with 3:1 ethanol: acetic acid (Tarkowski 1966), stained with 5% (v/v) Giemsa (Merck, Darmstadt, Germany) for 15

minutes and observed under a light microscope at magnifications of x100 and x400. Oocytes were considered mature when a metaphase II chromosome configuration was present.

Evaluation of oocyte cytoplasmic maturation

Immature COCs were divided into three groups for in vitro maturation in the media described above (positive control), without supplementation of gonadotrophins (negative control) and only supplemented with AMP 10 mM. After 21 h of maturation, in vitro fertilisation was performed using frozen–thawed semen from a Holstein bull of proven fertility. Semen was thawed at 37°C in modified synthetic oviduct fluid (mSOF; Takahashi and First 1992), centrifuged twice at 500 x*g* for 5 min and then resuspended in fertilisation medium to a final concentration of 2×10^9 motile spermatozoa L^{-1} after 30 min of sperm selection by swim up (Hallap *et al*. 2004). Fertilisation was performed in IVF-mSOF, consisting of mSOF supplemented with bovine serum albumin (BSA) and heparin under mineral oil at 39◦C, in 5% CO_2 in air and 100% humidity for 20 h. Zygotes were denuded by repeated pipetting and placed in 500µL of *in vitro* culture (IVC)-mSOF, consisting of mSOF supplemented with Minimum Essential Medium (MEM) amino acids (GIBCO), MEM non-essential amino acid (GIBCO), glutamine, 6 g L^{-1} BSA and 5% (v/v) FBS (GIBCO), under mineral oil at **39°C** in 90% N_2 : 5% CO_2 : 5% O_2 and 100% humidity for 24 h. The cytoplasmic maturation was evaluated by the ratio of cleaved embryos. An additional cohort of 10 oocytes from each replicate was maintained through the fertilisation procedure without exposure to sperm to test for parthenogenesis.

Evaluation of oxidative activity, mitochondrial activity and mitochondrial distribution

From the total number of ~~the~~ oocytes ~~used~~recovered, 2/3 were ~~fated~~ utilised to determine the oxidative activity, mitochondrial activity and mitochondrial distribution at 0, 9, 15 and 22 h of maturation. These time points were chosen as they correlate to key events of the maturation process: GVBD (6 – 8 h), metaphase I (12 – 14 h) and extrusion of the first polar body (18 – 21 h).

The cumulus cells were removed mechanically by repeated pipetting in PBS with 1 g L^{-1} hyaluronidase before the zona pellucida was dissolved with 5 g L^{-1} pronase for 1 min.

Fluorescent probes and confocal microscopy were used to analyze the parameters mentioned above. The dual stains of RedoxSensor red CC-1 and MitoTracker green FM were used in this experiment. Oocytes were coincubated with a final concentration of 1nM RedoxSensor red CC-1 and 0.5 nM MitoTracker green FM, for 30 min at 37˚C in the dark and then washed twice in PBS. Stained oocytes were then placed between slide and coverslip for the observation in a laser confocal microscope (Nikon C1 confocal scanning head, Nikon TE2000E). One optical section was examined for each oocyte. The images obtained were saved and then analyzed using ~~the~~ Adobe Photoshop CS2 (version 9).

Both red and green fluorescence emission intensities were determined in four different regions (squares) within three areas (1 - 3) of the oocyte, as depicted in Fig 1A?? (Wakefield et al. 2007).

The oxidative activity was calculated as the sum of the average red fluorescence intensity in the three areas of the same oocyte. The mitochondrial activity was calculated as the sum of the average green fluorescence intensity in the three areas of the same oocyte. Ratios of green fluorescence intensity between areas 3 and 1 were then calculated to compare the distribution of active mitochondria.

Evaluation of oocyte nuclear morphology

The remaining 1/3 of the oocytes were used to evaluate the nuclear morphology at 0, 9, 15 and 22 h of maturation. Denuded oocytes were fixed in a 40 mg L^{-1} paraformaldehide solution for an hour and then incubated in a permeabilizing solution for an hour and a half. Finally the fixed oocytes were stained with 10 mg L^{-1} Hoechst 33342 solution for 15 min.

Oocyte nuclear status was observed at x400 under a Jenamed II epifluorescence microscope (Carl Zeiss Jena) with a 410 nm filter at each time point.

Statistical analysis

The results of glucose uptake, lactate production, oxidative activity, mitochondrial activity and the ratio of green fluorescence intensity between area 3 and 1 to evaluate mitochondrial distribution are expressed as the mean ± s.e.m. In the studies evaluating glucose uptake and lactate production the comparisons were made by analysis of variance (ANOVA) followed by Bonferroni post-test. The oxidative activity, the mitochondrial activity and the mitochondrial distribution were compared using a 2 x 4 or 3 x 4 factorial design. Meiotic and cytoplasmic maturation rates between treatments were compared using a chi-square analysis for non-parametric data. In all tests, significance was set at $p<0.05$.

Results

Glycolytic activity of COCs and oocyte maturation

In order to study the glycolytic pathway, COCs were incubated during maturation with increasing concentration of the pathway modulators. COCs matured in the presence of ATP showed a dose dependant inhibition in glucose uptake and lactate production

240 ($p<0.05$). A co-dependent inhibition on their progression to metaphase II was also observed
241 ($p<0.05$) (Fig 2 a).

242 The addition of increasing concentrations of NaF in the maturation medium
243 produced a dose-dependent inhibition of both lactate production and nuclear maturation
244 ($p<0.05$). However, the inhibition of glucose uptake was not dose-dependent ($p<0.05$) (Fig
245 2 b).

246 The addition of AMP to the maturation media had no effect on glucose uptake and
247 lactate production or on rates of nuclear maturation compared with the control group (Fig 2
248 c). In order to test if the gonadotrophins (FSH, LH) were masking an effect of AMP, the
249 experiment was repeated with maturation media devoid of gonadotrophins. In the absence
250 of FSH and LH, AMP had both stimulatory effect on glucose uptake and lactate production
251 ($p<0.05$) with no effect on the nuclear maturation (Fig 2 d).

252 As the rate of nuclear maturation had not been altered with the addition of AMP, we
253 evaluated the cytoplasmic maturation of the oocytes cultured with 10 mM AMP without
254 gonadotrophin supplementation. We chose this concentration because it was the lowest that
255 stimulated the glycolytic pathway in the COCs. A significant decrease in the cleavage rates
256 compared with both control groups (matured with and without gonadotrophins) was
257 observed in the group of COCs matured in the presence of AMP ($p<0.05$) (Fig 3).

258

259 *Oxidative activity of the oocyte*

260 To confirm the participation of the COC glycolysis in the oxidative metabolism,
261 oocytes were stained with Redox Sensor red to quantify mitochondrial oxidative activity at
262 different time points (0, 9, 15 and 22 hs). In these experiments, 10 mM ATP and 3 mM

NaF were at the minimum inhibitory concentrations observed for glucose uptake and lactate production. For glycolytic pathway stimulation we used 10 mM AMP to stimulate glucose uptake and lactate production.

Oocytes showed changes in their oxidative activity throughout maturation, an increase in the oxidative activity was observed at 15 and 22 h of maturation compared with time 0 h ($p<0.05$). Although similar changes in oxidative activity were also observed in the oocytes matured in the presence of ATP or NaF, overall activity was significantly lower than the values obtained for the control group ($p<0.05$) (Fig 4 a and 4 b). In contrast, AMP (in the absence of gonadotrophins) stimulated oxidative activity at 15 and 22 h maturation ($p<0.05$) compared to 0 h. Nevertheless, at 15 h the increase was significantly lower for the oocytes matured with AMP and the control without gonadotrophins respect to the control with FSH + LH ($p<0.05$). Additionally, oocytes matured with AMP showed the highest oxidative activity at hour 22 of maturation ($p<0.05$) (Fig 4 c).

Mitochondrial activity of the oocyte

To study the effect of manipulating glycolytic activity on mitochondrial activity within oocytes, fluorescence intensity of Mitotracker green was analyzed at the same time points and using the same ATP, NaF and AMP concentrations described in the experiment above.

The intensity of green fluorescence varied throughout maturation. A significantly increase in intensity was observed at hour 15 and 22 of maturation compared with hour 0 ($p<0.05$). A partial increase was observed in the ATP group at hour 15 ($p<0.05$) while no changes were observed for the NaF group (Fig 5 a and 5 b). Stimulation of glycolysis with AMP revealed an increase in mitochondrial activity at 15 and 22 h of maturation compared

with 0 h ($p<0.05$), and an increase in the same parameter compared with the respective controls at hours 9 and 22 of maturation ($p<0.05$); (Fig 5 c).

A high positive correlation between oxidative activity and mitochondrial activity of oocytes was observed for each treatment ($r>0.82$, $n=199-211$, $p<0.05$).

Mitochondrial distribution in the oocyte

Changes in the mitochondrial distribution were observed during maturation. The distribution in immature oocytes was cortical (Picture 1), however a progressive mitochondria migration to the central area was observed (Picture 1) during maturation. This observation was confirmed analyzing the ratio between the intensity of green fluorescence between area 3 and area 1 of the oocyte ($p<0.05$). On the other hand, oocytes treated with ATP, NaF or AMP showed a partial migration compared with their respective controls ($p<0.05$);(fig 6 a, 6 b and 6 c).

Oocyte nuclear morphology

To determine which stage of nuclear maturation is affected by glycolysis inhibition, COCs were also analyzed with the fluorochrome Hoechst 33342 at the different time points. In the control group 70.8% of the oocyte had passed GVBD state after 9 hours of maturation, while the oocytes matured with ATP or NaF showed significantly lower rates ($p<0.05$). The rates of oocytes in MI was also significantly lower in the treatments with ATP and NaF at hour 15 of maturation ($p<0.05$). The extrusion of the first polar body was observed in the 81.5% of the control oocytes at hour 22 but diminished in the treated oocytes ($p<0.05$), mostly staying arrested at the germinal vesicle stage (Table 1). Oocytes treated with AMP did not show differences in metaphase II rates at hour 22 of maturation

311 respect to controls, so we did not include the analysis of oocyte nuclear morphology in this
312 experiment.
313
314 **Discussion**
315 The present study describes the effect of the addition of glycolytic modulators
316 during bovine oocyte IVM on glucose uptake and lactate production of COCs, and on the
317 maturation rate, oxidative activity, mitochondrial activity and mitochondrial distribution of
318 the oocytes.
319 When ATP or NaF was added in the maturation media, a reduction in glucose
320 uptake and lactate production was observed, as were the meiotic maturation rate, suggesting
321 a relationship between COC glycolytic activity and nuclear maturation capacity of the
322 oocyte. Although a dose-dependent inhibition in lactate production was observed with both
323 modulators, only COCs matured with ATP showed a dose-dependent inhibition of glucose
324 uptake; this difference might be related to a difference in the intracellular mechanisms of
325 action. NaF is a specific pharmacological inhibitor of the glycolytic pathway by inhibiting
326 the enzyme enolase, whereas ATP is both a physiological modulator of
327 phosphofructokinase 1 as well as other glucose-dependent metabolic pathways. Both have
328 their effects at different levels within the glycolytic pathway: ATP inhibits the pathway at
329 the preparatory phase, whereas NaF acts at the payoff phase of the pathway (Nelson and
330 Cox 2005). The distal inhibition of glycolysis by NaF might allow a flux of intermediary
331 metabolites to enter other pathways.
332 The dose-dependent inhibition of oocyte nuclear maturation in presence of either
333 ATP or NaF may be attributed a reduced contribution of oxidative substrates, such as
334 pyruvate and lactate, by the cumulus cells. These substrates are essential for the bovine

oocyte and reflects the very low capacity for glucose metabolism by the oocyte (Cetica et al. 1999; Zuelke and Brackett 1992), as demonstrated by the considerably lower activity of phosphofructokinase 1 in denuded oocytes than in cumulus cells (Cetica et al. 2002).

In a previous report, we demonstrated that FSH/LH caused a significant (30.5%) increase in glucose uptake by COCs (Gutnisky et al. 2007). In the present work, we found that the dose-dependent stimulation of glycolysis by AMP is not synergistic with FSH/LH stimulation. Furthermore, although the kinetics of meiotic maturation was not altered when AMP was added to the culture media, the subsequent cleavage rates following IVF were diminished significantly, suggesting that stimulation of the glycolytic pathway by AMP may affect cytoplasmic maturation of bovine oocytes. In line with these results, Preis et al. 2005 suggested that glucose uptake and lactate production by the COC is related to the capability of the oocyte to be fertilized. Moreover, AMP may be affecting the AMP/ATP ratio activating the AMP kinase. The activity of this enzyme is known to affect the nuclear maturation of the bovine oocyte (Bilodeau-Goeseels et al. 2007).

In the present study it was detected a fluctuation in the oxidative activity and the mitochondrial activity of bovine oocytes during IVM. Additionally, both parameters are highly correlated and they depict similar variations during maturation. Interestingly, the oxidative fluctuations observed here are not coincident with the changes in reactive oxygen species concentration detected bovine oocyte maturation in vitro (Morado et al. 2009). Our results reveal that oxidative activity increases in bovine oocytes during maturation at 15 and 22 hours of culture. The increase in the oxidative activity is coincident with key meiotic events of maturation, such as the formation of the metaphase plate of the first meiotic division and extrusion of the first polar body (Gordon 1994), respectively. Protein synthesis increases three fold during metaphase I compared to GV (reviewed by Ferreira et

al. 2009), suggesting that the increase in the oxidative activity at hour 15 of maturation is related to new protein synthesis.

The reduced glycolytic activity induced by ATP and NaF is coincident with a low oocyte oxidative activity at 15 and 22 hours of maturation, compared to untreated COCs. We also observed that inhibiting the glycolytic pathway arrested most of the oocytes at the GV stage. Although ~~from~~ these results ~~it could be~~ suggest~~ed~~ that glycolytic activity of the COC may be decreased due to the inhibition of GVBD and oocyte maturation, we previously demonstrated that glycolytic activity in cumulus cells is not influenced by the presence of the oocyte (Sutton et al. 2003). Therefore, the pattern of oxidative activity would be dependent on the changes in glycolytic activity of the COCs, and therefore would reflect ATP demand. On the other hand, ~~the~~ stimulation of the pathway by AMP affected the pattern of oocyte oxidative activity with respect to the control group. The maintenance of a higher oxidative activity in the oocyte during IVM ~~might~~ in some way might be affecting the cytoplasmic maturation of the female gamete.

Quantification of mitochondrial activity in untreated COCs increased significantly at 15 and 22 hours of maturation. Inhibition of COC glycolysis prevented this increase in mitochondrial activity, most likely as a consequence of the inhibition in glycolytic activity in the COCs, causing a decrease in the supply of oxidative substrates to the oocyte. A similar observation was made for mitochondrial distribution~~:~~; inhibition of glycolysis prevented mitochondrial migration during maturation. Stimulating the glycolytic pathway by AMP altered the pattern of mitochondrial activity compared to the control group. As discussed already in regard to the oxidative activity, the mitochondrial activity remained higher during IVM in the presence of AMP, suggesting the increased glycolytic activity of

cumulus cells may increase the contribution of reduced coenzymes and/or oxidative substrates to the oocyte, augmenting mitochondrial activity. Nevertheless, this does not appear to improve cytoplasmic maturation.

The Redox-Sensor Red staining (oxidative levels) and Mitotracker Green (mitochondrial activity) were closely co-localised and within the cytosolic compartment of the oocyte, this being congruent with the characteristic pattern of metabolically active cells (Chen and Gee; 2000). This relationship might be the result of an increase in cytosolic reductive agents, like lactate and malate, within the oocyte. These metabolites can act as reductive compounds when they are substrates of the lactate dehydrogenase and malate dehydrogenase, respectively (Cetica et al. 1999; 2003). **It has been reported that mit~~h~~och**ondrial reorganization and burst of ATP production during oocyte maturation were completely inhibited if cell cycle progression is inhibited (Yu et al. 2010). In agreement with these findings, we ~~found out~~determined that alterations of oxidative and mitochondrial activity patterns are related with modifications in normal mitochondrial migration, suggesting that the same is true in bovine oocytes.

In conclusion, we report that the glycolytic pathway activity in COCs is necessary for successful IVM of the bovine oocyte. Modifications in the oocyte's oxidative and mitochondrial activities are associated with increases at 15 and 22 hours of incubation during maturation. Inhibiting glycolysis reduced these parameters in the oocyte at these time points. Furthermore, an inhibition of mitochondrial migration in oocytes was detected. The stimulation of the glycolytic pathway by AMP in the absence of gonadotrophins also changes the oxidative behavior, which reduces oocyte cytoplasmic competence.

406 ▪ Arzondo, M., Caballero, J., Marin Briggiler, C., Dalvit, G., Cetica, P., and Vazquez-
407 Levin, M. (2012). Glass wool filtration of bull cryopreserved semen: a rapid,
408 effective method to obtain a high percentage of functional spermatozoa.
409 *Theriogenology* (in press).
410
411 ▪ Barhan, D., and Trinder, P. (1972). An improved colour reagent for the
412 determination of glucose by the oxidase system. *Analyst.* **97**, 142.
413
414 ▪ Bilodeau-Goeseels, S., Sasseville, M., Guillemette, C., and Richard, F. (2007).
415 Effects of adenosine monophosphate- activated kinase activators on bovine oocyte
416 nuclear maturation in vitro. *Mol Reprod Dev*. **74**, 1021-1034.
417
418 ▪ Brackett, B., and Zuelke, K. (1993). Analysis of factors involved in the *in vitro*
419 production of bovine embryos. *Theriogenology.* **39**, 43-64.
420
421 ▪ Cetica, P., Pintos, L., Dalvit, G., and Beconi, M. (1999). Effect of lactate
422 dehydrogenase activity and isoenzyme localization in bovine oocytes and utilization of
423 oxidative substrates on *in vitro* maturation. *Theriogenology.* **51**, 541-550.
424
425 ▪ Cetica, P., Pintos, L., Dalvit, G., and Beconi, M. (2002). Activity of key enzymes
426 involved in glucose and triglyceride catabolism during bovine oocyte maturation in
427 vitro. *Reproduction.* **124(5)**, 675-81.
428

- Cetica, P., Pintos, L., Dalvit, G., and Beconi, M. (2003). Involvement of enzymes of amino acid metabolism and tricarboxylic acid cycle in bovine oocyte maturation *in vitro*. *Reproduction.* **126(6)**, 753-763.

- Chen, C., and Gee, K. (2000). Redox-dependent trafficking of 2,3,4,5,6- of pentafluorodihydrotetramethylrosamine, a novel fluorogenic indicator of cellular oxidative activity. *Free Radic Biol Med*. **28(8)**, 1266–1278.

- Clarenburg, R. (1992). Carbohydrate metabolism. In: Physiological chemistry of domestic animals. (Ed R. Reinhardt) pp. 239-291. (Mosby Year Book Inc, Saint Louis)

- Cran, D. (1985). Qualitative and quantitative structural changes during pig oocyte maturation. *J Reprod Fertil.* **74**, 237-245.

- Cummins, J. (2004). The role of mitochondria in the establishment of oocyte functional competence. *European Journal of Obstetrics, Gynecology and Reproductive Biology*, **115S**, 23-29.

- Downs, S., and Mastropolo, A. (1994). The participation of energy substrates in the control of meiotic maturation in murine oocytes. *Dev Biol.* **162**, 154-168.

- Downs S., and Utecht A. (1999). Metabolism of radiolabeled glucose by mouse oocytes and oocyte-cumulus cell complexes. *Biol Reprod.* **60(6)**, 1446-52.

- Dumollard, R., Ward, Z., Carroll, J., and Duchen, M. (2007). Regulation of redox metabolism in the mouse oocyte and embryo. *Development.* **134(3)**, 455:465.

- Ferreira, E., Vireque, A., Adona, P., Meirelles, F., Ferriani, R., and Navarro, P. (2009). Cytoplasmic maturation of bovine oocytes: Structural and biochemical modification and acquisition of developmental competence. *Theriogenology*. **71**, 836- 848.

- Fleming, W., and Saacke, R. (1972). Fine structure of the bovine oocytes and early embryos. *J Reprod Fertil.* **29**, 203-213.

- Gordon, I. (1994). Oocyte recovery and maturation. In: Laboratory production of cattle embryos. pp. 31: 142. (Cab International, Wallingford, UK).

- Gutnisky, C., Dalvit, G., Pintos, L., Thompson, J., Beconi, M., and Cetica, P. (2007). Influence of hyaluronic acid synthesis and cumulus mucification on bovine oocyte in vitro maturation, fertilisation and embryo development. *Reproduction, fertility and development.* **19**, 1-10.

- Harvey, A., Kind, K., and Thompson, J. (2002). REDOX regulation of embryo development. *Reproduction.* **123**, 479-486.

- Herrick, J., Brad, A., and Krisher, R. (2006). Chemical manipulation of glucose metabolism in porcine oocytes: effects on nuclear and cytoplasmatic maturation *in vitro*. *Reproduction.* **131**, 289-298.

- Hyttel, P., Xu, K., Smith, S., and Greve, T. (1986). Ultrastructure of *in vitro* oocyte maturation in cattle. *J Reprod Fertil.* **78**, 615- 625.

- Khurana, N., Niemann, H. (2000). Effect of oocyte quality, oxygen tension, embryo density, cumulus cells and energy substrates on cleavage and morula/blastocyst formation of bovine embryos. *Theriogenology*. **54**, 741-756.

- Kruip, T., Cran, D., Beneden, T., and Dieleman S. (1983). Structural changes in bovine oocytes during final maturation *in vivo*. *Gamete Res.* **8**, 29-47.

- Lim, J., Lee, B., Lee, E., Chung, H., Ko, J., Park, S., Cha, K., and Hwang, W. (1999). In *vitro* maturation and *in vitro* fertilization of bovine oocytes cultured in a chemically defined, protein-free medium: effects of carbohydrates and amino acids. *Reproduction, Fertility and Development.* **11**, 127-132.

- Mayes, P., and Bender, D. (2004). Glucólisis y la oxidación del piruvato. In: Harper. Bioquímica Ilustrada. 16º edición. pp. 153-161. (Editorial El Manual Moderno, México D.F.).

- Morado, S., Cetica, P., Beconi, M., Dalvit, G. (2009). Reactive oxygen species in bovine oocyte maturation in vitro. *Reproduction, Fertility and Development* **21**, 608-614.

- Nelson, D., and Cox, M. (2005). Glycolysis, gluconeogenesis and the pentose phosphate pathway. In: Lehninger. Principles of biochemistry. pp. 522-534. (4˚ edition. (W.H. Freeman and Company, New York)

- Nishi, Y., Takeshita, T., Sato, K., and Araki, T. (2003). Change of the mithocondrial distribution in mouse ooplasm during *in vitro* maturation. *J Nippon Med Sch.* **70(5)**, 408-415.

- Preis, K., Seidel, G. Jr., and Gardner, D. (2005). Metabolic markers of developmental competence for in vitro- matured oocytes. *Reproduction.* **130**, 475-483.

- Roberts, R., Stark, J., Iatropoulou, A., Becker, D., Franks, S., and Hardy, K. (2004). Energy substrate metabolism of mouse cumulus-oocyte complexes: response to follicle-stimulating hormone is mediated by the phosphatidylinositol 3-kinase pathway and is associated with oocyte maturation. *Biol Reprod.* **71,** 199–209.

- Rose-Hellekant, T., Libersky-Williamson, E., Bavister, B. (1998). Energy substrates and amino acids provided during in vitro maturation of bovine oocytes after acquisition of developmental competence. *Zygote.* **6**, 285-294.

- Schirmer, T, and Evans, P. (1990). Structural basis of the allosteric behaviour of phosphofructokinase. *Nature*. **343**, 140-145.

- Stojkovic, M., Machado, S., Stojkovic, P., Zakhartchenko, V., Hutzler, P., Gonçalves, P., Wolfi, E. (2001). Mitochondrial distribution and adenosine triphosphate content of bovine oocytes before and after in vitro maturation: correlation with morphological criteria and developmental capacity after in vitro fertilization and culture. *Biol Reprod*. **64**, 904-909.

- Sun, Q., Wu, G., Lai, L., Park, K., Cabot, R.., Cheong, H., Day, B., Prather, R., and Schatten, H. (2001). Translocation of active mitochondria during pig oocyte maturation, fertilization and early embryo development in vitro. *Reproduction.* **122**, 155-163.

- Sutton, M., Cetica P., Beconi, M., Kind, K., Gilchrist, R., and Thompson, J. (2003). Influence of oocyte-secreted factors and culture duration on the metabolic activity of bovine cumulus cell complexes. *Reproduction.* **126**, 27-34.

- Sutton Mc-Dowall, M., Gilchrist, R., Thompson, J. (2005). Effect of hexoses and gonadotrophin supplementation on bovine oocyte nuclear maturation during in vitro maturation in a synthetic fluid medium. *Reproduction, Fertility and Development*. **17**, 407-415.

- Sutton-McDowall, M., Gilchrist, R., and Thompson, J. (2010). The pivotal role of glucose metabolism in determining oocyte developmental competence. *Reproduction*. **139**, 685–695.

- Takahashi, Y., and First, N. (1992). *In vitro* development of bovine one-cell embryos: influence of glucose, lactate, pyruvate, amino acid and vitamins. *Theriogenology*. **37**, 963-978.

- Tarrazona, A., Rodriguez, J., Restrepo, L., and Olivera- Angel, M. (2006). Mitochondrial activity, distribution and segregation in bovine oocytes and in embryos produced *in vitro. Reprod Domest Anim.* **41**, 5-11.

- Trimarchi, J., Liu, L., Porterfield, D., Smith, P., and Keefe, D. (2000). Oxidative phosphorylation-dependent and independent oxygen consumption by individual preimplantation mouse embryos. *Biol Reprod.* **62**, 1866- 1874.

- Trinder, P. (1969). Determination of glucose in blood using glucose oxidase with an alternative oxygen acceptor. *Ann. Clin. Biochem.* **6**, 24-27.

- Wakefield, S., Lane, M., Schulz, S., Hebart, M., Thompson, J., and Mitchell, M. (2007). Maternal supply of omega-3 polyunsaturated fatty acids alter mechanisms involved in oocyte and early embryo development in the mouse. *Am J Physiol Endocrinol Metab*. **294**, E425: E434.

- Zuelke, K., and Brackett, B. (1992). Effects of luteinizing hormone on glucose metabolism in cumulus-enclosed bovine oocytes matured in vitro. *Endocrinology*. **131(6)**, 2690-6.

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The authors thank~~s~~ The Japanese International Cooperation Agency (JICA) for technology transfer and equipment, Deltacar abattoir for ovaries, Mr. V.H. Chaves for recovering the ovaries and Astra Laboratories for ultra-pure water.

Caption of the figures:

Fig 1:

Schematic representation of the four different regions (squares) within three areas (1 - 3) of ~~the~~ an oocyte (1 a) used for determining mitochondrial fluorescence.

Oocytes stained with ~~the dual~~two fluorescent probe~~s, of~~ MitoTracker Green (A) and RedoxSensor Red (B) at the differen~~ne~~t time points (Original magnification x 400). Oocytes stained with Hoechst DNA fluorochrome to ~~study~~ determine nuclear maturation (C) at the different time points: Hour 0 (germinal vesicle), Hour 9 (GVBD), ~~Hora~~ Hour 15 (metaphase I) and Hour 22 (1st polar body extrusion). The arrows ~~show~~ indicate the presence of metaphase II chromatin configuration and the lower arrow indicates the 1st polar body. (Original magnification x 1000) (1 b).

Comment [a1]: I too am confused by this....

Comment [a2]: The Little map has to be aligned in the same diagram as the actual representative micrographs, so the whole thing is one figure, with letters indicating each represntative figure.

Comment [a3]: ¿??

Comment [a4]: Where are the micrographs?

Fig 2:

Glucose uptake and lactate production in COCs matured with different concentrations of ATP (2 a), NaF (2 b), AMP (2 c) and AMP without gonadotrophin supplementation (2 d).

[a, b, c] Bars of the same color with different super index differ significantly (p<0,05).

n=30-40 COCs for each treatment.

Fig 3:

Cleavage ~~percentage~~ rate ~~in~~ following IVF from oocytes matured with AMP 10 mM without gonadotrophins.

[a, b, c] Bars with different super index differ significantly (p<0,05).

n=101-116 COCs for each treatment.

Fig 4:

Oxidative activity ~~of the~~within oocytes matured in the presence of ATP 10 mM (4 a), NaF 3 mM (4 b) or AMP 10 mM (4 c).

[a, b, c] Bars of the same color with different super index differ significantly (p<0,05).

*^ Significant difference **between** treatments at the same time.

n=30-40 COCs for each treatment.

Fig 5:

Mitochondrial activity ~~of the~~within oocytes matured in the presence of ATP 10 mM (5 a), NaF 3 mM (5 b) or AMP 10 mM (5 c).

[a, b, c] Bars of the same color and different super index differ significantly (p<0,05).

*^ Significant difference **between** treatments at the same time.

n=30-40 COCs for each treatment.

Fig 6:

Active mitochondria distribution ~~of~~ within ~~the~~ oocytes matured in the presence of ATP 10 mM (6 a), NaF 3 mM (6 b) or AMP 10 mM (6 c).

[a, b, c] Bars of the same color and different super index differ significantly (p<0,05).

* Significant difference **between** treatments at the same time.

n=30-40 COCs for each treatment.

Table 1:

Effect of ATP 10 mM and ~~Naf~~ NaF 3 mM on nuclear morphology

[a, b, c] Different superscript indicates ~~index differ~~ significant~~ly~~ differences in percentage of oocytes between treatments at the same time point and same nuclear stage.

n=15-20 oocytes for each treatment.

GV: Germinal vesicle, **GVBD**: Germinal vesicle Breakdown,

MI: Metaphase I y **PBE**: Polar body extrusion.

Table 1:

	Control				ATP				NaF			
	GV	GVBD	MI	PBE	GV	GVBD	MI	PBE	GV	GVBD	MI	PBE
Hour 0	100[a]				100[a]				100[a]			
Hour 9	29,2[a]	70,8[a]			100[b]	0[b]			88,9[b]	11,1[b]		
Hour 15	9,5[a]		90,5[a]		87,5[b]		12,5[b]		84,2[b]		15,8[b]	
Hour 22	11,1[a]		7,4[a]	81,5[a]	100[b]		0[a]	0[b]	58,8[c]		17,7[a]	23,5[c]

Figure 1:

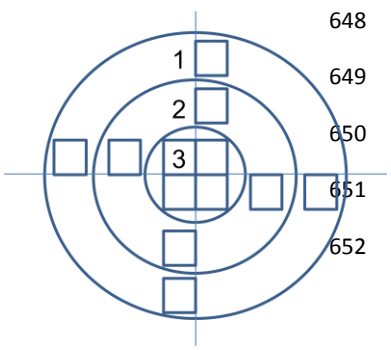
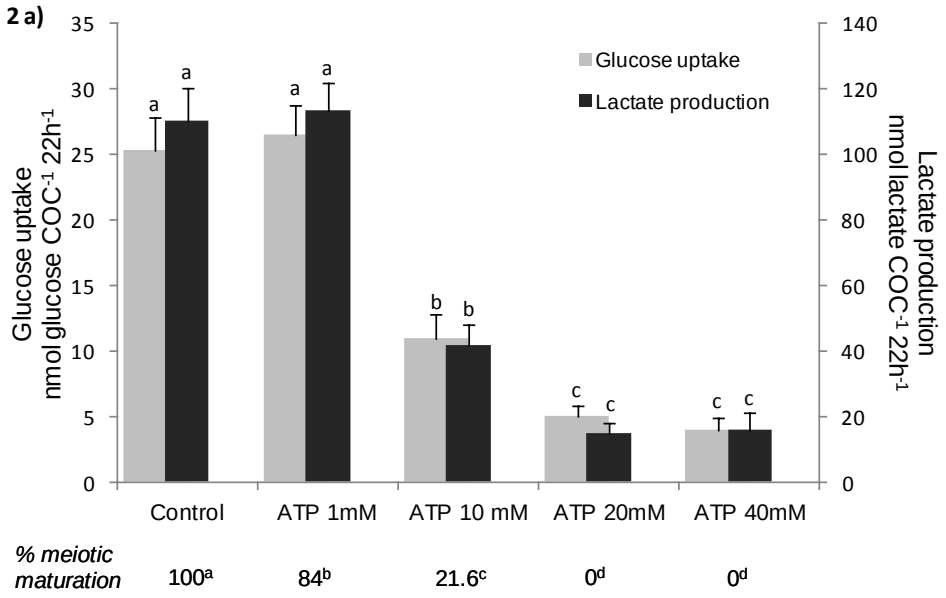
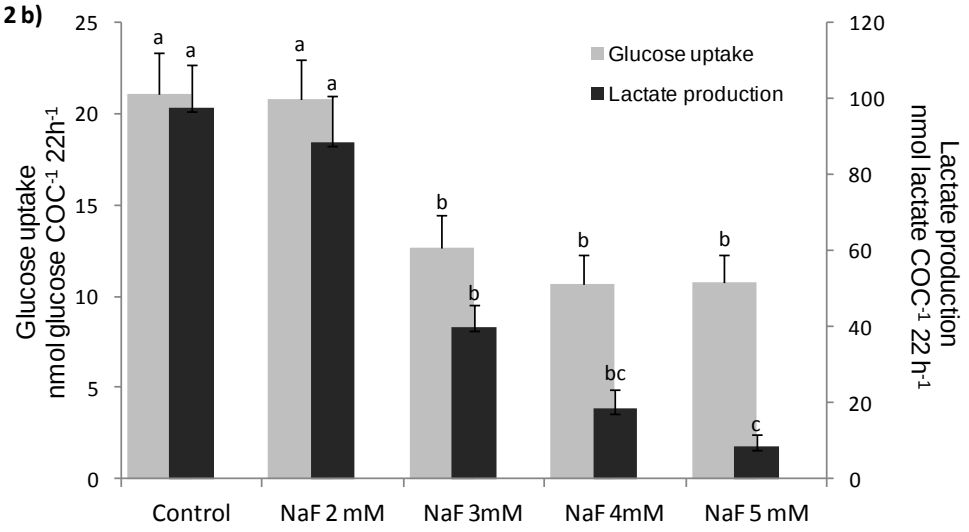


Figure 2:



Comment [a5]: Where are the micrographs – this needs to be combined with the figure above and therefore the labelling will change.

2 b)

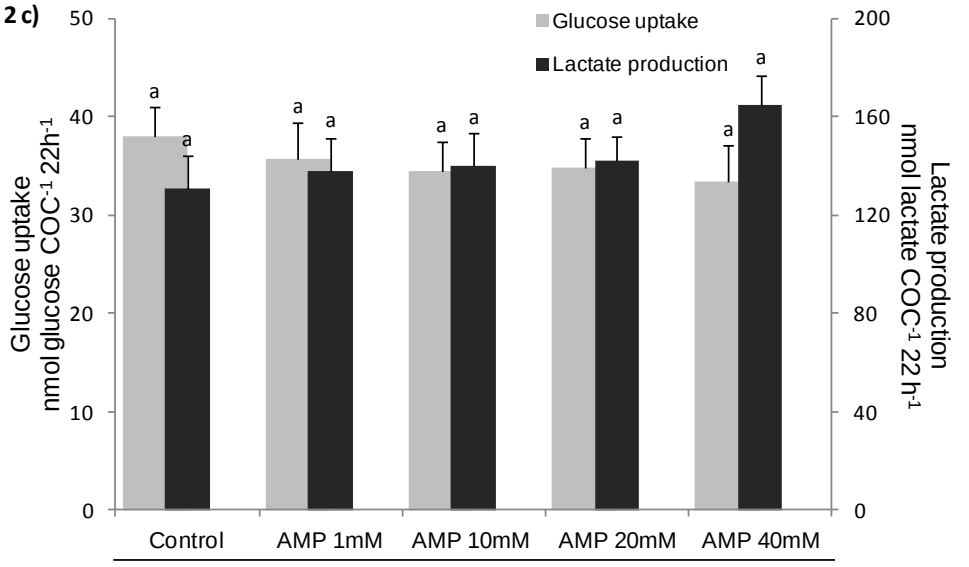


	Control	NaF 2 mM	NaF 3mM	NaF 4mM	NaF 5 mM
% meiotic maturation	85.2[a]	65.2[ab]	52.4[b]	28.6[c]	0[d]

658

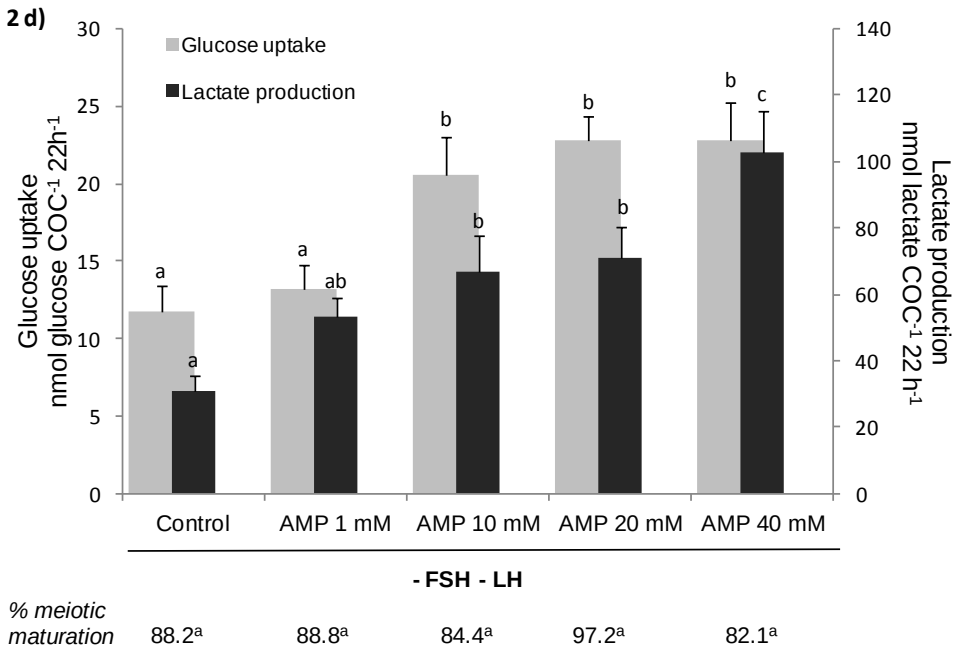
659

2 c)



	Control	AMP 1mM	AMP 10mM	AMP 20mM	AMP 40mM
% meiotic maturation	89.8[a]	87.7[a]	85.2[a]	86.5[a]	85.9[a]

660



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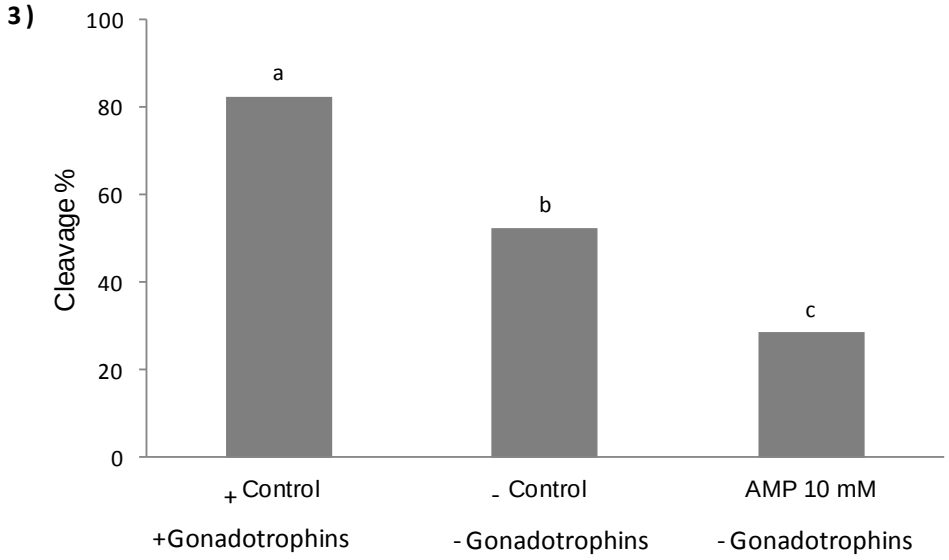
662 Figure 3:



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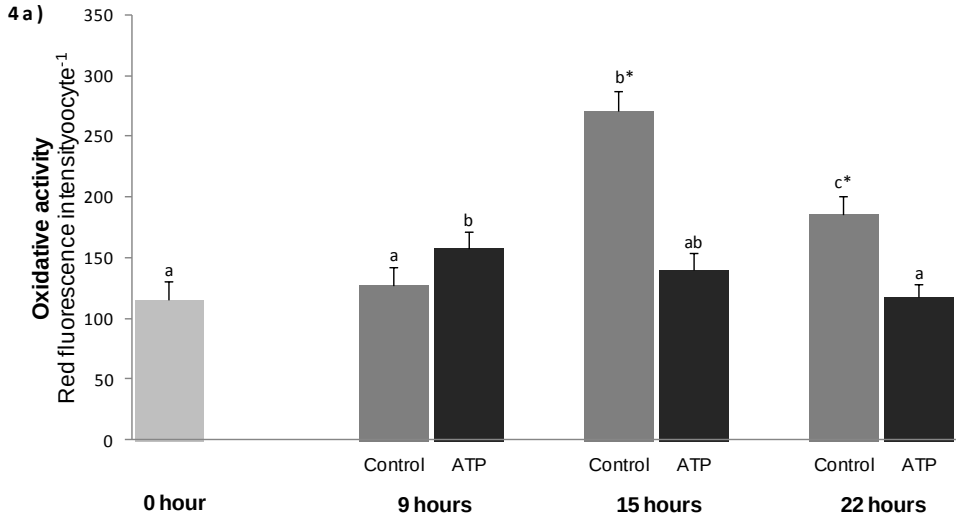
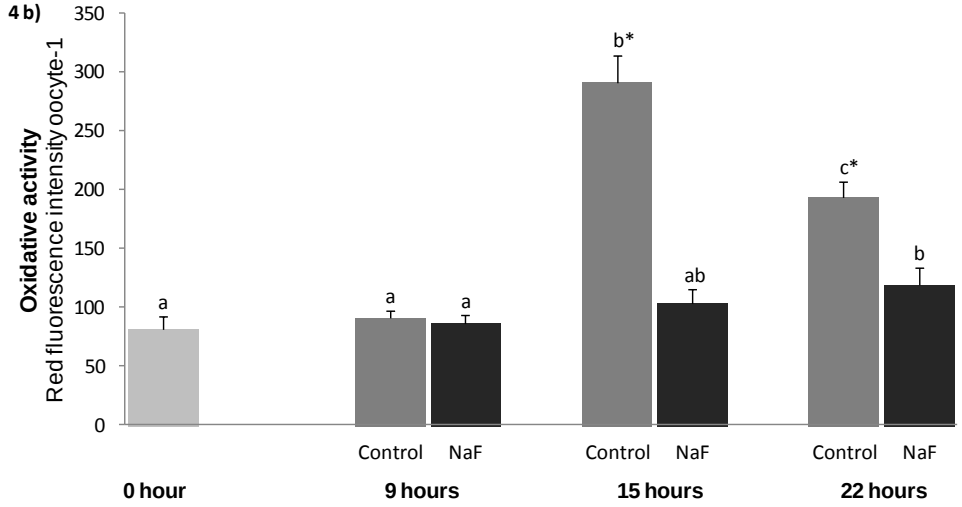
664 Figure 4:

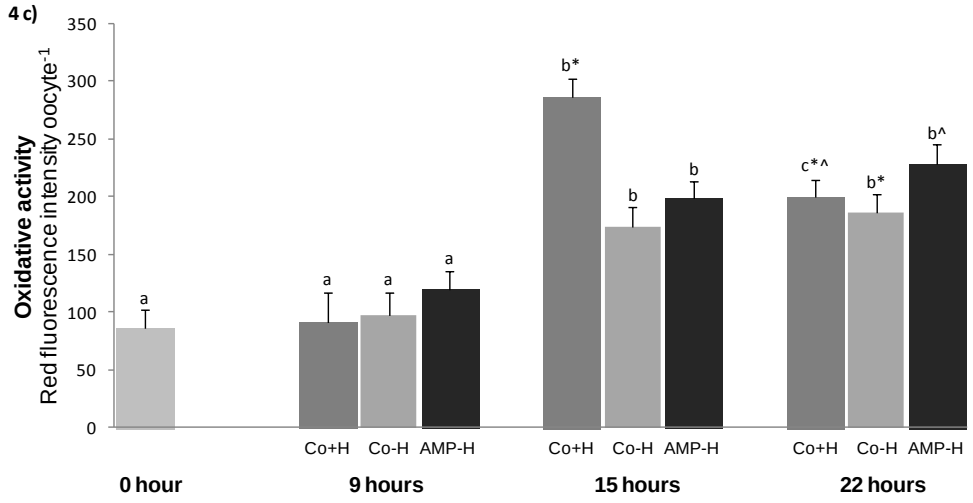

4 a)
Oxidative activity
Red fluorescence intensityoocyte-1
350
300
250
200
150
100
50
0
a
a
b
b*
ab
c*
a
Control
ATP
Control
ATP
Control
ATP
0 hour
9 hours
15 hours
22 hours

665


4 b)
Oxidative activity
Red fluorescence intensity oocyte-1
350
300
250
200
150
100
50
0
a
a
a
b*
ab
c*
b
Control
NaF
Control
NaF
Control
NaF
0 hour
9 hours
15 hours
22 hours

666

667



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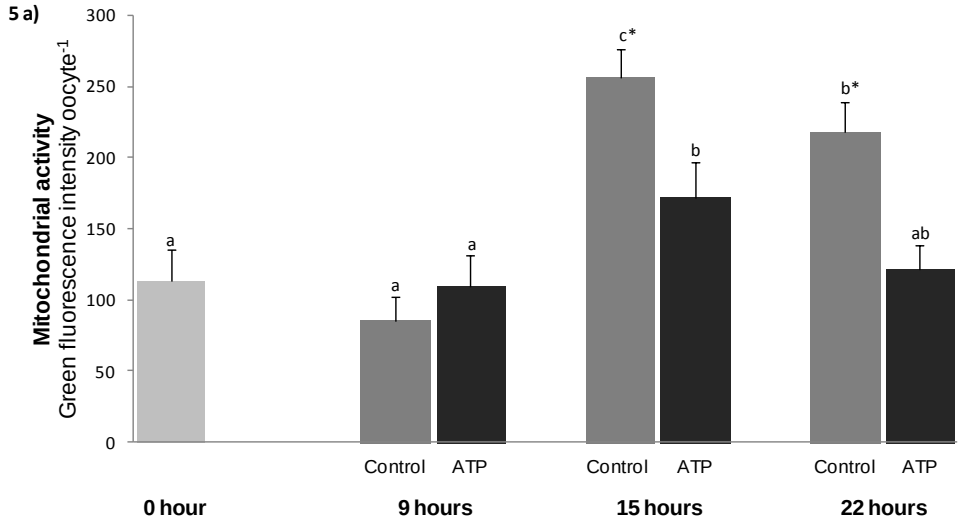
670 Figure 5:



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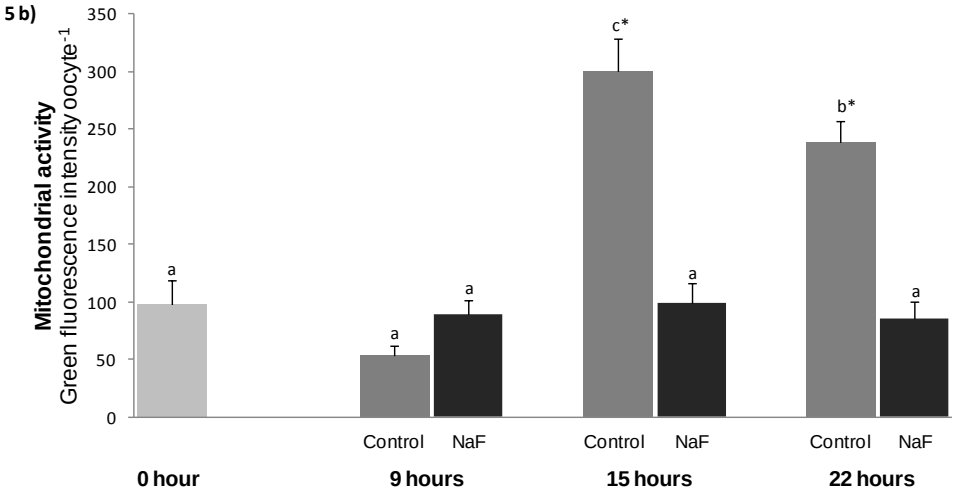

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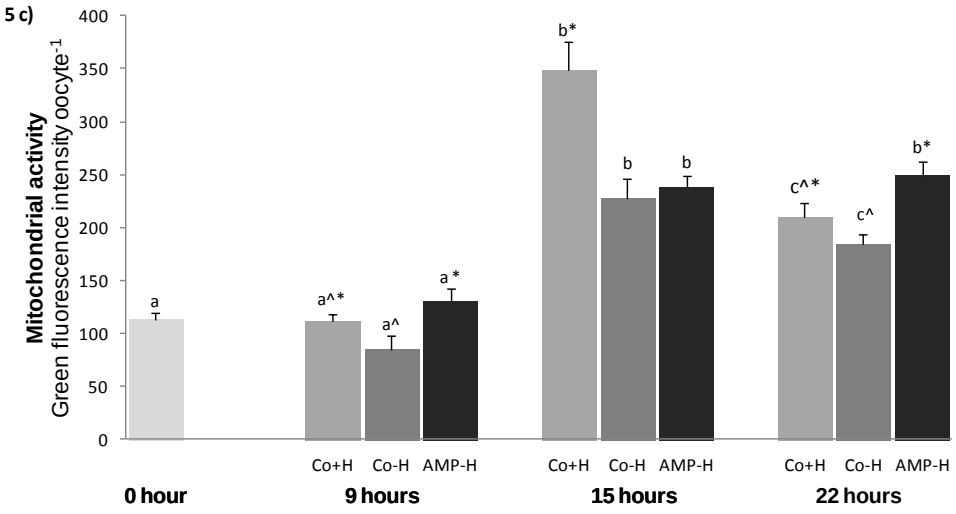

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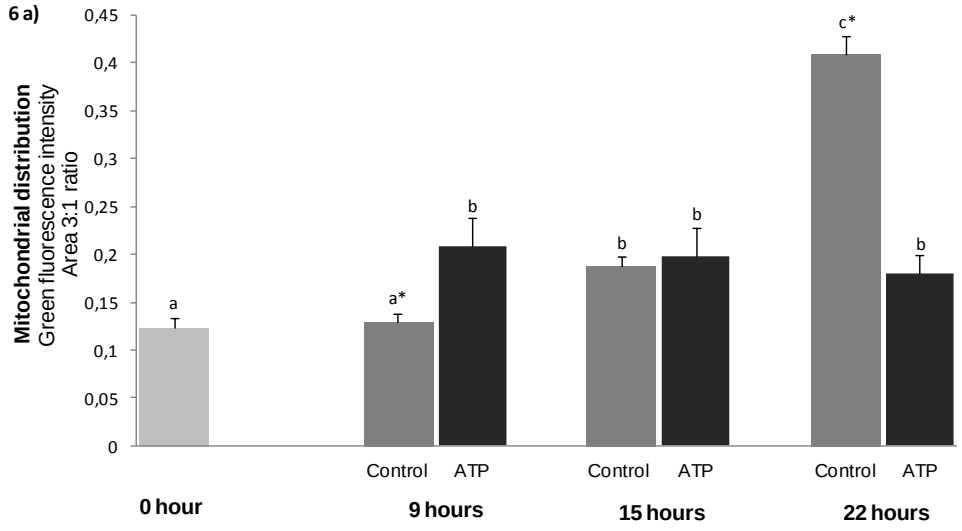
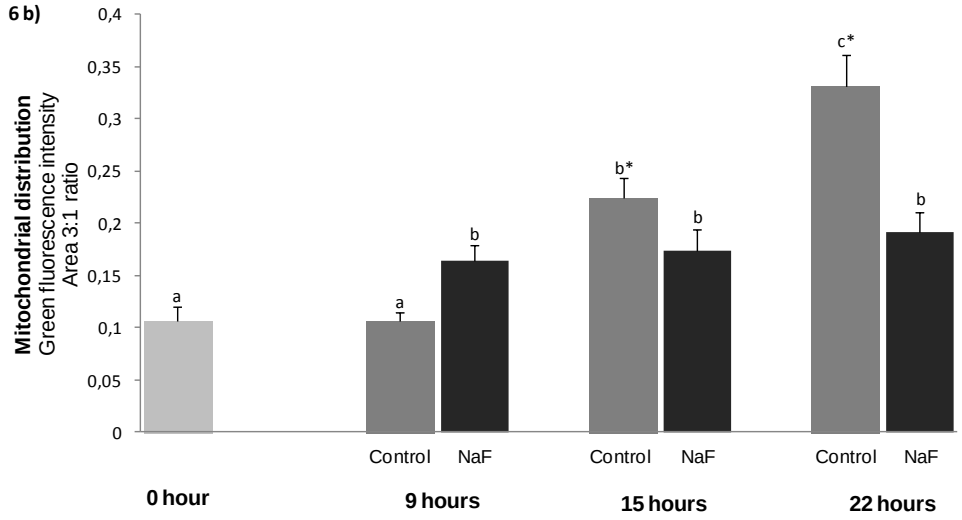
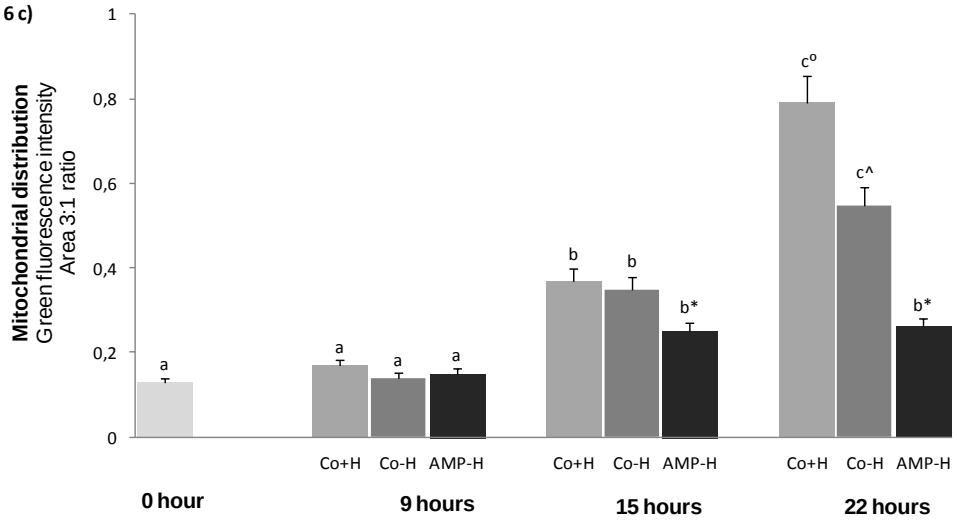
675 Figure 6:


6 a)
Mitochondrial distribution
Green fluorescence intensity
Area 3:1 ratio
0,45
0,4
0,35
0,3
0,25
0,2
0,15
0,1
0,05
0
a
a*
b
b
b
c*
b
Control
ATP
Control
ATP
Control
ATP
0 hour
9 hours
15 hours
22 hours

676


6 b)
Mitochondrial distribution
Green fluorescence intensity
Area 3:1 ratio
0,4
0,35
0,3
0,25
0,2
0,15
0,1
0,05
0
a
a
b
b*
b
c*
b
Control
NaF
Control
NaF
Control
NaF
0 hour
9 hours
15 hours
22 hours

677


6 c)
Mitochondrial distribution
Green fluorescence intensity
Area 3:1 ratio
1
0,8
0,6
0,4
0,2
0
a
a
a
a
b
b
b*
c°
c^
b*
Co+H
Co-H
AMP-H
0 hour
9 hours
15 hours
22 hours

678