

# Energy metabolism in pig oocytes and early embryos

R. G. Sturmey and H. J. Leese

Department of Biology (Area 3), University of York, PO Box 373, York YO10 5YW, UK

**Pig oocytes and embryos differ from those of other species in having a large quantity of endogenous lipid, a potential role for which has yet to be identified. In the present study, the hypothesis that endogenous triglyceride acts as a metabolic substrate during *in vitro* maturation and early embryo development was tested. Embryos were produced by *in vitro* fertilization (IVF) of *in vitro*-matured, abattoir-derived immature oocytes, cultured in medium NCSU23 up to the blastocyst stage. The triglyceride content of single oocytes and embryos was measured throughout development. Oxygen and glucose consumption and the formation of lactate were measured non-invasively over the same period, enabling total ATP production to be calculated. The triglyceride content of oocytes before maturation ( $135 \pm 4.9$  ng) decreased by 13 ng ( $P < 0.05$ ) during *in vitro* maturation, but there was no apparent change in triglyceride**

**content during embryo development (117.68 ng). Oxygen consumption was low throughout embryo cleavage before reaching a peak at the blastocyst stage ( $P < 0.01$ ), a pattern similar to that seen in other mammals studied. Glucose consumption and lactate production were also at a maximum at the blastocyst stage ( $P < 0.05$ ). These data indicate that pig oocytes may use endogenous triglyceride as an energy source during *in vitro* maturation and that most (91–97%) of the ATP produced during embryo development comes from oxidative phosphorylation. The high exogenous glucose concentration in NCSU23 ( $5.5 \text{ mmol l}^{-1}$ ) may be needed to form pyruvate, which in turn, produces oxaloacetate, which is required to prime the tricarboxylic acid cycle. However, the reason for the high lipid content in early pig embryos remains to be elucidated.**

## Introduction

Pig oocytes and early embryos appear dark, due to a high quantity of endogenous lipid. An immature pig oocyte typically contains 156 ng lipid (McEvoy *et al.*, 2000), a value much higher than that of oocytes of mice, cows and sheep, which typically contain 4 (Lowenstein and Cohen, 1964), 58 (Ferguson and Leese, 1999) and 89 ng (Coull *et al.*, 1998), respectively. Homa *et al.* (1986) reported that triglyceride was the major component of intracellular lipid in immature, *in vivo*-derived pig oocytes, indicating that the presence of lipid is not an artefact of *in vitro* culture. The most likely role of triglyceride is as an intracellular energy store. However, there has been no detailed study of the role of triglyceride during oocyte maturation and subsequent embryo development. Energy is derived from triglyceride by  $\beta$ -oxidation, a process that requires oxygen. Measurement of oxygen consumption and triglyceride content potentially provides a means of determining whether triglyceride is metabolized during development and its contribution, if any, to ATP production.

With respect to exogenous energy sources in mammalian embryos, a major focus has been on glucose, the uptake of which by pig embryos was first characterized

by Flood and Wiebold (1988). Techniques to measure energy metabolism in early pig embryos have included the use of radiolabelled substrates (Swain and Krisher, 2001; Swain *et al.*, 2001, 2002) and non-invasive assays to measure glucose consumption and the production of lactate (that is, glycolysis) (Gandhi *et al.*, 2001). Together with oxygen consumption, measurements of glucose consumption and lactate production allow the total production of ATP to be calculated.

Pig embryos are mostly produced *in vitro* using NCSU23 medium (Petters and Reed, 1991). However, by providing only glucose and glutamine as potential energy substrates, this medium differs greatly from those used to produce embryos in other species. Moreover, the concentration of glucose in NCSU23 ( $5.5 \text{ mmol l}^{-1}$ ) is not within the physiological range. In the oviduct, pig embryos are exposed to  $0.17 \text{ mmol glucose l}^{-1}$  (Nichol *et al.*, 1998), which is significantly lower than the concentration in NCSU23. High glucose concentrations have been implicated as inhibitory to the development of mouse (Chatot *et al.*, 1989) and hamster embryos (Seshagiri and Bavister, 1989); however, attempts to produce pig embryos in a lower glucose concentration have proved unsuccessful. Thus, KSOM and Gardner's G1.1/2.1 medium, both of which provide glucose at a lower concentration than NCSU23, have failed to give rates of blastocyst development comparable with those achieved in medium NCSU23 (Machaty *et al.*, 1998;

Email: rgs102@york.ac.uk

Gandhi *et al.*, 2001). It is clear that development of pig embryos is superior in media containing high concentrations of glucose and, as such, it is important to characterize their metabolism in terms of glucose uptake and lactate production.

In the present study, the role of different energy sources during maturation of pig oocytes and early embryo development was examined. The triglyceride content of pig eggs during *in vitro* maturation and of the resulting embryos throughout development up to the blastocyst stage was determined. This approach was combined with measurements of the uptake of oxygen and glucose, and the formation of lactate, thereby allowing calculation of total ATP production.

## Materials and Methods

### Supplier

All chemicals were purchased from Sigma-Aldrich (Poole) unless otherwise stated.

### Embryo production

Reproductive tracts obtained from an abattoir were placed in PBS supplemented with 400  $\mu\text{l}$  of antibiotic-antimycotic solution (10 000 U penicillin G  $\text{ml}^{-1}$ , 10 000  $\mu\text{g}$  streptomycin sulphate  $\text{ml}^{-1}$ , 25  $\mu\text{g}$  amphotericin  $\text{ml}^{-1}$ ; Invitrogen Life Technologies, Paisley) and maintained at 25°C in a Thermos flask during transportation to the laboratory. Upon arrival, ovaries were removed from the tracts and rinsed in luke-warm water before being rinsed four times in sterile PBS containing antibiotic-antimycotic solution. Oocytes were harvested by aspirating medium-sized follicles (3–6 mm in diameter), using an 18.5-gauge needle attached to a 10 ml disposable syringe containing 2 ml pre-warmed TCM199 supplemented with 5.0 mmol  $\text{NaHCO}_3$   $\text{l}^{-1}$ , 15.0 mmol Hepes  $\text{l}^{-1}$  ( $\text{Na}^+$  salt/free acid), 0.05 g kanamycin sulphate  $\text{l}^{-1}$ , 0.4 g BSA  $\text{l}^{-1}$  (fraction V) and 0.04 g heparin  $\text{l}^{-1}$ . Viable cumulus–oocyte complexes (COCs) were selected under a stereomicroscope. Selection was on the basis of at least two complete layers of cumulus cells and an intact, evenly granulated ooplasm. Selected COCs were washed twice in holding medium without heparin and washed a further three times in chemically defined oocyte maturation medium (TCM199 plus 0.1% (w/v) PVA containing 0.5  $\mu\text{g}$  FSH  $\text{ml}^{-1}$ , 0.5  $\mu\text{g}$  LH  $\text{ml}^{-1}$ , 0.57 mmol cysteine  $\text{l}^{-1}$  and 10 ng epidermal growth factor (EGF)  $\text{ml}^{-1}$ ) (Abeydeera *et al.*, 2000). Groups of 50 COCs were placed into culture in 100  $\mu\text{l}$  droplets of maturation medium that had previously been covered in embryo-tested mineral oil and pre-equilibrated in 5%  $\text{CO}_2$  in air.

After 44 h of maturation, 0.1% (w/v) hyaluronidase was added to the droplets to facilitate loosening of the cumulus cells. The COCs were washed three times, without

any further selection, in IVF medium (that is, modified Tris-buffered medium (mTBM); Abeydeera and Day, 1997) supplemented with 2.5 mmol caffeine  $\text{l}^{-1}$  and 0.4% (w/v) BSA. Groups of 35 COCs were placed in 50  $\mu\text{l}$  droplets of IVF medium that had previously been covered in embryo-tested mineral oil and pre-equilibrated in a 5%  $\text{CO}_2$  incubator. Fertilization was performed using frozen–thawed spermatozoa from a boar of proven fertility. Spermatozoa were thawed in a waterbath at 45°C for 10 s. The motile spermatozoa were separated by centrifugation at 1000  $\text{g}$  in a 45%:90% Percoll gradient for 30 min (Jeong and Yang, 2001). The pellet was re-suspended in 4 ml pre-gassed mTBM and centrifuged at 500  $\text{g}$  for 10 min to remove remaining traces of Percoll. The resulting pellet was re-suspended in 500  $\mu\text{l}$  mTBM. After appropriate dilution, 50  $\mu\text{l}$  sperm suspension was added to the IVF droplets containing the oocytes, to give a final sperm concentration of  $1 \times 10^6$   $\text{ml}^{-1}$  in the IVF droplet. The oocytes and spermatozoa were co-incubated for 6 h.

After sperm–oocyte co-incubation, putative zygotes were collected into 1 ml of pre-equilibrated NCSU23 medium (Petters and Reed, 1991), supplemented with 0.4% (w/v) BSA and vortexed for 2 min to remove loosely attached spermatozoa and cumulus cells. Putative zygotes were washed twice through fresh, pre-equilibrated NCSU23 + 0.4% (w/v) BSA, before being cultured in groups of 20 in 20  $\mu\text{l}$  droplets of the same medium for 6 days. Rates of blastocyst development of  $32 \pm 6.3\%$  were maintained consistently for the duration of the study.

Embryos that were assayed non-invasively were returned to culture and their subsequent development was recorded to the blastocyst stage. Embryos were only subjected to one assay procedure to minimize any potential stress effects. Data from the embryos that developed successfully were included in the study; embryos that showed arrested development were excluded from the study. All experiments were performed using between 9 and 39 embryos for each stage and, for every assay, three readings per sample were recorded. Glucose and lactate assays were performed on single embryos, with oxygen assays performed on groups, ranging from 5 to 15 embryos per observation.

### Measurement of oxygen consumption

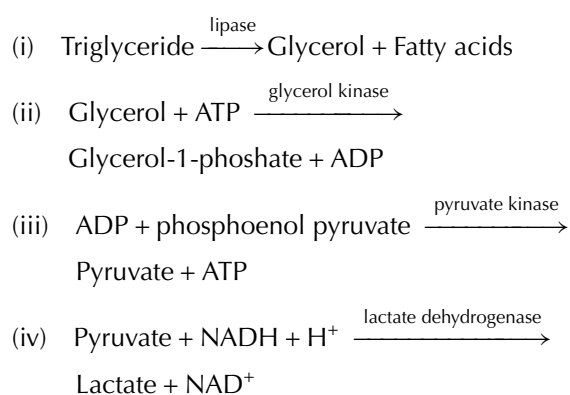
Oxygen consumption by oocytes and preimplantation embryos was measured as described by Houghton *et al.* (1996). In brief, small groups of oocytes or embryos (5–15 depending on developmental stage) were incubated in 2  $\mu\text{l}$  pre-equilibrated culture medium adjacent to a droplet of pyrene, in a 5  $\mu\text{l}$  PCR micropipette. The tube was rendered airtight by sealing the plunger end with wax, and placing rubber tubing over the open end, which was sealed using a clamp. Pyrene is a non-toxic highly fluorescent compound, the fluorescence of which

is quenched in the presence of oxygen. The pyrene is dissolved in mineral oil at a concentration of  $1 \text{ mmol l}^{-1}$ . Oxygen consumption was measured by assessing the change in fluorescence of the pyrene using a quantitative fluorescence microscope (Fluovert; Leica UK Ltd, Milton Keynes), with photomultiplier and photometer attachments (Leese and Barton, 1984; Gardner and Leese, 1986). Readings were taken at 15 min intervals for approximately 6 h. For each experiment, two controls were performed: (i) a 0% oxygen control, containing  $1 \mu\text{l}$  pyrene and  $2 \mu\text{l}$  of  $1 \text{ mg yeast ml}^{-1}$  dissolved in  $60 \text{ mmol glucose l}^{-1}$  pre-equilibrated for 12 h; and (ii) a 20% oxygen control, containing  $1 \mu\text{l}$  pyrene and  $2 \mu\text{l}$  oxygen-saturated culture medium. The fluorescence readings obtained were converted into values for oxygen depletion, using a mathematical model (Houghton *et al.*, 1996). On completion of the assay, oocytes or embryos were removed from the micropipette, placed into fresh medium and cultured to the blastocyst stage.

#### Assessment of triglyceride content of oocytes and embryos

**Preparation of samples.** Cumulus cells were removed from COCs by repeated pipetting through finely pulled Pasteur pipettes of decreasing diameter in the presence of 0.1% (w/v) hyaluronidase. All samples were incubated with 0.1% (w/v) pronase for approximately 30 s to remove the zona pellucida, thereby allowing the enzymes used in the triglyceride assay access to the intracellular triglyceride. Samples were frozen individually in  $5 \mu\text{l}$  microcaps in  $1.1 \mu\text{l}$  freezing buffer (PBS supplemented with 0.3% (v/v) fetal calf serum (FCS) and 0.1% (v/v) BSA). The FCS was necessary to provide a background amount of triglyceride that could be readily measured by the assay kit. The microcaps were sealed with parafilm and frozen at  $-80^\circ\text{C}$  until analysis.

**Determination of triglyceride content.** The triglyceride assay (Ferguson and Leese, 1999) is based on the enzymatic hydrolysis of triglyceride:



The amount of glycerol present in the sample was quantified in terms of the amount of NADH oxidized to  $\text{NAD}^+$  (reaction iv) measured using a Fluovert quantitative fluorescent microscope with photometer and photomultiplier attachments. The reagents necessary for the assay were derived from a triglyceride test kit (Sigma), scaled down to work on a microlitre scale. The assay used two reaction mixtures: mixture A contained ATP, lactate dehydrogenase, lipase, NADH, phosphoenol pyruvate, pyruvate kinase, buffer and non-reactive stabilizers and fillers; mixture B contained glycerol kinase and non-reactive stabilizers and fillers.

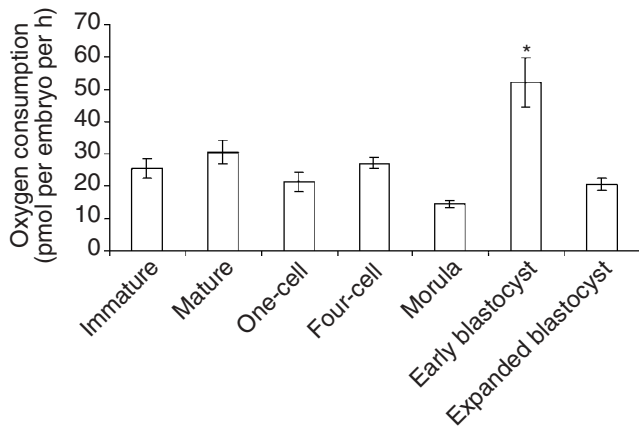
The fluorescence microscope was calibrated before each assay using  $5 \mu\text{l}$  mixture A in a  $10 \mu\text{l}$  microcap at  $37^\circ\text{C}$  to obtain a maximum reading, and  $5 \mu\text{l}$  of 0.9% NaCl to provide a value for background fluorescence.

A standard curve was established before each assay. Six triglyceride standards in the range of  $0.00\text{--}0.54 \mu\text{g ml}^{-1}$  ( $0, 0.135, 0.270, 0.405, 0.459, 0.540 \mu\text{g ml}^{-1}$ ) were prepared by diluting FBS of known triglyceride concentration (according to certified values of triglyceride in the FBS) in 0.9% (w/v) NaCl. All standard curves used yielded a correlation coefficient ( $r^2$ ) value of at least 0.98 between fluorescence and triglyceride.

Reactions were performed in  $10 \mu\text{l}$  microcaps. An aliquot ( $5 \mu\text{l}$ ) of cocktail A was placed on to a siliconized slide and immediately taken up into the microcap. The microcap was sealed with Parafilm and incubated at  $37^\circ\text{C}$  for 10 min. After incubation, the contents were expelled on to a siliconized slide and the sample was added. The droplet was immediately taken back into the microcap, re-sealed and incubated at  $37^\circ\text{C}$  for 10 min to allow reaction (i) to occur. Any endogenous ADP was consumed by pyruvate kinase and lactate dehydrogenase reactions, (iii) and (iv). Fluorescence was measured at three different regions along the tube to provide initial readings, and the tube contents were expelled on to a siliconized slide. An aliquot ( $1 \mu\text{l}$ ) of cocktail B was added; the droplet was taken back into the microcap, re-sealed with Parafilm and incubated for a further 7 min at  $37^\circ\text{C}$  to allow reactions (ii), (iii) and (iv) to proceed. This protocol ensured that changes in fluorescence resulted only from the conversion of NADH to  $\text{NAD}^+$  derived from ADP resulting from the hydrolysis of triglyceride. Final fluorescence readings were taken in triplicate and averaged. The amount of triglyceride was calculated by relating changes in fluorescence to those given by the standards. Blank samples, containing freezing buffer but no embryos, were also assayed and the values were subtracted from the unknowns to account for dilution effects and triglyceride present in the freezing buffer.

#### Measurement of glucose consumption and lactate production

At specific stages of development, individual embryos were cultured for 3 h in  $76.55 \text{ nl}$  droplets of modified



**Fig. 1.** Oxygen consumption of pig oocytes and embryos during *in vitro* development. Number of observations = 5 (one- and four-cell stages) or 6 (all other stages). \*Indicates significant difference from other stages of development ( $P < 0.05$ ).

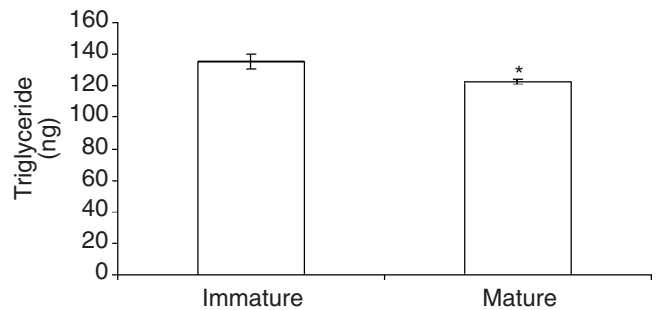
NCSU23. After preliminary investigations (data not shown), it proved necessary to reduce the glucose concentration in NCSU23 from  $5.5 \text{ mmol l}^{-1}$  to  $1.0 \text{ mmol l}^{-1}$  for uptake to be detected readily. The culture period was limited to 3 h to ensure that glucose did not become a limiting factor to embryo development. After this period, the embryos were removed and spent culture droplets were frozen at  $-80^\circ\text{C}$  until assayed. The embryos were returned to group culture and their development to the blastocyst stage was monitored. Glucose utilization and lactate production were determined by ultrafluorometric assays as previously described by Leese and Barton (1984), and Gardner and Leese (1990). Assays were carried out on a Fluovert quantitative fluorescence microscope (Leica, Milton Keynes). Before each assay, a standard curve was produced in the range  $0.0\text{--}1.1 \text{ mmol glucose l}^{-1}$  or  $0.0\text{--}4.4 \text{ mmol lactate l}^{-1}$ . The loss of glucose and formation of lactate in individual droplets were calculated by relating changes in fluorescence to those given by the standards.

#### Calculation of ATP production

Total ATP production was calculated on the basis that the consumption of one mole of oxygen produces six moles of ATP, and that the conversion of one molecule of glucose to two molecules of lactate produces two molecules of ATP (Donnay and Leese, 1999).

#### Data presentation and statistical analysis

Oxygen consumption by oocytes and embryos is presented as  $\text{pmol O}_2$  per embryo per h. The triglyceride content of oocytes and embryos is shown as mean  $\pm$  SEM. Glucose consumption and lactate production are expressed as  $\text{pmol}$  per embryo per h. Oxygen consump-



**Fig. 2.** Triglyceride content of pig oocytes before (immature) and after (mature) maturation. Number of oocytes assayed = 13 (immature) and 17 (mature). \*Indicates significant difference compared with immature oocytes ( $P < 0.05$ ).

tion and glucose consumption/lactate production across all stages was compared using a one-way ANOVA, with a Fisher's least square difference pair-wise comparison (Minitab V12.2). The triglyceride content of oocytes was compared using a sample *t* test, with analyses of embryo triglyceride content using ANOVA with Fisher's least square difference pair-wise comparison. The same tests were used for glucose consumption and lactate production.

## Results

### Oxygen consumption

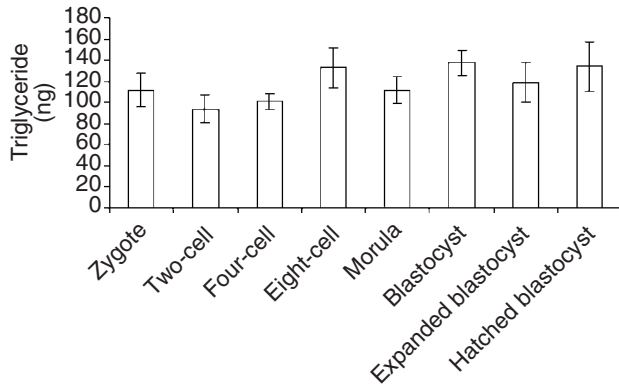
The oxygen consumption of pig oocytes before and after maturation and during early embryo development is shown (Fig. 1). There was considerable variation in the value for oxygen consumption at all stages measured, such that there were no significant differences for the values for oocytes before and after maturation, or from the zygote up to the morula stage. However, there was a highly significant ( $P < 0.01$ ) increase in oxygen consumption at the blastocyst stage, before a return to lower consumption at the expanded blastocyst stage.

### Triglyceride content

The triglyceride content of pig oocytes decreased during *in vitro* maturation from  $135.5 \pm 4.9 \text{ ng}$  in immature oocytes ( $n = 12$ ) to  $122.5 \pm 1.36 \text{ ng}$  after maturation ( $n = 12$ ) ( $P \leq 0.002$ ) (Fig. 2). This decrease of  $13 \text{ ng}$  during the 44 h maturation period is equivalent to a mean usage of  $0.296 \text{ ng}$  per oocyte per h. During embryo development there was no significant change in triglyceride content although there was considerable variation within sample groups (Fig. 3).

### Glucose consumption and lactate production

Glucose consumption did not vary from the zygote to the eight-cell stage, but increased slightly at the morula



**Fig. 3.** Triglyceride content of *in vitro*-derived pig embryos during development. Number of embryos assayed = 7 (expanded and hatched blastocyst stages), 10 (two-cell and morula stages), 12 (four-cell and zygotes), 13 (eight-cell) and 17 (blastocyst stage).

**Table 1.** Glucose use and lactate production by pig embryos

Stage of development	<i>n</i>	Glucose use (pmol per embryo per h)	Lactate production (pmol per embryo per h)
Zygote	23	4.1 ± 0.6 <sup>x</sup>	6.2 ± 1.9 <sup>ad</sup>
Two-cell	16	2.3 ± 0.2 <sup>x</sup>	1.8 ± 0.2 <sup>b</sup>
Four-cell	15	1.9 ± 0.4 <sup>x</sup>	4.1 ± 0.8 <sup>abd</sup>
Eight-cell	23	1.6 ± 0.2 <sup>x</sup>	3.9 ± 0.8 <sup>ab</sup>
Morula	39	6.7 ± 0.7 <sup>xz</sup>	6.2 ± 0.8 <sup>ad</sup>
Day 6 blastocyst	33	14.9 ± 2.7 <sup>y</sup>	8.4 ± 0.9 <sup>de</sup>
Day 7 blastocyst	26	9.2 ± 2.8 <sup>z</sup>	10.8 ± 2.6 <sup>e</sup>

Values are mean ± SEM.

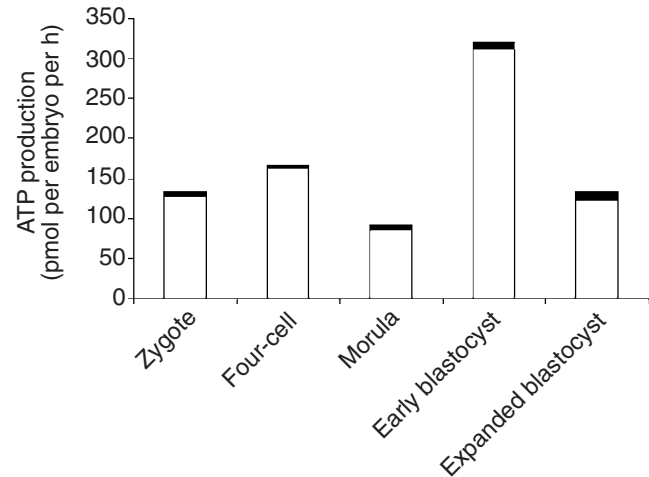
<sup>a-e</sup> and <sup>x-z</sup> Different superscripts within a column indicate significant differences ( $P < 0.05$ ).

stage. Glucose use had significantly increased ( $P < 0.05$ ) in day 6 blastocysts, before decreasing at the day 7 blastocyst stage ( $P < 0.05$ ) to a level similar to that of the morula stage (Table 1).

There was a significant decrease in lactate production from the zygote to the two-cell stage ( $P < 0.05$ ; Table 1). Lactate production then remained unchanged up to the morula stage before significantly increasing ( $P < 0.05$ ) at both the early and expanded blastocyst stages (Table 1).

#### Total ATP production

The total ATP production of pig embryos, as calculated from the production of lactate and the consumption of oxygen, is shown (Fig. 4). The total amount of ATP production remained low during the cleavage stages, reached a peak at the early blastocyst stage and decreased to amounts similar to those at the morula stage in expanded blastocysts. Throughout all stages of development, oxidative metabolism was the major producer of ATP, with formation resulting from the conversion of glucose to lactate making a small contribution (2.6–8.7% of total ATP production).



**Fig. 4.** ATP production by pig embryos during development. □: ATP from aerobic metabolism; ■: ATP from anaerobic glycolysis.

## Discussion

Oxygen consumption provides a measure of total energy production and a means of assessing the potential contribution of endogenous substrates, such as triglyceride (Guppy *et al.*, 2002). In the present study, the consumption of oxygen was measured at the beginning and end of maturation of pig oocytes, and during embryo development. The measurements were made on small groups of oocytes and embryos, thereby allowing accurate selection on the basis of developmental stage. As the method used was non-invasive, measurements could be taken at various stages, and the embryos could subsequently be cultured up to the blastocyst stage, thereby allowing realization of developmental potential.

The values for oxygen consumption of denuded pig oocytes did not differ significantly before and after maturation; they were slightly lower than those for bovine oocytes at comparable stages of development (Ferguson, 1999). When converted to conventional units of  $QO_2$  (that is,  $\text{mg} (\text{dry weight})^{-1} \text{h}^{-1}$ ), the oxygen consumption of pig oocytes was between  $4.48 \text{ mg}^{-1} \text{ dry weight h}^{-1}$  and  $5.26 \text{ mg}^{-1} \text{ dry weight h}^{-1}$ . These values are similar to those given by skin and bone (Newsholme and Leech, 1989), two tissues that have a relatively low metabolic rate, indicating that maturation of pig oocytes may represent a quiescent period, with only a modest requirement for ATP.

Values for oxygen consumption did not differ significantly during the early cleavage stages of pig embryo development, a pattern similar to that in mice (Houghton *et al.*, 1996), cows (Thompson *et al.*, 1996) and humans (Butcher *et al.*, 1998). However, at the blastocyst stage there was a marked increase in oxygen consumption, again following the pattern in other species. When the

blastocyst had completed expansion, oxygen consumption decreased to a value similar to that of pre-cavitation embryos. This observation may be explained in terms of the  $\text{Na}^+ - \text{K}^+ - \text{ATPase}$ , or sodium pump, which is one of the main consumers of energy in early embryos (Donnay and Leese, 1999) and somatic cells (Rolfe and Brown, 1997). Donnay and Leese (1999) measured oxygen consumption in bovine blastocysts in the presence and absence of ouabain (an inhibitor of the  $\text{Na}^+ - \text{K}^+ - \text{ATPase}$ ), and showed that 44% of  $\text{O}_2$  consumption is accounted for by the activity of this enzyme during blastocyst expansion. As the embryo completes formation of the blastocoel, a steady state is reached and it is likely that the sodium pump needs only to remain active enough to balance the leakage of ions from the blastocoel. This corresponds to a decrease in oxygen consumption at the expanded blastocyst stage.

Previous studies have shown that pig oocytes are rich in lipid (McEvoy *et al.*, 1997). Triglyceride is the major component of intracellular lipid in the oocytes (Homa *et al.*, 1986) and provides a large potential energy reserve. Techniques reported in earlier work have relied on large numbers of oocytes to detect intracellular lipid (from  $1 \times 10^3$  to  $1 \times 10^5$  per sample). In the present study, it was possible to quantify the triglyceride content of individual oocytes and embryos, which enabled the stage of development to be visualized precisely.

McEvoy *et al.* (2000) reported that the triglyceride content of immature, freshly aspirated pig oocytes was 74 ng, a value almost half that reported in the present study for oocytes at the same stage of development ( $135.5 \pm 4.9$  ng triglyceride in an immature oocyte). In the present study, the amount of triglyceride in immature oocytes ( $135.5 \pm 4.9$  ng) decreased to  $122.5 \pm 1.36$  ng after maturation ( $P < 0.05$ ), which represents a total decrease of 13 ng triglyceride or 0.3 ng triglyceride per oocyte per h. It can be calculated that the oxidation of 0.3 ng triglyceride requires 0.6 nl or 26.9 pmol of oxygen; that is, the oxygen consumed is more than sufficient to account for the metabolism of triglyceride equivalent to the decrease observed in the present study (13 ng). Ferguson and Leese (1999) showed a similar pattern in bovine oocytes, in which the decrease in triglyceride content during maturation was 13 ng, which is the same as the value for pig oocytes. These data provide strong support for a metabolic role for triglyceride in the *in vitro* maturation of pig and bovine oocytes. Kruip *et al.* (1983) reported that mitochondria are translocated from a peripheral to a cortical region and are associated with lipid droplets during maturation of bovine oocytes *in vivo*. Smooth endoplasmic reticulum (SER) also becomes associated with lipid droplets and with mitochondria, forming 'metabolic units' (Kruip *et al.*, 1983). This finding indicates that bovine oocytes may use lipid as an energy source, providing ATP for the protein synthesis that is necessary for continuation of cytoplasmic maturation and meiosis.

In the present study, triglyceride content did not differ significantly during embryo development. However, the variation between triglyceride values was such that the possibility of metabolism of triglyceride during embryo development cannot be excluded. Thus, it may be calculated that the oxygen consumption during the cleavage stages could be satisfied by the usage of only 5 ng triglyceride per day, increasing to 13.7 ng at the blastocyst stage.

Pig embryos consumed glucose and produced lactate at all stages of development. Glucose use was low during the early cleavage stages, increased slightly at the morula stage and reached a peak at the early blastocyst stage. At the expanded blastocyst stage glucose uptake decreased to similar values to those found at the morula stage. The marked increase in glucose uptake at the blastocyst stage has previously been reported in mouse (Houghton *et al.*, 1996), bovine (Thompson *et al.*, 1996), pig (Swain *et al.*, 2001; Swain and Krisher, 2001), rat (Brison and Leese, 1991), rabbit (Brinster, 1969) and human embryos (Gott *et al.*, 1990) and appears to be a universal feature of early mammalian development. Gandhi *et al.* (2001) investigated glucose consumption and lactate production in pig embryos at four specific stages of development (two-cell, eight-cell, morula and blastocyst) using non-invasive techniques. However, the present study is the first in which the metabolism of *in vitro* produced pig embryos at all stages of development, cultured in NCSU23, has been characterized. It was necessary to reduce the glucose concentration for the assay culture period, but no other modifications to NCSU23 were made. Gandhi *et al.* (2001) performed assays in a metabolic assessment medium, which not only differed from NSCU in terms of glucose concentration ( $0.5 \text{ mmol l}^{-1}$  versus  $5.5 \text{ mmol l}^{-1}$  in NCSU23), but also provided pyruvate ( $0.5 \text{ mmol l}^{-1}$ ), and  $1 \times$  non-essential amino acids, which are not normally present in NCSU23. However, the values reported for glucose consumption were similar to those found in the present study. This finding indicates that the metabolism of pig embryos may be different from that of other species, although this may not be the case *in vivo* as the embryo will be exposed to a wider range of potential metabolites (Iritani *et al.*, 1974; Khandoker *et al.*, 1997; Nichol *et al.*, 1998). It has previously been shown that the metabolism of *in vivo*-derived embryos differs significantly from that of *in vitro*-derived embryos, and that *in vivo*-derived embryos have higher rates of glycolysis at all stages investigated, and an increase in glycolytic activity at the blastocyst stage (Swain *et al.*, 2002).

Lactate formation was low and quite variable during the cleavage stages, but increased steadily after compaction, reaching a peak in the expanded blastocyst. This general trend was also reported by Swain *et al.* (2002). At the zygote and two-cell stages, loss of glucose from the medium was in excess of that which could be accounted for in terms of lactate production. At the

four- and eight-cell stages, all of the loss of glucose can be accounted for by lactate production, implying that glycolysis is the dominant pathway of glucose metabolism, as found by Gandhi *et al.* (2001). At compaction, the proportion of glucose consumption that could be accounted for by lactate production decreased to approximately 50%, and remained at this value for the remainder of development. It is possible that the glucose unaccounted for by lactate formation is metabolized by the pentose phosphate pathway or via complete oxidation. Flood and Wiebold (1988) proposed that the pentose phosphate pathway was the dominant pathway before the eight-cell stage in *in vivo*-derived pig embryos, with a shift to a largely glycolytic metabolism at the morula stage. The data from the present study indicate that glycolysis probably occurred throughout development. The pentose phosphate pathway may be important in the provision of NADPH, which can generate glutathione, which provides protection for the embryo against oxidative damage. This role may be particularly important in pig embryos because of the high proportion of unsaturated lipid (Homa *et al.*, 1986; Youngs *et al.*, 1994).

Total ATP production could be estimated from the data on oxygen consumption, and from the conversion of glucose to lactate. Two moles of ATP are produced for each mole of glucose converted to lactate; six moles of ATP are generated for each mole of oxygen consumed. Total ATP production did not differ significantly during the cleavage stages, but doubled at the early blastocyst stage. This increase in ATP production coincides with the increase in energy requirements as a result of relocalization and increased activity of the Na<sup>+</sup>-K<sup>+</sup>-ATPase (Macphee *et al.*, 1994). Machaty *et al.* (2001) used 2,4-dinitrophenol and sodium azide to inhibit ATP production by mitochondria and demonstrated that oxidative phosphorylation is vital for the development of early pig embryo pre-cavitation. Embryo development from the zygote stage was completely arrested by inhibiting oxidative phosphorylation. This finding was not surprising as we have shown that at all stages of development, the proportion of ATP generated by oxidative metabolism is high (about 95%). However, Machaty *et al.* (2001) reported that the addition of sodium azide, an inhibitor of oxidative phosphorylation, and 2,4-dinitrophenol, an uncoupler of oxidative phosphorylation, to culture medium at the morula stage resulted in an increase in the number of cells in pig blastocysts. The mechanism of this intriguing effect is not known.

Nichol *et al.* (1992) reported that pig oviduct fluid collected on day 1 after ovulation contained 0.17 mmol glucose l<sup>-1</sup>, 6.48 mmol lactate l<sup>-1</sup> and 0.22 mmol pyruvate l<sup>-1</sup>. In contrast, medium NCSU23 contains 5.5 mmol glucose l<sup>-1</sup> and 1.0 mmol glutamine l<sup>-1</sup>, but no lactate or pyruvate. Despite this, NCSU23 has repeatedly been shown to be the most successful culture medium for the

production of pig blastocysts *in vitro* (Machaty *et al.*, 1998; Gandhi *et al.*, 2001; Swain *et al.*, 2001). This apparent preference for a high glucose concentration is unusual as such concentrations can inhibit development of mouse (Chatot *et al.*, 1989) and hamster (Seshagiri and Bavister, 1989) embryos. A possible explanation for the preference of *in vitro*-produced pig embryos for 5.5 mmol glucose l<sup>-1</sup> is in terms of its potential role in lipid metabolism. If the free fatty acids derived from the triglyceride are oxidized, small amounts of carbohydrate are required to provide oxaloacetate to prime the TCA cycle. Oxaloacetate can be derived from pyruvate via the enzyme pyruvate carboxylase. As NCSU23 does not contain pyruvate, pig embryos grown in NCSU23 are likely to derive their pyruvate from glucose via the glycolytic pathway, although it is not clear why pig embryos should prefer such a high concentration of glucose (5.5 mmol l<sup>-1</sup>) when produced in NCSU23 medium. It is possible that the transport systems for glucose or pyruvate or both are not sufficiently developed.

In conclusion, there is good evidence for a role for endogenous triglyceride in maturation of pig oocytes; the evidence is less compelling for a role at the early stages of embryo development. Further study is required to determine why pig oocytes and embryos contain such large quantities of lipid.

The authors would like to acknowledge Grampian Country Pork for the donation of pig ovaries, PIC Fyfield and GTC (Mains of Murthy) for provision of the boar semen, and L. Abeydeera and F. Houghton for their helpful comments and discussion relating to this manuscript. R. G. Sturmey is funded by the BBSRC and Sygen International.

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Received 10 January 2003.

First decision 18 February 2003.

Revised manuscript received 24 April 2003.

Accepted 1 May 2003.