New approaches for non-invasive embryo quality assessment
ESHRE Special Interest Group Embryology

Embryo viability and metabolism: obeying the quiet rules

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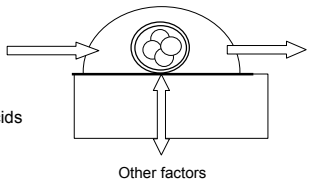
Embryo viability and metabolism: obeying the quiet rules

- Non-invasive assessment of embryo metabolism in relation to the outcome of embryo transfer:
  - Glucose
  - Glycolysis
  - Pyruvate
  - Amino acids
- *Quiet embryo hypothesis*
- Categories of quietness:
  - Functional
  - Due to individual differences between cells
  - In response to different environments
- Molecular & cellular determinants of a quiet phenotype
- Somatic cells/Speculations

Metabolism of early embryos:
candidates for non-invasive assessment

Depletion	Appearance
Pyruvate	H_20
Lactate	CO_2
Glucose	Lactate
Lipid	Amino acids
Amino acids	NH_4^+
Oxygen	Enzymes
	Cytokines
	Proteins



Early history of non-invasive nutrient assessment in relation to outcome of transfer

Renard et al (1980) *J Reprod Fertil* **58**, 161
Day 10 bovine blastocysts with glucose uptake >5 µg/h
have greater viability post-transfer

Rieger et al (1984) *Theriogenology* **21**, 138
Measurement of metabolic activity as an approach to evaluating viability and diagnosing sex in early embryos

Gardner & Leese (1987) *J Exp Zool* **242**, 103
Glucose consumption by day 4 mouse
blastocysts correlated with success of embryo transfer

Original Hypothesis:

A viable embryo has a high metabolism*

* ability to give rise to live offspring following transfer

Glucose

In-vitro uptake of glucose by bovine blastocysts
Renard JP, **Philippon A**, **Menezo Y**.

Blastocysts, obtained from cows on Day 10-11 after oestrus, were cultured for 20 h. Most (81.3%) blastocysts grew in culture and about 50% took up glucose.
There was no morphological difference between the blastocysts which did or did not take up glucose but development in vivo was better for blastocysts which had taken up glucose (69.2%) than for those which did not (14.2%).

Journal of Reproduction & Fertility **58**:161 -164.

Uptake of glucose (pmol/embryo/h) by single mouse blastocysts prior to transfer into pseudopregnant recipients
Gardner & Leese (1987) *Journal of Experimental Zoology* **242**: 103-105

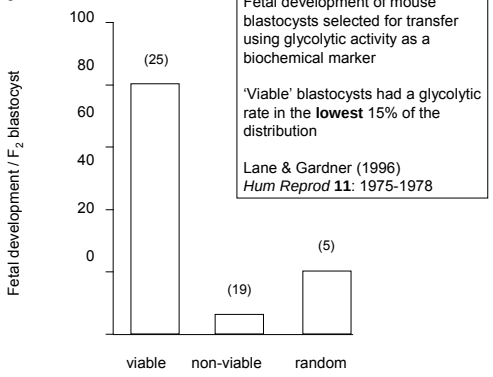
Viable		Non-viable
Male	Female	
4.37	4.92	3.57
(26)	(13)	(12)

Conclusion:

Mouse embryos with capacity to develop after transfer have a **higher** glucose uptake at the blastocyst stage

However - - *glycolysis* (conversion to glucose to lactate) presents a different picture:

Glycolysis

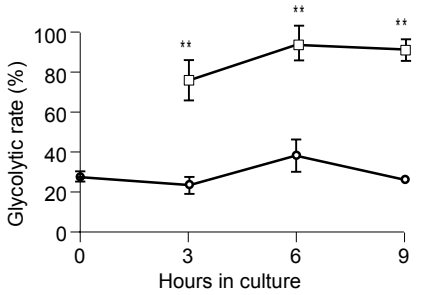


Fetal development of mouse blastocysts selected for transfer using glycolytic activity as a biochemical marker

'Viable' blastocysts had a glycolytic rate in the **lowest** 15% of the distribution

Lane & Gardner (1996)
Hum Reprod **11**: 1975-1978

Glycolytic activity is a marker of embryo stress



Gardner & Leese (1990): *Journal of Reproduction & Fertility* **88**, 361-368

Pyruvate

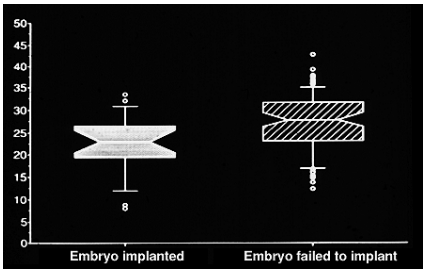
Conaghan J, Hardy K, Handyside, AH, Winston RML & Leese HJ (1993)

Selection criteria for human embryo transfer: a comparison of pyruvate uptake and morphology

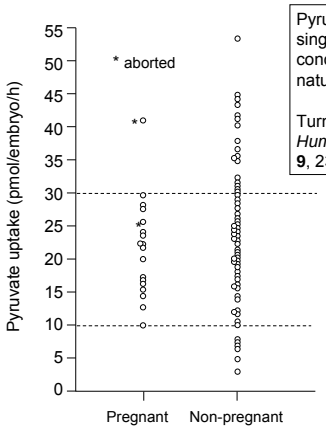
Journal of Assisted Reproduction and Genetics **10**, 21

590 stimulated IVF cycles: live birth rates normal

Pyruvate uptake as a marker of embryo health



Conaghan et al, 1993



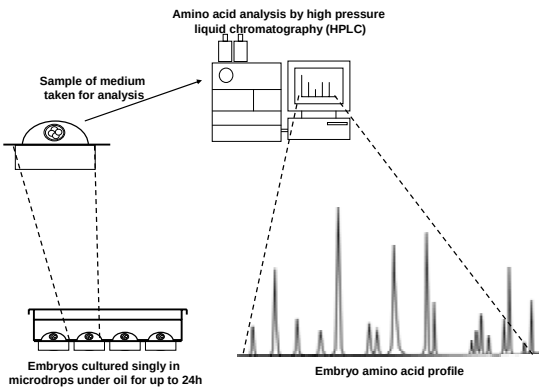
Pyruvate uptake by single embryos conceived by natural cycle IVF

Turner et al (1994) *Human Reproduction* **9**, 2362-2366

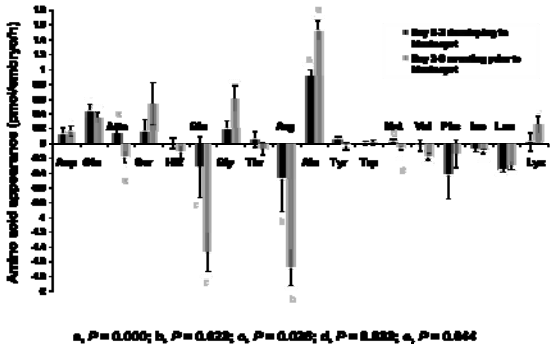
Amino Acids

Perform a variety of important physiological functions

- *Protein synthesis*
- *Energy sources*
- *Nucleotide synthesis*
- *Osmolytes*
- *Antioxidants*
- *pH regulation*
- *Chelators*
- *Signalling molecule precursors*



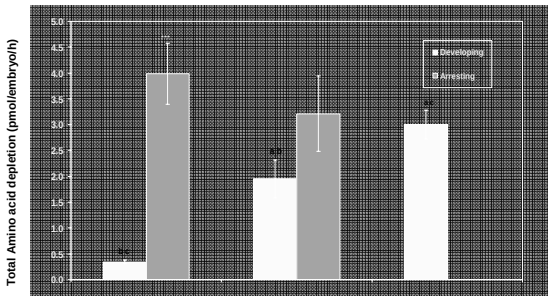
Amino Acid metabolism of single human embryos



a, $P = 0.000$; b, $P = 0.022$; c, $P = 0.026$; d, $P = 0.032$; e, $P = 0.044$

Houghton et al (2002) Human Reproduction 17: 999-1005

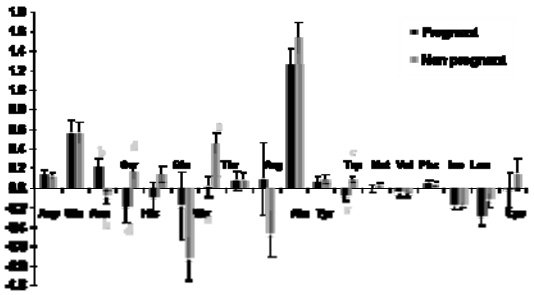
Amino acid depletion by single human embryos



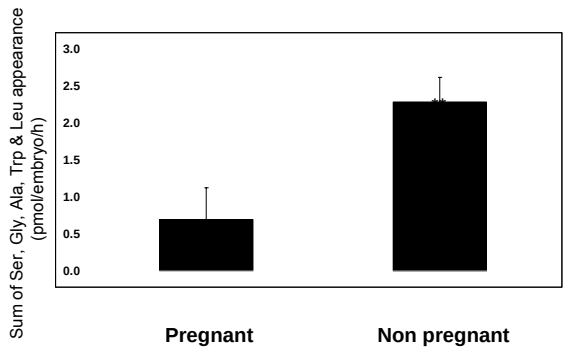
a, *P* = 0.05; b, *P* = 0.01; c, *P* = 0.001

Houghton et al (2002) *Human Reproduction* **17**: 999-1005

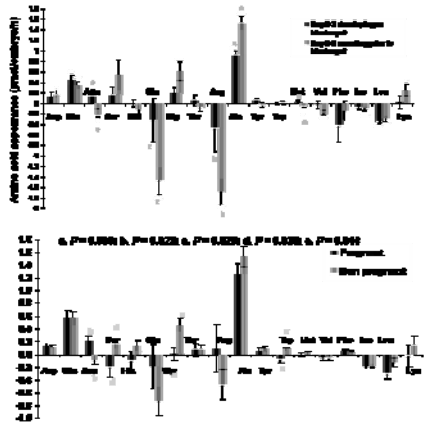
Amino acid profiles and IVF pregnancy outcome



Cleavage-stage human embryos with the potential to give a pregnancy have a lower turnover of amino acids: Brison et al (2004) *Hum Reprod* **19**: 2319



Brison et al (2004) *Hum Reprod* **19**: 2319-2324



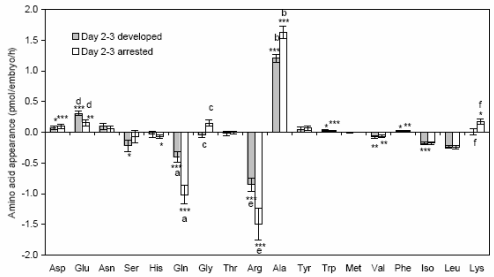
Amino Acid metabolism of single human embryos

Conclusion

Amino acid turnover (sum of depletion and appearance) is **reduced** in cleavage-stage human embryos which have the potential to develop to the blastocyst stage in culture and to give rise to a pregnancy following transfer

The same conclusion – with regard to development in culture - applies to cryopreserved embryos

Amino acid profiling of cryopreserved embryos



Stokes et al (2007) *Human Reproduction* **22**:829-35.

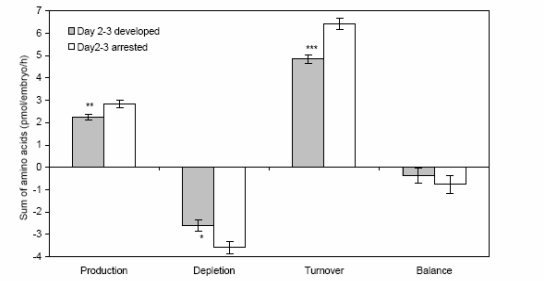


Figure 2 Total amino acid production, depletion, turnover and balance by frozen thawed human embryos from day 2 to day 3 of development. **P*<0.05; ***P*<0.01; ****P*<0.001 significantly different from embryos which arrest.

Stokes et al (2007) *Human Reproduction* **22**:829-35.

Conclusion:

Early embryos prefer to survive with a relatively low level of metabolism

Hypothesis:
Quiet please, do not disturb:
a hypothesis of embryo metabolism and viability

Leese: *Bioessays* **24**, 845-849 (2002)

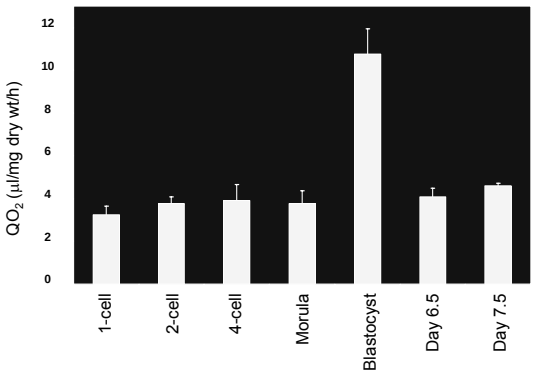
Categories of quietness

Functional
- Cleavage stages quiescent vs blastocyst
- Inner cell mass quieter than Trophectoderm (Houghton, 2006 *Differentiation* **74**: 11-18)
- Maintenance of quietness by nitric oxide (Manser et al (2005)
- Gamete development at reduced temperature
- Somatic tissues: contribution to metabolic rate
- Embryonic diapause and hibernation

Individual differences between embryos and cells:
- Metabolic differences in relation to viability
- Do quiet Inner Cell Mass cells form stem cells?
- Are quiet blastomeres more likely to survive?
- Are putative tumour cells more active than host cells?

Response to environmental stress:
- In vivo vs in vitro +/-serum
- Ammonia
- Accelerated development
- Maternal feeding

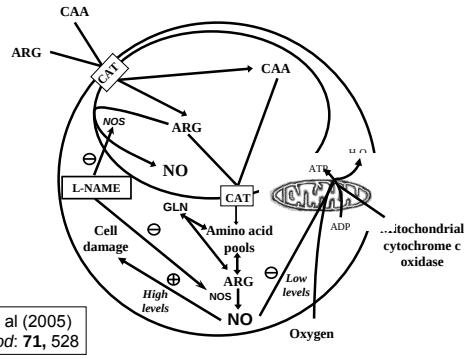
Oxygen consumption by early mouse embryos



Houghton et al (1996) *Molecular Reproduction & Development* **44**:476-85.

Regulation of oxygen consumption: maintenance of quietness?

Nitric oxide production reduces blastocyst oxygen consumption by inhibiting cytochrome c oxidase.



Manser et al (2005) *Biol Reprod*: **71,** 528

Tissue distribution of metabolic rate

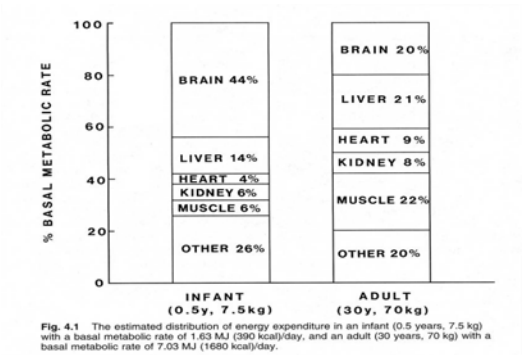


Fig. 4.1 The estimated distribution of energy expenditure in an infant (0.5 years, 7.5 kg) with a basal metabolic rate of 1.63 MJ (390 kcal)/day, and an adult (30 years, 70 kg) with a basal metabolic rate of 7.03 MJ (1680 kcal)/day.

From Garrow, James & Ralph: Human Nutrition & Dietetics (2000) Churchill Livingstone.

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Response to environmental stress: testing the quiet embryo hypothesis
- In vivo vs in vitro +/-serum
- Ammonia following urea feeding
- Accelerated development
- Maternal feeding

Testing the quiet embryo hypothesis: *in vivo* vs *in vitro*

In vitro produced (IVP) bovine embryos are less viable than *in vivo*-derived and have:

- two-fold higher glycolytic rate than *in* vivo[a]
- increased glycolytic rate with serum[b]
- higher rate of protein synthesis[c]
- higher rate of apoptosis[d]

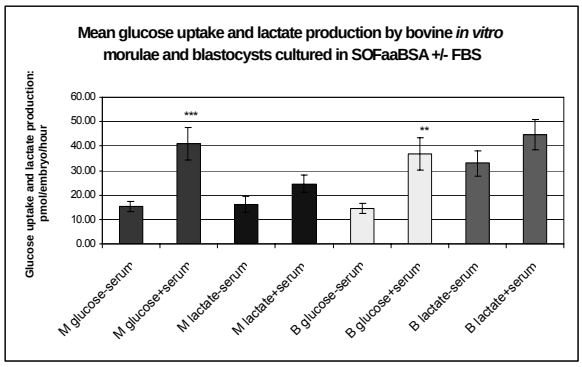
[a]Khurana & Niemann (2000) *Biol Reprod* **62**; 847
[b]Hall & Leese unpublished
[c]Morris et al (2002) Teagasc Agriculture and Food Development Authority, Report No. 4627. ISBN No. 1 84170 300 1.
[d]Pomar et al (2005)*Theriogenology* **63**:2254

Energy Metabolism in Preimplantation Bovine Embryos Derived In Vitro or In Vivo

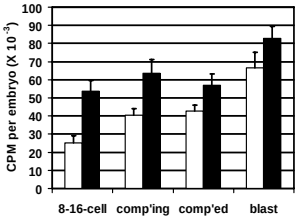
Stage of embryonic development	Biochemical parameter/source of embryos*					
	CO_2 production (pmoles)		Lactate accumulation (pmoles)		Carbon uptake (pg atoms)	
	IVP	In vivo	IVP	In vivo	IVP	In vivo
Immature oocyte	—	0.0	—	0.0	—	0.1 ± 0.0^{a}
Matured oocyte	0.3 ± 0	0.1 ± 0.0^{aB}	1.1 ± 0.1^{a}	0.0	0.6 ± 0.0^{aA}	0.2 ± 0.1^{aB}
1-Cell	0.5 ± 0.1^{a}	0.3 ± 0.0^{b}	0.0	0.0	1.7 ± 0.3^{bA}	0.6 ± 0.1^{bB}
2-Cell	0.2 ± 0.0^{b}	0.2 ± 0.0^{b}	0.0	0.0	0.8 ± 0.1^{a}	0.7 ± 0.1^{b}
8-Cell	0.4 ± 0.1^{a}	0.3 ± 0.1^{b}	0.0	0.0	3.2 ± 0.8^{c}	4.6 ± 0.3^{c}
12-Cell	0.6 ± 0.1^{a}	0.5 ± 0.1^{b}	2.8 ± 1.3^{b}	0.0	4.8 ± 0.4^{c}	5.4 ± 0.7^{c}
16-Cell	1.3 ± 0.1^{c}	2.2 ± 0.4^{c}	9.2 ± 0.8^{cA}	5.4 ± 0.9^{cB}	7.4 ± 1.1^{cA}	11.6 ± 1.3^{dB}
Early morula	3.8 ± 0.5^{d}	4.4 ± 0.3^{d}	8.7 ± 2.4^{c}	6.0 ± 1.1^{a}	11.0 ± 0.8^{dA}	15.3 ± 0.9^{eB}
Morula	7.3 ± 1.5^{eA}	3.2 ± 0.6^{dB}	25.9 ± 5.6^{dA}	11.9 ± 1.4^{bB}	18.7 ± 4.1^{e}	14.7 ± 1.8^{e}
Early blastocyst	8.8 ± 2.0^{eA}	4.7 ± 0.3^{dB}	27.4 ± 6.4^{dA}	11.4 ± 1.8^{bB}	19.0 ± 5.1^{e}	17.5 ± 0.8^{e}
Mid-blastocyst	8.1 ± 0.7^{e}	7.5 ± 0.5^{e}	48.0 ± 7.6^{eA}	24.6 ± 6.0^{cB}	20.0 ± 2.1^{e}	22.0 ± 0.6^{f}
Blastocyst	9.9 ± 1.1^{e}	8.7 ± 1.0^{e}	70.2 ± 4.6^{eA}	40.6 ± 8.7^{cB}	24.7 ± 4.5^{e}	31.0 ± 3.7^{fg}
Hatching blastocyst†	9.7 ± 1.5^{e}	10.8 ± 0.7^{e}	79.0 ± 4.4^{e}	57.6 ± 7.0^{d}	23.4 ± 2.7^{e}	28.5 ± 1.6^{g}
Hatched blastocyst†	11.9 ± 1.4^{e}	11.5 ± 0.8^{e}	70.1 ± 10.6^{e}	58.1 ± 23.0^{d}	25.9 ± 3.0^{e}	34.2 ± 11.0^{g}

* Values are means ± SEM per embryo per hour pooled from 5–15 replicates.
† Values for in vivo hatching/hatched blastocysts are for those embryos which were obtained after 48–72 h in vitro culture of freshly obtained in vivo embryos.
a–g Values with different superscripts within same column differ significantly ($P < 0.05$ at least).
A,B IVP vs. in vivo within same biochemical parameter and stage of embryonic development ($P < 0.05$ at least).

Khurana and Niemann *Biology of Reproduction* **62**, 847-856 (2000)



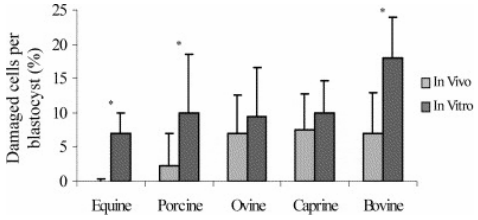
Hall and Leese, unpublished



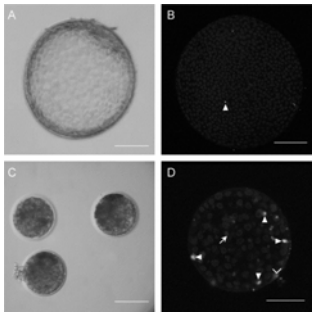
D. G. Morris, M. G. Diskin and J. M. Sreenan
Teagasc, Research Centre,
Athenry, Co. Galway
Project No. 4627
Beef Production Series No. 40

Incorporation of ^{35}S methionine by *in vivo* (□) and IVP (■) embryos
Incorporation increased (P<0.05) from the 8-16-cell stage to the blastocyst stage in both *in vivo* and IVP embryos.
Incorporation, however, was not different between the 8-16-cell, compacting and compacted stages.
Incorporation was similar for *in vivo* and IVP blastocysts and was higher in IVP embryos at all other stages (P<0.05)

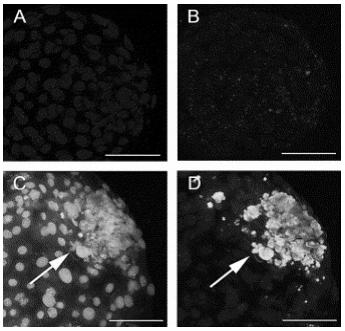
Pomar, Teerdsc, Kidsona, Colenbrandera, Tharasanitb, Aguilard and Roelena (2005)
Differences in the incidence of apoptosis between in vivo and in vitro produced blastocysts of farm animal species: a comparative study



Mean percentage of damaged cells (Ethidium + TUNEL positive nuclei + Hoechst positive fragmented nuclei) in in vivo and in vitro produced equine, porcine, ovine, caprine and bovine blastocysts.
Within species bars with asterisks differ significantly ($p \leq 0.05$).
Theriogenology **63**: 2254



Normal light microphotographs (A and C) and multiphoton laser scanning images (B and D) of equine in vivo produced (A and B) and in vitro produced (C and D) blastocysts. Nuclei were stained (B and D) with Hoechst (blue). Fragmented nuclei (arrow), nuclei from cells in which the membrane integrity was lost, stained with EthD-1 (red, v line) and fragmented DNA as visualized by TUNEL staining (green, arrow head) are indicated (B and D).



Multiphoton laser scanning images (A and B) and epifluorescence microphotographs (C and D) of bovine in vivo produced (A and B) and in vitro produced (C and D) blastocysts. Nuclei were stained (A and C) with Hoechst (blue) and fragmented DNA is visualized by TUNEL staining (B and D). Signs of apoptosis are visible in the ICM cells (arrow). Bars represent 50 µm.

Testing the quiet embryo hypothesis:

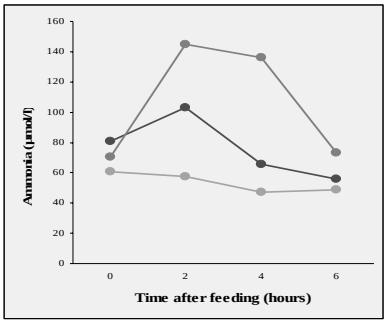
'Active' metabolism due to high ammonia following urea feeding

Evidence for active metabolism in livestock early embryos is provided in a study where superovulated donor ewe diets containing 3% urea (high urea, HU) generated elevated ammonium concentrations in vivo. The resultant embryos proved less capable of surviving to term … than embryos from conventionally fed (control, C) donor ewes (McEvoy et al., 1997: *Animal Reproduction Science* **47**: 71-90)

In a complementary experiment in the same paper, Day 3 embryos collected from analogous C and HU ewes differed to the extent that the latter were more advanced and more metabolically active. However, the HU donor-derived embryos tended to have inferior survival rates during subsequent culture in vitro, hinting that dietary-mediated up-regulation of metabolism was harmful rather than beneficial to these embryos.

Leese, Baumann, Sturmey & McEvoy (2007) *Human Reproduction* **22**:3047-50

Post-prandial plasma ammonia concentrations in ewes offered low (○), moderate (○) or high (○) ammonia-producing diets



McEvoy et al (1997)
Animal Reprod Sci
47: 71-90

In vivo study: ammonia from diet with 3% urea

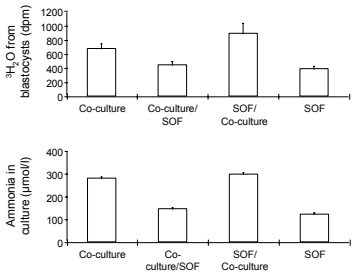
Ovine blastocyst yields were reduced,
but blastocyst metabolism was upregulated
and subsequent fetal growth increased

McEvoy et al (1997)
Animal Reprod Sci
47: 71-90

Testing the quiet embryo hypothesis

Ammonia in culture and metabolism of embryos

Metabolism of glucose (indicated by ^3H_2O production) was greater among ovine blastocysts cultured in conditions that generated high concentrations of ammonia, a toxin associated with aberrant development.



Negrin Pereira *et al* (1997) Theriogenology 47:377

***Testing the quiet embryo hypothesis*:**
Accelerated development

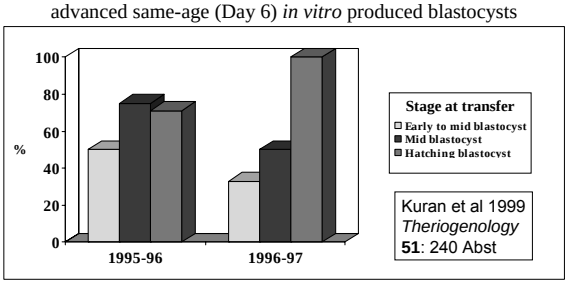
One likely consequence of active metabolism, unless devoted almost solely to 'running repairs' in adverse conditions, is accelerated development of embryos, a process that can be counter-productive. As well as undermining synchrony between an embryo and its environment, there is evidence that 'advanced' embryos exhibit compromised development.

Kuran *et al* (1999: *Theriogenology* 240), surveying the outcomes of large offspring studies in sheep (total of 219 singleton pregnancies), concluded that embryos transferred as early/mid blastocysts had a lower incidence of fetal oversize (38%) than same-age expanding/expanded blastocysts (58%) or hatched blastocysts (80%).

Leese, Baumann, Sturmey & McEvoy (2007) *Human Reproduction* **22**:3047-50

Large Offspring Syndrome

Incidence of fetal oversize is higher following transfer of more advanced same-age (Day 6) *in vitro* produced blastocysts



Data based on 120 fetuses (Day 125); graph indicates % heavier than heaviest in vivo-derived control for that year.

More recent data from Powell et al. (2006: *Theriogenology* **66**: 1901-1912) support the idea that **accelerated development** of embryos can predispose them to exhibit aberrations during subsequent development.

For example, circumstances associated with excessive dietary nitrogen provision to donor ewes yielded a set of outcomes that included accelerated early embryo development, low survival following embryo transfer, and altered fetal development among survivors.

Leese, Baumann, Sturmey & McEvoy (2007) *Human Reproduction* **22**:3047-50

The 'quiet range'

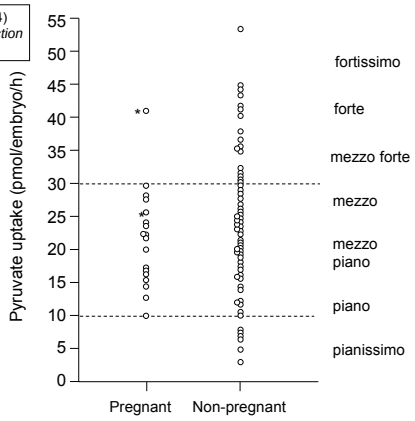
We propose - - - '*that the concept of quiet metabolism is not about 'one size fits all' but rather that - - - there is an optimal **range** of embryonic activity consistent with successful developmental progression*'

Caution against the notion that 'up-regulation' (e.g. of genes) is indicative of a healthy embryo

' *- - the challenge is to identify the 'range' of values for a given marker within which an embryo has a high probability of giving a healthy offspring. Our contention is that this range is likely to be in the quiet region of the scale*'

Leese, Baumann, Sturmey & McEvoy (2007)
Human Reproduction **22**:3047-50

Turner et al (1994)
Human Reproduction
9, 2362-2366



Lopes, Greve & Calleson (2007) *Theriogenology* 67: 21-31

Pregnancy status according to respiratory category (high vs. medium vs. low) of bovine in vivo-produced embryos

Respiratory category	**Pregnant (%)**	**Non-pregnant (%)**
High (>1.10 nl/h)	25 (*n* = 1)	75 (*n* = 3)
Medium (0.78–1.10 nl/h)	100 (*n* = 13)	0 (*n* = 0)
Low (<0.78 nl/h)	48 (*n* = 11)	52 (*n* = 12)

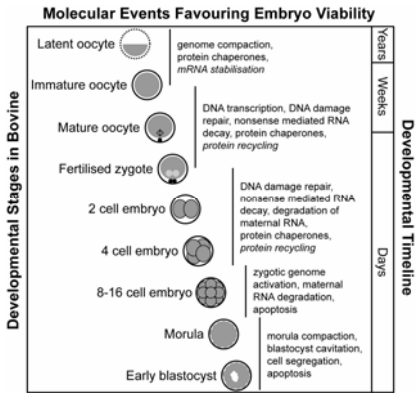
Molecular determinants of a quiet phenotype

The quiet embryo hypothesis: molecular characteristics favoring viability. Baumann, Morris, Sreenan & Leese (2007)
Molecular Reproduction and Development 74:1345-53

Quiet embryos operate at lower error rate (molecular and cellular), higher overall efficiency, and therefore utilise fewer resources:

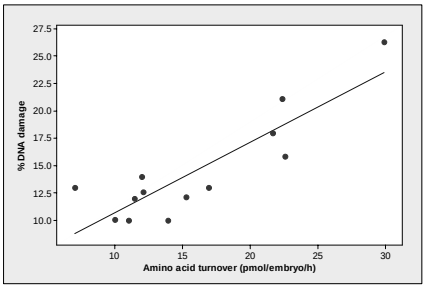
Candidate pathways:

- potential roadblocks in key biosynthetic/biodegradation pathways
- fluctuations in the profile of the transcriptome and/or proteome
- nucleic acid damage and repair
- apoptosis in the early embryo



Baumann et al (2007) *Molecular Reproduction and Development* 74:1345-53

DNA damage and amino acid metabolism by porcine blastocysts developed *in vitro*. The data indicate a positive correlation between the proportion of DNA damage and the metabolic activity of preimplantation embryos (Pearson Correlation 0.872, p<0.001)



Sturmey & Leese (2007) *Human Fertility* **10**: 264

Somatic cells/speculations

Tumour cells break the quiet rules

Caloric restriction – quieter metabolism?

Human athletic performance

Maternal feeding

Caloric restriction

- Extends lifespan of animals (McCay *et al*, 1935) - and of *C.elegans*, Drosophila, spiders, fish and non-human primates
- Slows progression of age-related disorders
- Due to restriction of energy rather than specific nutrients
- Strong link to reduced mitochondrial reactive oxygen species production

Human Athletic Performance
A fit person has a quiet resting metabolism

	Sedentary Male		World class endurance runner
Variables	pre-training	post-training	
Heart rate min/max (beats/min)	75/185	65/183	**45**/174
Ventilation min/max (litres/min)	7/110	6/135	6/195
VO2 min/max (ml/kg/min)	3.5/41	3.5/50	3.5/82

Maternal feeding:

High level of maternal feeding in obesity and diabetes

'Enrichment' of the periconceptual environment

Loss of quietness: up-regulation of egg and embryo metabolism

Potential long-term effects on the conceptus and offspring

Leese, Baumann, Sturmey & McEvoy (2007)
Human Reproduction **22**:3047-50

Original Hypothesis:
A viable embryo has a high metabolism
intuitive and governs the expectation

Quiet embryo hypothesis
A viable embryo has a quiet metabolism
counter-intuitive

Modified quiet embryo hypothesis
Quiet range of embryonic activity consistent with successful developmental progression

What next?

Examples of:

Theory-dependence of data: experimental design and observation

Hypothesis testing, Paradigm shifts or Solving puzzles?

Maintenance of quietness

- Promote embryo metabolism which is 'quiet' rather than 'active'
- Limit the concentrations of nutrients
- Mimic nutrient concentrations in female reproductive tract
- Trust the autonomy of the embryo
- Select the 'quietest' embryos for transfer

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