Biological optimization, the Goldilocks principle, and how much is *lagom* in the preimplantation embryo

HENRY J LEESE*[1], FABRICE GUERIF[2] VICTORIA ALLGAR[1], DANIEL BRISON, KERSTI LUNDIN[3] AND ROGER G STURMEY[1]

Hull York Medical School, Centre for Cardiovascular and Metabolic Research, Hertford Building, University of Hull, Hull, UK.

[1]Hull York Medical School, Hertford Building, University of Hull, Hull HU6 7RX, UK

[2] Medecine et Biologie de la Reproduction, CHRU de Tours; UMR83 PRC, Université de Tours, Tours, France

Department of Reproductive Medicine, Central Manchester University Hospitals NHS Foundation Trust, St Mary's Hospital, Manchester M13 9WL, UK.

[3]Reproductive Medicine, Sahlgrenska University Hospital, 413 45 Gothenburg, Sweden

*Corresponding author:
Hull York Medical School
Hertford Building
University of Hull
Hull HU6 7RX, United Kingdom
Email: henry.leese@hyms.ac.uk

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Abstract

The quiet embryo hypothesis postulates that early embryo viability is associated with a relatively low metabolism (Leese. 2002. *BioEssays* 24**:** 845-849). This proposal is re-visited here using retrospective and prospective data on the metabolic activity and kinetics of preimplantation development alongside the concept that an optimal range of such indices and of energetic efficiency influences embryogenesis. It is concluded that these considerations may be rationalised by proposing the existence of a 'Goldilocks zone', or as it is known in Sweden, of *lagom* –meaning "just the right amount"– within which embryos with maximum developmental potential can be categorised.

INTRODUCTION

Leese (2002) proposed that early embryo viability was best served by a relatively low metabolism, which later became known as the 'quiet embryo hypothesis'. The premise was further developed by Baumann et al. (2007), in terms of potential molecular determinants of 'quiet' metabolism; by Leese et al. (2007), who introduced the idea of a 'quiet range' of nutrient turnover; and by Leese et al. (2008), who considered categories of quietness: (i) 'functional' quietness, the contrasting levels of intrinsic metabolic activity in different cell types; (ii) inter-individual embryo/cell differences in metabolism; and (iii) loss of quietness in response to environmental stress. With hindsight, the original quiet embryo hypothesis was too rigid in its distinction between 'quiet' and 'active' metabolism – indeed, metabolism that is too quiet most likely represents an embryo about to arrest. The aim of this essay is to develop the hypothesis based on two aspects discussed by Johnson (2013) considered below: the idea of an optimal range of metabolic activity and the concept of energy efficiency. The concepts inherent in the hypothesis will also be compared with those in the 'Goldilocks Principle'.

The 'Goldilocks principle' states that "something must fall within certain margins, as opposed to reaching extremes" (en.wikipedia.org/wiki/Goldilocks_principle), which is derived from *Goldilocks and the Three Bears* (en.wikipedia.org/wiki/Goldilocks_and_the_Three_Bears). Within this fairy tale, largely attributed to the Victorian-era British Romantic author Robert Southey, a little girl named Goldilocks wanders into a house owned by three bears and discovers three bowls of porridge, three chairs, and three beds. Each set of objects is characterised by a distribution of two extremes plus a middle option; thus, the porridge was 'too hot', too cold', or 'just right', which is the one Goldilocks chooses. After consuming the porridge, sitting in the 'just right' chair, and sleeping in the 'just right' bed, Goldilocks manages to escape the bears when they return to their house. The 'just right' concept is found across languages and cultures; for example, the term *lagom* is widely used in Sweden, where it means "just enough" or "just the right amount" as well as "moderation" and "in balance" (en.wikipedia.org/wiki/Lagom).

The Goldilocks principle has been applied to many phenomena in economics, astronomy, physics, psychology, the social sciences, and biology (e.g. Liu et al., 2012; Drake et al., 2014),

including a few examples in reproductive biology and medicine. Fowler and O'Shaughnessy (2013), for example, highlighted the way in which fetal androgen production, especially testosterone, needs to be 'just right' to ensure the appropriate developmental trajectory of the fetus and offspring; conversely, inappropriate fetal androgen or androgen signalling – both too little and too much – is associated with disorders of male reproductive development, and are implicated as a cause of polycystic ovarian syndrome in women. In another example, Clancy (2013) considered what is 'just right' in balancing fetal needs versus maternal supply during pregnancy in great apes and humans in terms of inflammation, determining that this process is essential during implantation but potentially predisposes the mother to disorders such as gestational diabetes and choriodecidual inflammatory syndrome.

An overarching question in how the Golidlocks Principle is applied to biological systems is: What determines 'just right' or *lagom*? Here, we address this at the cellular level in the context of the developing preimplantation embryo, proposing that 'just right' is the capacity to develop successfully at the highest efficiency – i.e. to carry out faithfully the developmental programme while expending the minimum amount of energy. Initially, we re-interpret? data on energy homeostasis/pyruvate consumption in early cattle embryos from Guerif et al (2013). Considerable use is made of the review by Johnson (2013), entitled *Teaching the principle of biological optimization*, which provides a valuable guide to the need for energy efficiency, the uses to which energy is put, and the factors that drive the optimization of energy use at all levels – from genes, proteins, and physiological systems, to whole organisms and ecosystems. Before presenting these analyses, it is necessary to consider briefly the energy metabolism of the early embryo.

NUTRITION AND METABOLISM OF THE EARLY MAMMALIAN EMBRYO

The nutritional needs of mammalian embryos through the preimplantation stage are remarkably simple. Simple physiological salts solutions supplemented with a few nutrients and serum albumin are the minimum requirements for culture (reviewed by Biggers, 1998). Further,

energy production throughout preimplantation development is largely aerobic (reviewed by Smith and Sturmey, 2012): Pyruvate is the preferred energy substrate for the first cleavage (from 1 to 2 cells), and is obligatory for many species. A variety of nutrients – notably, pyruvate, lactate, amino acids and endogenous fatty acids – can also be utilised as early development progresses. Cleavages to the morula stage are relatively quiescent in terms of oxygen consumption, which is widely accepted as the best overall metric of metabolism. As the embryo continues to the blastocyst stage, glucose consumption rises significantly – a large proportion of this glucose is converted to lactic acid, at least in vitro – while oxygen consumption also rises. This change in metabolism during blastocyst formation is largely due to the energy demands of the sodium pump required to form the blastocoel cavity and of protein synthesis, which is associated with the first increase in the mass of the embryo that occurs at this stage.

THE EARLY EMBRYO AS A MODEL SYSTEM

The early embryo, aside from its biological fascination, has a special advantage as a model system for considering energy homeostasis; namely, its availability as a discrete cellular entity. The molecular cell biology and biochemistry of early embryos are readily studied at the level of single cells (unfertilised or fertilised eggs) or small clusters of cells (cleavage stage preimplantation embryos) through to the blastocyst stage, which comprises about 100 cells. In marked contrast, most mammalian cells, apart from those in the extracellular compartments in the body, are rarely found individually, instead being present in highly organised, multicellular tissues. Such cells are routinely studied in very large numbers ($>10^6$), which severely limits the possibility to examine single-cell biochemistry. Thus, the early embryo is an excellent system for studying intra- and intercellular differences. Understanding the basis of this variation is essential to resolving one of the major challenges facing in vitro fertilisation and related technologies: How to devise a robust, non-invasive test of cellular health with which to select single embryos for transfer into the uterus.

FACTORS INFLUENCING THE EFFICIENCY OF EARLY EMBRYOS AND CELLS

Competition for resources

Early embryos can exist with complete autonomy, as demonstrated by their capacity to develop in vitro. Their solitary existence obviates the need to compete with other cells for resources as their nutritional needs are provided, in vivo, by the oviduct and uterus and their own endogenous reserves, or by the in vitro culture medium – although the notion of autonomous preimplantation development needs to be revisited based on the increasing awareness of embryo-maternal interactions, whose roles are only beginning to be clarified (reviewed in Leese and Brison, 2015). In marked contrast, somatic cellular systems – cells, tissues, and whole organisms – operate under the limited resources, therefore/such that the most efficient and successfully competitive survive (Johnson, 2013). Cells in tissues and tissues within the body are metabolically constrained from becoming autonomous or 'rogue' by a variety of mechanisms; for example, gap junctions between cells in tissues and hormonal and neuronal regulation between tissues both maintain homeostasis (Brison et al 2014).

Intrinsic factors

Even if the drive to compete is minimised, cells, tissues, and organisms still possess an *intrinsic* capacity for survival whereby those that make more efficient use of resources will be at an advantage (Johnson 2013). 'Efficiency', in an energetic sense, implies carrying out a defined action with the minimum input of energy. Illustration of this concept requires data from a system in which input and output are well-defined and can be measured quantitatively – e.g. a study by Guerif et al. (2013) on the relationship between the consumption of the essential nutrient pyruvate by 2-cell bovine embryos and their capacity to reach a subsequent stage of development (i.e., the 4-cell stage) or the blastocyst stage. Pyruvate is an appropriate nutrient to use as a metric of energy input since it is largely oxidised to produce ATP in the embryo. These data were also chosen because they are quite detailed and include prospective as well as retrospective studies. Guerif et al. (2013) conducted two types of experiments in which bovine

144 embryos were produced in an identical manner, via in vitro fertilisation of in vitro-matured
145 immature oocytes obtained from abattoir ovaries.

146 *Experiment I*. Zygotes (fertilised eggs) were allowed to develop to the 2-cell stage before
147 being incubated individually in 5 µl of culture medium for 24 hours under an environment of 5%
148 CO_2, 5% O_2, 90% N_2. The embryos were removed and allocated into two groups: those that had
149 developed to the 4-cell stage (n=40) and those that showed no development, i.e. remained at the
150 2-cell stage (n=30). The individual droplets in which the embryos had been incubated were then
151 analysed retrospectively for their pyruvate content, enabling the relationship between embryo
152 development and metabolism (the consumption of pyruvate) to be determined. A significant
153 difference in pyruvate consumption was measured between the groups; those which exhibited
154 development having higher values on average than those with no development (P=0.016). These
155 data may be presented in a number of different ways: Traditionally, they would be tabulated as
156 values of pyruvate consumption (pmol per embryo per hour) (Fig. 1A). A more striking way would
157 be to illustrate the data and statistics as a plot of mean values with confidence intervals (Fig. 1B).
158 However, in order to examine individual cellular efficiency and discover whether the Goldilocks
159 Principle applies, the data should instead be visualised as distributions, i.e. the spread of data for
160 pyruvate consumption by each embryo (Fig. 1C).

161 A number of conclusions may be drawn from the pyruvate data, independent of presentation.
162 (i) A high attrition rate was observed, wherein only 57% (40/70) of the 2-cell embryos developed
163 to the 4-cell stage. (ii) Considerable variation in pyruvate consumption was measured, whether
164 or not development occurred. (iii) Considerable overlap exists between the two cohorts, so these
165 data do not support the Goldilocks Principle – which predicts clustering of the data into defined,
166 but overlapping, categories (Fig. 2B). (iv) A considerable range of input was present in the
167 developed group, as the pyruvate values fell between 2 pmol pyruvate (very high efficiency) and
168 16 pmol pyruvate (low efficiency) consumed per embryo per hour. These differences
169 nevertheless could lead to the hypothesis that 'low efficiency' embryos, which use a large
170 amount of pyruvate to reach the next stage, might struggle to maintain such high consumption
171 throughout development compared to the more efficient developing embryos with a lower

pyruvate consumption. Conversely, highly efficient embryos that developed with very low pyruvate consumption might struggle to continue to develop through subsequent cleavage divisions if such a low rate of pyruvate consumption is maintained.

A caveat to these conclusions is the capacity of the embryo to use and to switch among other metabolic substrates. The obligatory nature of pyruvate as a nutrient was the logical first metric, and the lack of data on the relative contribution of all other potential nutrients – ideally determined simultaneously, which is still technically challenging and has yet to be overcome – would have made interpreting such additional data difficult. The best marker of metabolic capacity would be oxygen consumption (Lopes et al., 2007; Tejera et al., 2011), but this parameter is difficult to measure in such a small amount of material, and comprises several components that have yet to be quantified at all the preimplantation stages (Leese 2012). Given these constraints and the unique data set available, pyruvate consumption presently provides the best proxy for energy efficiency throughout preimplantation embryo development.

In order to test the proposition that early developing embryos utilising 'too low' or 'too high' a rate of metabolism will encounter a crisis phase later in development, a prospective experiment needed to be devised that longitudinally monitored metabolic profiling from the 2-4 cell stage through to the blastocyst stage, which takes about 6 cleavage divisions over 6 days in the bovine. The difficulty underlying this type of experiment is that bovine (as well as ovine and porcine) embryos are less viable if cultured singly in vitro, especially in extended culture; they prefer to be grown in groups (Stokes et al., 2005, Gopichandran and Leese, 2006). This problem was overcome by Guerif et al., (2013).

Experiment II. As in their first experiment, thirty bovine embryos were then incubated singly from Day 2 to Day 3 in small droplets of medium, and pyruvate uptake was measured. On the basis of the results, the embryos were allocated into tertiles with 10 embryos per group, representing 'high' (>10pmol/embryo/h [T3]); 'intermediate' (4-10pmol/embryo/h [T2]); and 'low' (<4pmol/embryo/h [T1]) pyruvate uptake during the 24 hours of culture (Fig. 2B). The embryos were then cultured to the blastocyst stage (Day 8) to test the applicability of the Goldilocks Principle to preimplantation development directly. Such monitoring was repeated six times.

The relationship between pyruvate uptake and blastocyst formation can be tabulated (Fig. 2A) or plotted by individual embryo, showing the full distribution of pyruvate uptake between 24-48 hours against blastocyst formation (Fig. 2B). The following conclusions may be drawn from this second data set: (i) In line with the first experiment, there is considerable variability in the capacity of in vitro-produced bovine 2-cell embryos to develop to the blastocyst stage. This is well known, and the overall blastocyst rate (~35%) is consistent with the data of others. (ii) The highest blastocyst rates were obtained with pyruvate consumption in the intermediate range; embryos in the higher and lower ranges were much less likely to form blastocysts. The data are therefore consistent with the Goldilocks Principle, in that a *lagom* range of pyruvate uptakes predicts a high blastocyst rate. (iii) Pyruvate uptake is not an all-or-nothing metric of bovine 2-cell embryo developmental capacity; the overlap between the categories was considerable, especially between the intermediate (T2) and higher (T3) ranges. The value of plotting these results as distribution of individuals lies in the identification of optimal ranges, and, in this particular example, of the long time interval between metabolic assessment (Day 2-3) and the measurement of development outcome (Day 8).

The end point in these bovine studies was blastocyst formation (Guerif et al., 2013), although determining whether these embryos have the same potential for implantation and the capacity to give rise to live offspring will be of particular interest. One example for which long-term analysis was performed is in the study by Turner et al. (2004), for which pyruvate uptake of single human embryos generated via natural cycle in vitro fertilization was measured. Pyruvate consumption was quantified over the first 24 hours following fertilisation prior to transfer on Day 2 (40-50 hours post-insemination). Pyruvate values were then related retrospectively to the pregnancy outcome (Fig. 3). These longitudinal data also indicate an optimal range of pyruvate uptake (between about 10 and 30 pmol per embryo per hour) within which a pregnancy can occur; embryos in the higher and lower ranges are less likely to lead to the establishment of pregnancy.

Gardner et al (2011) questioned the quiet embryo hypothesis largely on the basis that blastocyst formation is associated with a dramatic *increase* in glucose consumption – i.e. a highly active, as opposed to quiet, metabolism. In response, Leese (2012) proposed that what was

required as a test of the quiet embryo hypothesis was not the 'functional' demand for high glucose, but the overall metabolic cost of this process; the challenge was to measure energetic efficiency alongside nutrient uptake, and to relate the data to developmental competence, as has been done in this paper. An alternative interpretation of the Gardner and Wale data is to propose that the minimum threshold for glucose consumption required to make a blastocyst is set at a high level, but that within the range of values conducive to blastocyst formation exist sub-ranges of 'too high' and 'just right' that are consistent with viable pregnancy in the long term.

KINETICS OF EARLY EMBRYO DEVELOPMENT

The Goldilocks principle could also be applied to the speed of preimplantation development. In the early days of in vitro fertilization, when embryos were grown under what were likely to have been severely suboptimal culture conditions, a high speed of development was taken as an indicator of quality. As culture conditions and success rates improved, however, numerous studies were conducted, many of them large, correlating cleavage speed to implantation and live birth rates; more recently, the utilization of the time-lapse techniques allowed these associations to be investigated in a more precise manner. These compiled data are consistent with the proposition that the speed of development needs to be 'just right', and that both too slow and too fast development results in lower success rates, presumably indicating a non-optimal metabolic and/or genetic phenotype.

Early studies also observed that the sooner embryos underwent the first cleavage, the better their prognosis for blastocyst development, pregnancy, and live birth than for their later-cleaving counterparts (Lundin et al., 2001; Salumets et al., 2003; Van Montfoort et al., 2004). The time used for determining the early-versus-late cut-off was 25-27 hours. Implementation of time-lapse imaging plus the ability to observe embryo development continuously revealed that the optimal time to first cleavage was indeed intermediate: embryos that cleaved too rapidly (<24.3h) also showed poor developmental potential (Meseguer et al 2011). Similar conclusions that a tighter time distribution exists for implanting than for non-implanting embryos have been reached for a

number of morphokinetic variables, such as number of cells and length of cell cycles (Meseguer et al 2011, Cruz et al., 2012). These points are best illustrated by plotting the distribution of biomarkers of embryo health, as performed in the retrospective analysis by Meseguer et al. (2011), who recorded the time taken for individual human in vitro-fertilized embryos to divide to 5 cells and related this to their subsequent capacity to implant following transfer (Fig. 4): Consistent with the Goldilocks Principle, embryos more tightly distributed in the *lagom* intermediate range are more likely to give a positive outcome.

CONCLUSION: THE GOLDILOCKS ZONE

In light of the data appraised in this paper and the model of a 'quiet range' of metabolic activity (Leese 2007), we propose that embryos with maximum developmental potential will be located in a 'Goldilocks zone'. The lower limits of this zone are determined by the minimum, or threshold, value that nutrient / metabolic activity has to reach to ensure the fidelity of homeostatic energy mechanisms while the upper limit is balanced by the physiological capacity to increase cellular metabolism versus *the energy parsimony in almost everything they do* (Johnson, 2013). The existence of 'ranges' or 'zones' is best revealed by plotting data as distributions of individual embryos rather than as averages – indeed, we believe that other areas of biology and medicine could benefit from this approach as it provides critical visualisation of the averages and rough statistics without collapsing the individual data. The challenge is to discover where the boundaries lie for other cell types, tissues, and whole organisms under different situations.

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Figure 1. Pyruvate consumption (pmol per embryo per hour) by 2-cell bovine embryos that developed to or showed no development to the 4-cell stage. **A**: Tabulated values, shown as mean ± standard deviation. **B**: Plotted values are shown as mean ± 95% confidence intervals. **C**: Distribution of individual values. Adapted from Guerif et al (2013).

Figure 2. Rate of blastocyst development according to the level of pyruvate consumption (pmol per embryo per hour) measured between Days 2 and 3. **A**: Tabulated values, shown as mean ± standard error of the mean. **B**: Individual values for pyruvate consumption by bovine embryos assigned prospectively to one of 3 categories representing 'low' (<4 pmol per embryo per hour [T1]), 'intermediate' (4-10 pmol per embryo per hour [T2]), and 'high' (>10 pmol per embryo per hour [T3]) pyruvate uptake, and then cultured to the blastocyst stage. The terms *optimum*, *pejus*, and *pessimism* illustrate the hypothetical response of an embryo to stress: When the stress is mild, embryo metabolism shifts up or down from within the *optimum* to the *pejus* range in order to minimise or rectify the damage. Under modest damage, metabolism can return to the *optimum* range when it has been corrected, whereas under severe stress, metabolism shifts irreversibly into the *pessimum* range from which it cannot recover. For further discussion, see Guerif et al (2013).

Figure 3. Pyruvate uptake of single human embryos generated via natural-cycle in vitro fertilization. Pyruvate consumption was measured over the first 24 hour following fertilisation prior to transfer on Day 2 (40-50 hours post-insemination). The values for pyruvate were related retrospectively to the outcome; pregnant or non-pregnant. Adapted from Turner et al (1994).

Figure 4. The time taken for individual human in vitro fertilized embryos to divide to the 5-cell stage in relation to their subsequent capacity to implant following transfer. Adapted from Meseguer et al (2011).