

# Kinetics of early *in vitro* development of bovine *in vivo*- and *in vitro*-derived zygotes produced and/or cultured in chemically defined or serum-containing media

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The kinetics of the *in vitro* development of early embryos from bovine zygotes derived *in vitro* and *in vivo* were compared, investigating the effect of serum during *in vitro* maturation and fertilization (IVM–IVF) and in culture. Zygotes were collected from superovulated heifers or produced *in vitro* from immature oocytes with or without serum supplementation, and cultured subsequently in defined culture medium (SOFaaci) with or without serum supplementation. Time-lapse images were recorded every 0.5 h throughout the culture period. More *in vivo*- than *in vitro*-derived zygotes developed to the compact morula or blastocyst stages (87% versus 47–54%, respectively;  $P < 0.05$ ). Embryo development was blocked predominantly at the second or fourth cell cycles (28 and 29%). However, blastomeres degenerated at all cleavage stages. Serum supplementation during IVM–IVF resulted in abnor-

mally sized blastomeres at first cleavage (defined serum: 20–22% versus *in vivo*-derived: 8%,  $P < 0.05$ ). The duration of the second, third and fifth cell cycles of *in vivo*-derived zygotes were 1–5 h shorter compared with those of *in vitro*-derived zygotes cultured under similar conditions ( $P < 0.05$ ). However, the kinetics of embryo development was affected by serum during IVM–IVF and culture. The first and fourth cell cycles were prolonged by 4–5 h in the absence of serum during IVM–IVF, whereas the presence of serum during culture decreased the duration of the fourth cell cycle and triggered premature blastulation. The results of this study illustrate the differences and similarities between the morphology and developmental kinetics of *in vivo*- and *in vitro*-derived zygotes, and show how serum supplementation during IVM–IVF and culture can alter these parameters.

## Introduction

It is generally accepted that bovine embryos produced *in vitro* have a lower developmental capacity than do embryos produced *in vivo*. Large field trials under commercial conditions have shown that pregnancy rates after transfer of both frozen–thawed and fresh *in vitro*-produced embryos are generally reduced significantly (Hasler *et al.*, 1995; Galli and Lazzari, 1996; Merton, 1997; van Wagtenonk-de Leeuw *et al.*, 2000). Furthermore, studies of, for example, embryo morphology, metabolism, chromosome configuration and gene expression have pinpointed specific dissimilarities between *in vivo*- and *in vitro*-produced embryos (for reviews, see van Soom and de Kruif, 1992; Thompson, 1997; Holm and Callesen, 1998).

Production of bovine embryos *in vitro* is generally referred to as a three-step procedure, namely oocyte maturation, fertilization (including *in vitro* sperm preparation) and culture of the *in vitro*-derived zygote. Deviations compared with *in vivo*-derived embryos may originate from any of these steps. Embryo culture conditions can influence *in vitro* embryo development significantly. This is exemplified

by the general improvement of bovine *in vitro* embryo production systems that has taken place, which has resulted in blastocyst developmental rates of 40–50% from inseminated oocytes in both routine experimental and commercial settings (Holm *et al.*, 1999; Marquant-Le Guenne *et al.*, 2001; Watanabe *et al.*, 2001). In particular, serum supplementation of the *in vitro* culture medium alters both embryo morphology and kinetics (Gardner, 1994; Pinyopummintr and Bavister, 1994; van Langendonck *et al.*, 1997; Yoshioka *et al.*, 1997), but other factors may also influence the kinetics of embryo development directly (for example, growth factors: Yoshioka *et al.*, 2000) or in more complex ways (for example, glucose and sex: Peippo *et al.*, 2001). In sheep, *in vitro* maturation and fertilization (IVM–IVF) of oocytes compromises subsequent development of the zygotes compared with *in vivo*-matured and fertilized zygotes (Holm *et al.*, 1996). Likewise, *in vivo*-derived bovine zygotes have a higher *in vitro* developmental capacity than do *in vitro*-derived zygotes (van Soom and de Kruif, 1992; van de Leemput *et al.*, 1999). These results illustrate the importance and influence of maturation and fertilization processes on the developmental capacity of the zygote.

The aim of the present study was to investigate: (i) the influence of *in vivo* versus *in vitro* maturation and fertilization on the *in vitro* developmental kinetics of the resulting

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**Table 1.** Experimental design

Experimental group	<i>In vivo</i>	<i>In vitro</i>		
		Defined IVC	Defined IVP	Standard IVP
Maturation	<i>In vivo</i>	IVM medium with serum	IVM medium (no serum or BSA)	IVM medium with serum
Fertilization	Artificial insemination	IVF medium with serum	IVF medium (no serum or BSA)	IVF medium with serum
Culture	IVC medium with PVA	IVC medium with PVA	IVC medium with PVA	IVC medium with serum
Number of trials (number of cows)	4 (16)	4	3*	4

IVP: *in vitro* production; IVM (*in vitro* maturation) medium: bicarbonate-buffered medium 199 with hCG and eCG; IVF (*in vitro* fertilization) medium: modified Tyrode's medium without glucose and BSA; IVC (*in vitro* culture) medium: modified SOF with amino acids, myo-inositol and sodium citrate; PVA: polyvinylalcohol.

\*One trial was excluded from the data as a result of technical failure in the time-lapse recording.

zygotes; and (ii) to compare the effect of presence versus absence of serum (and serum proteins) during (a) IVM–IVF and (b) embryo culture conditions on subsequent kinetics of development of *in vitro*-derived zygotes.

## Materials and Methods

### *In vivo*-derived zygotes

A total of 15 Danish Holstein–Friesian heifers (1–5 per trial) were synchronized with 500 µg cloprostenol (Estrumate® vet.; Schering-Plough, Farum) and superovulated in the following oestrous cycle with 200 mg NIH-FSH-P1 (Folltropin®-V; Vetrepahm, Bellesville) administered over 4 days in decreasing doses. Oestrus was induced by two injections of 375 µg cloprostenol, administered morning and evening on day 3 of FSH treatment, and the heifers were subsequently inseminated twice and killed, approximately 48 and 56 h, and 72–75 h, respectively, after the first cloprostenol injection. Within 30 min after death, the genital tracts were transported to the laboratory and both oviducts were flushed twice with 20 ml Hepes-buffered medium 199 (Sigma, St Louis, MO) supplemented with 3 mg polyvinylalcohol ml<sup>-1</sup> (PVA; Sigma). The *in vivo*-derived zygotes were recovered, washed three times and transferred to the time-lapse culture system (Holm *et al.*, 1998) in a chemically defined synthetic oviduct fluid (SOF) medium supplemented with amino acids (30 µl basal medium Eagle (BME) ml<sup>-1</sup> and 10 µl minimal essential medium (MEM) ml<sup>-1</sup>; Sigma), 2.8 mmol myo-inositol l<sup>-1</sup> (Sigma) and 0.3 mmol sodium citrate l<sup>-1</sup> (Sigma) (Holm *et al.*, 1999; SOFaaci medium). The genital tracts, flushing medium and zygotes were kept at 30–35°C during handling.

### *In vitro*-derived zygotes

Immature oocytes were aspirated from 2–8 mm follicles of ovaries obtained from an abattoir. *In vitro* maturation (IVM), sperm preparation and *in vitro* fertilization (IVF) were performed as described by Holm *et al.* (1999). In brief, immature bovine cumulus–oocyte complexes (COCs) were collected from the ovaries and matured for 24 h in IVM

medium (bicarbonate-buffered M199 (Sigma) supplemented with 10 iu hCG ml<sup>-1</sup> and 5 iu eCG ml<sup>-1</sup> (Suigonan™ Vet; Intervet Scandinavia, Skovlunde)) in the presence or absence of 15% (v/v) cattle serum (Danish Veterinary Laboratory, Frederiksberg) at 38.5°C in 5% CO<sub>2</sub> in humidified air.

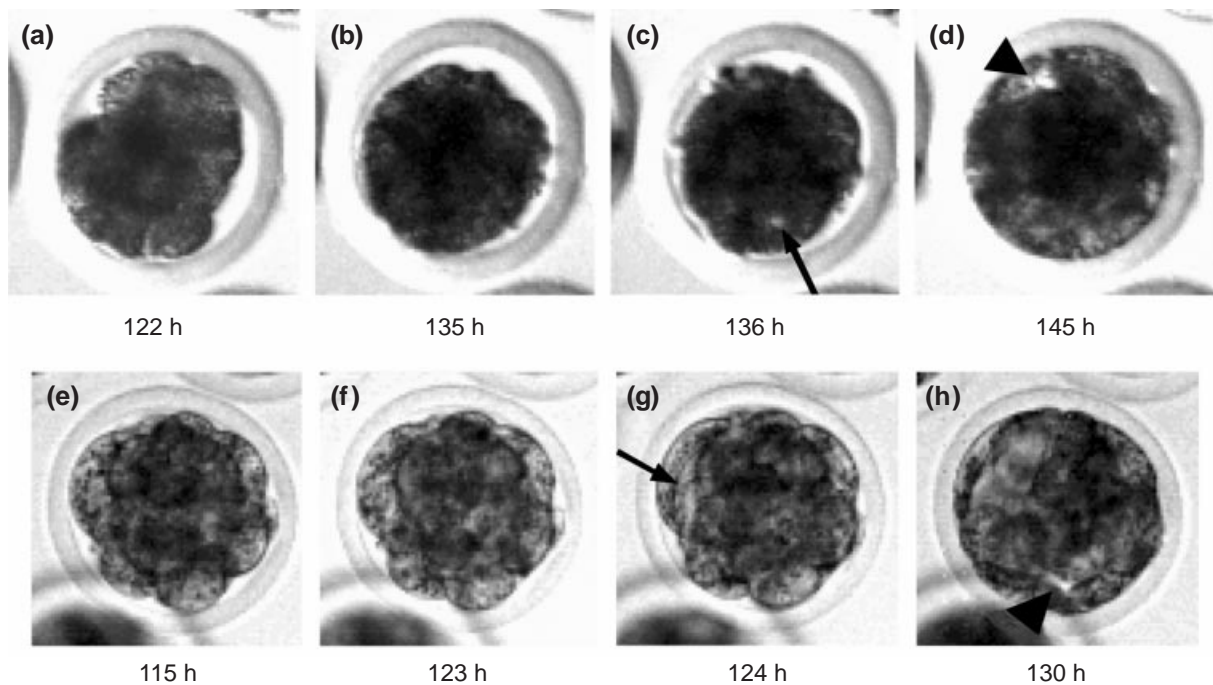
IVF was performed in medium consisting of modified Tyrode's solution without glucose and BSA, but supplemented with 5 iu heparin ml<sup>-1</sup> (Sigma), penicillamine (30 µmol l<sup>-1</sup>; Sigma), hypotaurine (15 µmol l<sup>-1</sup>; Sigma) and adrenaline (1 µmol l<sup>-1</sup>; Sigma). After maturation, the oocytes were inseminated with 2 × 10<sup>6</sup> Percoll-separated spermatozoa ml<sup>-1</sup> (Percoll®; Pharmacia AB, Uppsala) and the gametes were co-cultured for 20 h at 38.5°C in 5% CO<sub>2</sub> in humidified air. For oocytes matured in the presence of serum, the IVF medium was supplemented with approximately 1% (v/v) cattle serum.

Semen from the same bull of proven IVF ability was used in all trials. The basic sperm preparation medium was defined modified Tyrode's solution without serum or BSA supplementation. After 20 h of gamete co-incubation for IVF, the putative zygotes were denuded by vortexing (1 min at 2500 r.p.m.) and washed three times in Hepes-buffered M199 with 3 mg PVA ml<sup>-1</sup> before transfer to the time-lapse system.

### *In vitro* culture and time-lapse recording

In each trial, four groups of zygotes (10–40 in each group) were cultured in a four-well culture dish (Nunc®, Roskilde) until the blastocyst stage in 400 µl *in vitro* culture medium (SOFaaci supplemented with either 3 mg PVA ml<sup>-1</sup> or 5% (v/v) cattle serum) under oil (Uvasol®; Merck KGaA, Darmstadt) in an atmosphere of 5% O<sub>2</sub>, 5% CO<sub>2</sub> and 90% N<sub>2</sub> (Holm *et al.*, 1999; see Table 1 for experimental design).

The time-lapse incubator system was as described by Holm *et al.* (1998). In brief, each four-well culture dish was placed on the microscope stage (MultiControl 2000 Scanning stage; Märzhäuser, Pulheim) of an inverted Nikon TMD microscope (Diaphot; DFA A/S, Copenhagen). A black plexiglas incubator box regulated by an air temperature controller (Air-Therm™; World Precision Instruments, Aston) was fitted around the stage. A small *in vitro* culture



**Fig. 1.** Compaction and early blastulation in *in vitro*-produced bovine embryos cultured in (a–d) defined, serum-free SOFaaci medium and (e–h) standard serum-supplemented SOFaaci medium. Note the lack of proper compaction and premature blastulation in (e–h). Times are hours from insemination to the time that the micrograph was taken. Arrows point at first observed intracellular fluid accumulation. Arrowheads point at first observed stable confluent blastocoel.

chamber with a glass top and open bottom was placed over the culture dish and the humidified gas mixture was led into this culture chamber after passing through a gas washing bottle placed inside the incubator box. The time-lapse recording was directed by Image Analysis software (MicroVision™; Image House A/S, Copenhagen), which controlled both the movements of the scanning stage in the *x*, *y* and *z* axes, the operation of the connected highly light sensitive video camera (CF 8/1 DXC; Kappa, Gleichen), and the recording and storage of time-lapse sequences on the computer hard disc.

Images of each group (total magnification:  $\times 265$ ) were recorded sequentially in minimal light at intervals of 30 min throughout culture. The embryos were moved out of the light field between recordings.

Our weekly routine *in vitro* embryo production during the experimental period (Holm *et al.*, 1999) served as a control for the integrity of the basic culture system.

#### Experimental design, definitions and data analysis

The experimental design is shown (Table 1). Only zygotes that could be identified individually during the entire culture period were included in the analysis. Moreover, a variable proportion of zygotes had to be eliminated from the data set at the respective cleavage events owing to temporary movements out of the focal plane, blurring of the

viable blastomeres of identified embryos or to temporary technical problems with the image recording or storage. Blurring of blastomeres was caused by covering zygotes, cellular debris or other degenerated blastomeres.

Zygotes that developed to the compact morula or blastocyst stages within 7 days after insemination were regarded and designated as viable. In contrast, zygotes that did not reach these stages by that time were regarded as degenerated or retarded and designated as non-viable.

The time of the first appearance of the following cleavage or embryo stages was recorded for zygotes in focus with identifiable blastomeres: two-, three- to four-, five-, eight-, nine- and 16-cell stages; and for morulae and blastocysts with a visible coherent cell mass: early morula, compact morula, early blastocyst, expanded blastocyst and hatching blastocyst stages (Fig. 1). If proper evaluation of specific cleavage events was not possible (see above), the particular data points were treated as missing data.

The cleavage stages of embryos in which one or more blastomeres stopped further cleavage were categorized according to the number of cell cycles observed in the healthy blastomeres; for example, an embryo with three healthy, cleaving blastomeres and one arrested blastomere would be characterized as a five- to eight-cell stage embryo. From the fourth cell cycle onwards, not every individual blastomere could be observed directly. However, the passing from the fourth and fifth cell cycles, respectively, to the following

cycles was defined by combining the direct chronological observation of cleaving blastomeres or initiation of intensive movements within the cell mass created by blastomere cleavage, the cessation of all cleavages and movements followed by a resting period (presumably during the S-phase of the cell cycle), and then the start of a new cleavage round creating new movements. An embryo was defined to have reached the early morula stage when first signs of compaction could be observed, but with blastomeres still clearly distinguishable on the surface (an appearance that has been referred to as a 'cell ball' of small blastomeres; Lindner and Wright, 1983; see also Fig. 1). The compact morula was defined as the stage at which blastomeres had coalesced to form a smooth, tightly compacted cell mass with minimal diameter (Fig. 1). The appearance of the early blastocyst stage was characterized by the first appearance of a stable confluent blastocoel. Blastocysts were characterized as expanded when the diameter of the zona pellucida was increased as a result of expansion of the blastocoel. Finally, expanded blastocysts were identified as hatching when their zona pellucida cracked.

Abnormal cleavages leading to different sized or unequal numbers of blastomeres were recorded. Blastomere degeneration or exclusion of blastomeres from the cell mass of morulae resulting in embryos of reduced size (one-half, two-third or three-quarter embryos) was also recorded.

In one trial, observed reductions in blastocyst diameter (as a result of partial or total collapse of the blastocoel) were recorded, and the diameter and area of compact morulae and the diameter of expanded blastocysts at maximum expansion immediately before hatching were measured using the measuring features of the image analysis software.

The duration of the cell cycle and time intervals from the two-cell to subsequent stages were compared to provide a comparison between the cleavage events of *in vivo*- and *in vitro*-derived zygotes. The duration of the cell cycle and the time interval from insemination to subsequent cleavage stages were compared for *in vitro* zygotes produced in the absence or presence of serum (see Table 1).

### Statistical analysis

Time-lapse data were analysed by mixed linear model (SAS, 1992), using experimental group (see Table 1) and zygote category (viable versus non-viable) as independent parameters, and replicate (trial) as a random parameter. Possible statistical differences between individual treatment groups were compared on the basis of the respective least squared mean values calculated by the mixed procedure. Time intervals are given in h and are mean  $\pm$  SD unless otherwise noted. Cleavage and developmental data (for example, blastocyst rate, frequency of abnormal cleavages) were analysed by a generalized linear model (logistic regression of binomial distributed proportions; SAS, 1993) using experimental group, replicate and zygote category as independent parameters when appropriate. The level of significance was  $P \leq 0.05$ .

## Results

### *In vitro* development and cell death of viable and non-viable zygotes

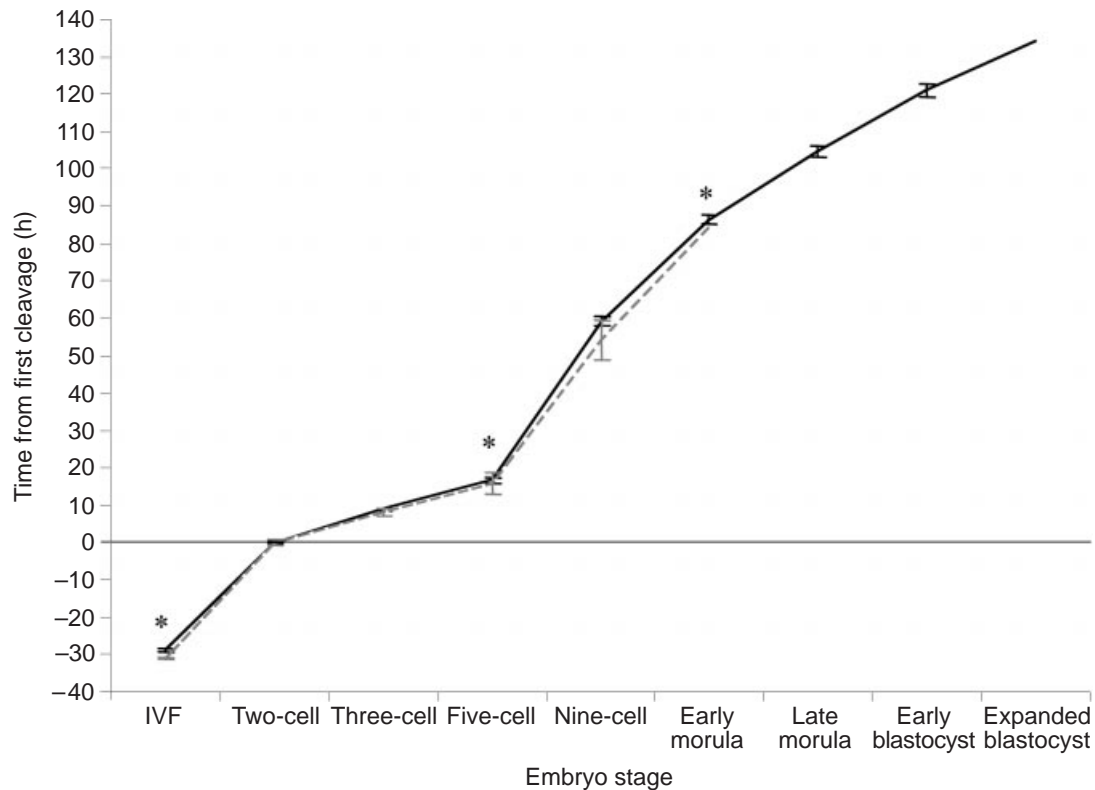
In total, 452 zygotes were observed in the time-lapse culture system and distributed as follows between the four experimental groups: (i) 143 *in vivo*-derived zygotes cultured in defined serum-free conditions (*in vivo* group); (2) 94 *in vitro*-derived zygotes matured, fertilized and cultured in the presence of serum (standard *in vitro* production (IVP) group); (iii) 142 *in vitro*-derived zygotes matured and fertilized with serum, but cultured in defined serum-free conditions (defined *in vitro* culture (IVC) group); and (iv) 73 *in vitro*-derived zygotes matured, fertilized and cultured entirely in defined, serum-free conditions (defined IVP). These zygotes represented (average per trial)  $84 \pm 10$ ,  $77 \pm 5$ ,  $80 \pm 10$  and  $60 \pm 20\%$ , respectively, of the recovered *in vivo* ova or inseminated *in vitro* oocytes in culture.

A significantly higher proportion of *in vivo*-derived zygotes ( $87 \pm 17\%$ ) developed to the compact morula or blastocyst stages compared with *in vitro*-derived zygotes (from  $47 \pm 12\%$  to  $54 \pm 10\%$ ).

In general, the non-viable embryos tended to develop at a slightly slower rate than did the viable embryos during most of the cell cycles studied (Fig. 2) and the duration of the first, second and third cell cycles of non-viable zygotes were prolonged significantly (Fig. 3). There was a significant interaction between experimental group and viability at the fourth cell cycle, as *in vivo*-derived non-viable embryos took a longer time and *in vitro*-derived non-viable embryos took a shorter time to complete this cell cycle than did their viable counterparts.

No significant influence of treatments on the kinetics of embryo attrition was detected in the present study, so the data were pooled. The highest rates of attrition were observed in the second and fourth cell cycles ( $P < 0.001$ ). Overall, development of  $29 \pm 6$ ,  $10 \pm 8$ ,  $28 \pm 5$ ,  $11 \pm 5$  and  $11 \pm 15\%$  of the 164 non-viable zygotes was blocked at the second, third, fourth, fifth and sixth cell cycles (at the early morula stage), respectively. In addition,  $12 \pm 9\%$  of these non-viable zygotes did not progress beyond the one-cell stage, but degenerated after distinct cleavage attempts between approximately 20 h and 30 h after insemination. These one-cell zygotes, which were all produced *in vitro* and, thus, represented 16% of the non-viable *in vitro*-derived zygotes, had been activated during fertilization, as intense activity was observed within their cytoplasm, leading to deformations or constrictions of the cell membranes but no cleavage. Similar behaviour was not observed for *in vivo*-produced one-cell ova.

Overall, 15% of all the zygotes included in the present study had an abnormal first cleavage; for example, cleavage into two cells of noticeably different sizes or directly into three or even four cells, often of irregular sizes. This abnormal first cleavage was observed in all treatment groups. However, the frequency of such abnormal cleavage was



**Fig. 2.** Overall developmental kinetics of viable ( $n = 281$ ; solid line) versus non-viable ( $n = 165$ ; dashed line) *in vitro*- and *in vivo*-derived bovine zygotes cultured *in vitro*, shown as the average time interval  $\pm$  SD from the cleavage to two-cell embryo to the respective cleavage event. Means  $\pm$  SD are pooled across treatments. Embryos that developed to the late morulae or blastocyst stages within 7 days after insemination were defined as viable. IVF: *in vitro*-produced zygotes only. \*Significant difference between zygote categories ( $P \leq 0.05$ ) by Mixed Procedure, SAS/STAT, model  $y = \text{treatment} + \text{viability} + \text{treatment} \times \text{viability}$ , random variable = trial. There were no significant differences between treatments or interactions between treatment and viability.

influenced significantly by the presence of serum during maturation and fertilization (8, 22, 21 and 8% of the *in vivo*, standard IVP, defined IVC and defined IVP zygotes, respectively;  $P < 0.001$ ). Furthermore, abnormal first cleavage was observed more frequently in non-viable than viable zygotes (across treatments: 22% ( $n = 164$ ) versus 10% ( $n = 281$ ), respectively;  $P < 0.001$ ). None of the redundant cells was observed to cleave during the subsequent cell cycles.

Death of individual blastomeres was observed directly or indirectly at all precompaction stages in every treatment group. Overall, in 4% of all observed cell cycles (based on a total of 1193 cleavage observations) one or more blastomeres died (cleavage of specific blastomeres ceased). Subsequently, these blastomeres were often observed to disintegrate or fragment during a later cell cycle. The proportion of blastomere death varied significantly between the cell cycles with the highest frequency observed at the third cell cycle (Fig. 4). Neither treatment nor *in vitro* viability influenced this parameter significantly. As a consequence of this precompaction cell death, 13% of the total of 281 observed compact morulae had noticeably reduced size, varying from 8% to 14% between treatments (not

significant). Cell death continued during compaction, frequently observed as exclusion of individual blastomeres from the compacted cell mass into the perivitelline space.

#### Duration of cell cycles of viable zygotes

The duration of the first cell cycle of *in vivo*-derived zygotes could not be measured, as the exact time of ovulation was unknown. For the *in vitro*-derived zygotes, the time from insemination to first cleavage was significantly longer for zygotes matured and fertilized in the absence of serum than in the other two *in vitro* groups ( $31.7 \pm 0.5$  h (defined IVP) versus  $27.8 \pm 0.5$  h (standard IVP) and  $27.4 \pm 0.4$  h (defined IVC);  $P < 0.01$ ). The duration of each of the subsequent cell cycles is shown (Table 2). The second and third cell cycles of *in vivo*-derived zygotes were about 1.5 h and just less than 1 h, respectively, which are shorter than those of *in vitro*-derived zygotes (defined IVC and defined IVP groups;  $P < 0.01$ ) cultured under defined conditions in the absence of serum. However, in the presence of serum during *in vitro* culture (standard IVP group), *in vitro*-derived zygotes tended to pass through the third cell cycle at a rate

**Table 2.** Duration of the cell cycle (h) of viable bovine zygotes developed *in vitro* from *in vivo* zygotes (*in vivo*) or *in vitro* in the presence of serum (standard IVP), in chemically defined medium without serum proteins (defined IVC) or in the presence of serum during *in vitro* maturation and fertilization (IVM-IVF), but in chemically defined *in vitro* culture (defined IVC)

Treatment	Presence of serum or serum proteins		Cycle 1 (one- to two-cell)	Cycle 2 (two- to three-cell)	Cycle 3 (three- to five-cell)	Cycle 4 (five- to nine-cell)	Cycle 5 (nine-cell to morula)	Morula to compact morula	Compact morula to early blastocyst	Early blastocyst to expanded blastocyst	Expanded blastocyst to hatched blastocyst
	IVM-IVF	IVC									
<i>In vivo</i> (n)	+	-	na	8.1 ± 0.3 <sup>a</sup> (50)	7.1 ± 0.2 <sup>ac</sup> (94)	45.1 ± 2.0 <sup>a</sup> (83)	21.8 ± 1.6 <sup>a</sup> (81)	18.5 ± 1.6 <sup>b</sup> (75)	23.5 ± 2.2 <sup>b</sup> (78)	19.8 ± 2.2 (90)	21.5 ± 2.4 (52)
Defined IVC (n)	+	-	27.8 ± 0.5 <sup>a</sup> (66)	9.6 ± 0.3 <sup>b</sup> (54)	8.0 ± 0.3 <sup>be</sup> (62)	43.1 ± 2.1 <sup>a</sup> (53)	26.8 ± 1.8 <sup>b</sup> (44)	16.3 ± 1.7 <sup>b</sup> (47)	25.7 ± 2.5 <sup>b</sup> (39)	17.2 ± 2.5 (31)	26.1 ± 3.1 (14)
Defined IVP (n)	-	-	31.7 ± 0.5 <sup>b</sup> (40)	9.7 ± 0.3 <sup>b</sup> (40)	8.2 ± 0.3 <sup>de</sup> (39)	48.6 ± 2.4 <sup>c</sup> (19)	27.7 ± 2.2 <sup>b</sup> (17)	16.4 ± 2.0 <sup>b</sup> (15)	22.4 ± 2.9 <sup>b</sup> (23)	19.9 ± 2.9 (18)	25.5 ± 4.9 (4)
Standard IVP (n)	+	+	27.4 ± 0.4 <sup>a</sup> (41)	9.2 ± 0.3 <sup>b</sup> (41)	7.5 ± 0.3 <sup>bc</sup> (43)	38.6 ± 2.3 <sup>b</sup> (31)	24.7 ± 2.2 <sup>ab</sup> (17)	10.6 ± 2.4 <sup>a</sup> (13)	13.4 ± 3.1 <sup>a</sup> (20)	22.0 ± 2.8 (24)	39.6 ± 4.7 (9)
P values			< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	0.02	< 0.01	ns	ns

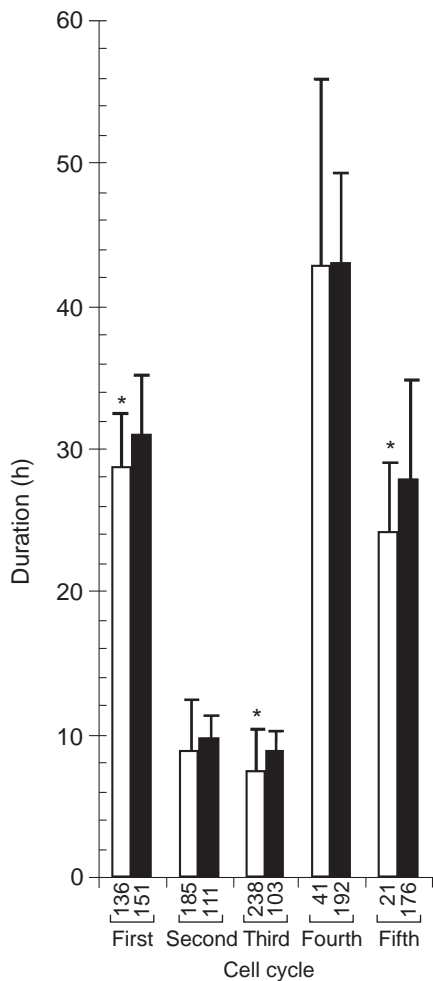
n = number of embryos.

Values are least squares means ± SEM.

na: not applicable; ns: not significant; IVP: *in vitro* production.

Effect of treatment on duration of cell cycle calculated by Mixed Procedure, model y = treatment, random variable = trial.

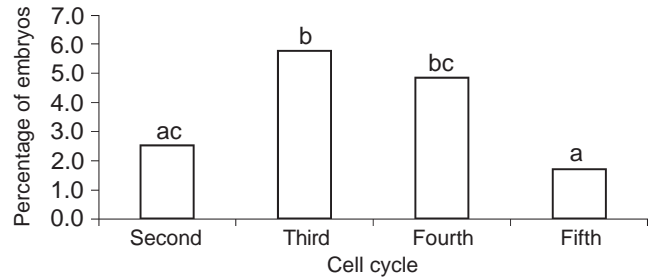
<sup>a-e</sup>Different superscripts within a column designate significant differences between individual treatments.



**Fig. 3.** Overall cell cycle duration of viable (□) versus non-viable (■) bovine zygotes cultured *in vitro*. Means ± SD are calculated across treatments. \*Significantly different from non-viable value ( $P \leq 0.05$ ) by Mixed Procedure, SAS/STAT, model  $y = \text{treatment} + \text{viability} + \text{treatment} \times \text{viability}$ , random variable = trial. There were no significant differences between treatments or interactions between treatment and viability. Data for ‘first’ are for *in vitro*-produced zygotes only. Values are number of zygotes in each group.

in between those observed for *in vivo*- and *in vitro*-derived zygotes in the absence of serum. The fourth cell cycle was the longest cycle in all groups. It was observed to be 3.5–5.5 h longer in zygotes matured and fertilized *in vitro* in the absence of serum proteins (defined IVP) compared with both *in vitro*-derived zygotes matured and fertilized in the presence of serum (defined IVC) and *in vivo*-derived zygotes (*in vivo* group;  $P < 0.01$ ). Moreover, addition of serum to the culture medium (standard IVP) decreased the duration of the fourth cell cycle further by about 5 h. The distribution of zygotes in relation to the duration of the fourth cell cycle is shown (Fig. 5).

The mean duration of the fifth cell cycle differed between *in vivo*- and *in vitro*-derived zygotes ( $P < 0.01$ ) in a similar way to the variation observed in the second and third cell



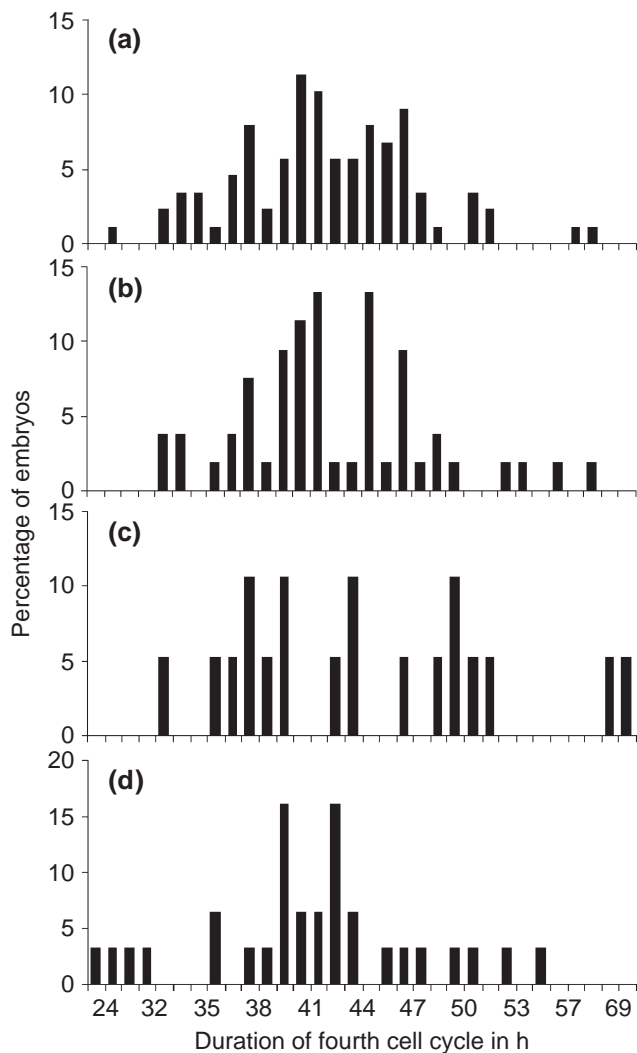
**Fig. 4.** Percentage of bovine embryos in second ( $n = 312$ ), third ( $n = 344$ ), fourth ( $n = 307$ ) and fifth ( $n = 230$ ) cell cycles in which blastomeres were dying. Frequencies are calculated across all treatment groups. <sup>a-c</sup>Different letters indicate significant differences between columns ( $P < 0.05$ ) by Genmod Procedure, SAS/STAT, model  $y = \text{treatment} + \text{cell cycle} + \text{treatment} \times \text{cell cycle}$ . There were no significant differences between treatments, but a significant interaction between treatment and cell cycle was observed.

cycles. *In vivo*-derived zygotes passed through the fifth cell cycle in the shortest time (a little less than 22 h and 5–6 h shorter than *in vitro*-derived zygotes (defined IVP and defined IVC) cultured similarly in the absence of serum. In the presence of serum, the duration of the cell cycle in *in vitro*-derived zygotes (standard IVP) was intermediate.

#### Morula compaction and blastocyst expansion and hatching

The shorter duration of the second, third and fifth cell cycles of *in vivo*-derived zygotes compared with the *in vitro*-derived zygotes cultured under similar defined conditions influenced their overall rate of development to the early morula and blastocyst stages (Table 3). Thus, *in vivo*-derived two-cell zygotes ( $n = 33$ ) reached the early morula stage after approximately 85 h, which was 7 and 12 h earlier than *in vitro*-derived two-cell zygotes produced in the presence of serum (defined IVC) or in chemically defined medium (defined IVP), respectively ( $P < 0.05$ ). *In vitro*-derived zygotes produced and cultured in the presence of serum (standard IVP) developed to the early morula stage at a similar rate, this being, on average, within 87 h from the two-cell stage. However, this was particularly a consequence of their short fourth cell cycle (Table 2).

Compaction and blastulation were not different between *in vivo*-derived zygotes and *in vitro*-derived zygotes cultured in chemically defined medium in the absence of serum (defined IVC and defined IVP): (i) the mean time intervals between the different morula and blastocyst stages did not differ (Table 2); (ii) the compact morulae of these zygotes appeared morphologically alike regarding tightness of compaction (Fig. 1); and (iii) the projected area of the cell mass at the compact morula stage was similar (see Table 4). In contrast, compaction and blastulation of *in vitro*-derived zygotes cultured in the presence of serum (standard IVP) were significantly different from the pattern described above. Firstly, the time interval from the early morula to the



**Fig. 5.** Distribution of the duration of fourth cell cycle (h) for bovine embryos in each treatment group. (a) *In vivo* group ( $n = 88$ ), *in vivo*-derived zygotes cultured in defined serum-free conditions; (b) defined *in vitro* culture (IVC) group ( $n = 52$ ), *in vitro*-derived zygotes matured and fertilized with serum, but cultured in defined serum-free conditions; (c) defined *in vitro* production (IVP) group ( $n = 18$ ), *in vitro*-derived zygotes matured, fertilized and cultured in the presence of serum; and (iv) standard IVP group ( $n = 30$ ), *in vitro*-derived zygotes matured, fertilized and cultured in the presence of serum.

compact morula with maximal compaction stage was about 6–8 h shorter (Table 2) in the presence of serum, and these zygotes did not compact to the same extent as did *in vitro*-derived zygotes cultured in the absence of serum (Fig. 1). This was also illustrated by their 10% larger cell mass area at the time of maximal compaction (Table 4). Secondly, blastulation had already occurred at 13 h after maximal compaction, which was 9–12 h earlier than in all the other zygotes cultured in the absence of serum (Table 2).

In all blastocysts, from the earliest to the hatched stages, expansion of the blastocoel *in vitro* occurred as irregular

pulses of sudden partial or occasionally total collapses followed by further re-expansion. In the present study, blastocysts completed on average 6–10 pulses from the start of blastulation to the rupture of the zona pellucida (with a variation from one to > 20). Blastocysts produced entirely in chemically defined media (defined IVP) underwent significantly more collapses than did blastocysts of the other groups (Table 4). A large proportion of expanded blastocysts experienced at least one total collapse, which often coincided with the rupture of the zona pellucida. The subsequent full re-expansion to the maximal diameter of about 200–220  $\mu\text{m}$  (Table 4) was normally accomplished within 2.0–2.5 h.

The diameter of *in vivo*-derived blastocysts was significantly smaller at the time of zona pellucida rupture compared with that of *in vitro*-derived blastocysts from all treatment groups (Table 4).

## Discussion

In the present study, time-lapse video analysis was used to describe and compare the developmental kinetics of *in vivo*- and *in vitro*-produced zygotes, and to investigate the influence of serum during IVM–IVF and culture on developmental kinetics of *in vitro*-derived zygotes. The experimental context of this study is of utmost importance for scientific interpretation and further possible generalization. Firstly, all the zygotes were subjected to *in vitro* conditions of variable duration and, thus, the behaviour of *in vivo*-derived zygotes is a reflection of their origin rather than a mirror of the behaviour of similar embryos *in vivo*. Secondly, the kinetics of embryo development are the outcome of very complex interactive processes that are influenced by many factors, some of which are difficult to control or are not known. An example of this is the multifaceted interaction between glucose, embryo sex and time-lapse conditions that influence the kinetics of embryo development (Peippo *et al.*, 2001).

As in other kinetic studies (Grisart *et al.*, 1994; van Langendonck *et al.*, 1997; Holm *et al.*, 1998), the time-lapse culture system used in the present study supported embryo development in a similar way to incubator controls. These standard control cultures comprise IVM–IVF and culture of about 100 immature oocytes in serum-supplemented media equivalent to those used in the standard IVP treatment in the present study. Rates of cleavage and development to the blastocyst stage of the incubator controls varied during the experimental period between 75–90% and 40–55%, respectively (data not shown). Hence, embryo development was comparable to earlier published results regarding our SOF culture system (Holm *et al.*, 1999).

Zygotes capable of developing beyond the early morula stage, thereby initiating the first blastomere differentiation, generally developed faster during the first three cell cycles than did the remaining zygotes. In the present study, this difference was about 1–2 h. This relationship between the duration of the first cell cycle(s) and *in vitro* viability has



**Table 3.** Intervals (h) from two-cell stage to different cleavage stages of viable bovine zygotes developed *in vitro* from *in vivo* zygotes (*in vivo*) or *in vitro* in the presence of serum (standard IVP), in chemically defined medium without serum proteins (defined IVP) or in the presence of serum during *in vitro* maturation and fertilization (IVM–IVF), but in chemically defined *in vitro* culture (defined IVC)

Treatment	Presence of serum or serum proteins		Three- to four-cell	Five-cell	Nine-cell	Morula	Compact morula	Early blastocyst	Expanded blastocyst*
	IVM–IVF	IVC							
<i>In vivo</i> (n)	<i>In vivo</i> (+)	–	8.1 ± 0.3 <sup>a</sup> (50)	15.4 ± 0.5 <sup>a</sup> (49)	61.1 ± 2.3 <sup>a</sup> (39)	85.1 ± 4.8 <sup>a</sup> (33)	104.0 ± 3.2 <sup>b</sup> (38)	126.4 ± 2.9 <sup>b</sup> (44)	142.4 ± 3.9 <sup>b</sup> (36)
Defined IVC (n)	+	–	9.6 ± 0.3 <sup>b</sup> (54)	17.9 ± 0.5 <sup>b</sup> (54)	60.7 ± 2.2 <sup>a</sup> (47)	92.4 ± 4.8 <sup>c</sup> (45)	105.8 ± 3.2 <sup>b</sup> (49)	129.0 ± 2.9 <sup>b</sup> (40)	139.0 ± 4.1 <sup>b</sup> (28)
Defined IVP (n)	–	–	9.7 ± 0.3 <sup>b</sup> (40)	17.9 ± 0.6 <sup>b</sup> (39)	67.2 ± 2.5 <sup>c</sup> (20)	97.6 ± 4.9 <sup>b</sup> (29)	108.7 ± 3.4 <sup>b</sup> (33)	132.0 ± 3.3 <sup>b</sup> (30)	144.3 ± 4.5 <sup>b</sup> (18)
Standard IVP (n)	+	+	9.2 ± 0.3 <sup>b</sup> (41)	16.6 ± 0.6 <sup>c</sup> (40)	54.7 ± 2.4 <sup>b</sup> (29)	86.6 ± 5.0 <sup>a</sup> (19)	94.1 ± 3.8 <sup>a</sup> (19)	107.5 ± 3.1 <sup>a</sup> (38)	118.1 ± 4.4 <sup>a</sup> (24)
<i>P</i> values			< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01

Results are least squares means ± SEM (n = number of embryos).

\*A substantial proportion of morphologically good quality defined IVP (*in vitro* production) early blastocysts had not yet started expansion at the end of culture at day 8 after insemination.

Effect of treatment on cell cycle duration calculated by Mixed Procedure, model y = treatment, random variable = trial.

<sup>abc</sup>Different superscripts within a column indicate significant differences between individual treatments.

been described previously (van Langendonck *et al.*, 1997; van Soom *et al.*, 1997; Holm *et al.*, 1998; Lonergan *et al.*, 1999). Chromosomal aberrations in early *in vitro*-derived bovine embryos are one cause of retarded early embryonic development (Kawarsky *et al.*, 1996; Viuff *et al.*, 2000). These aberrations are frequent, particularly in *in vitro*-compared with *in vivo*-derived embryos (Viuff *et al.*, 1999). Aberrant maternal inherited cytoplasm may also interfere with cell cycle kinetics, thus offering a possible explanation for the different kinetics of the viable and non-viable zygotes.

The results of the present study show that maturation and fertilization of bovine oocytes *in vivo* compared with *in vitro* influenced subsequent *in vitro* development of the zygotes. Firstly, *in vivo*-derived zygotes were more developmentally competent *in vitro* than were all groups of *in vitro*-derived zygotes, as 87% versus ≤ 54% of the respective zygotes developed to the compact morula or blastocyst stages. Secondly, the duration of the first cell cycles before the major genomic transition were shorter in *in vivo*-derived zygotes than in *in vitro*-derived zygotes (second, third and fifth cell cycles; the first cell cycle was not analysed). Thirdly, the average diameter of *in vivo*-derived zygotes at hatching was smaller than that of *in vitro*-produced zygotes.

Increased developmental competence is one of numerous characteristics that differentiates *in vivo*-derived zygotes from *in vitro*-derived zygotes (for a review, see Holm and Callesen, 1998) and was also observed by van Soom and de Kruif (1992). Whether the increased competence is caused solely by superior maturation or is a combination of maturation and fertilization *in vivo* has not been clarified properly. However, *in vivo*-matured oocytes are more developmentally competent than *in vitro*-matured oocytes (Greve *et al.*,

1987; Leibfried-Rutledge *et al.*, 1987; Thompson *et al.*, 1998; P. Humblot, P. Lonergan and P. Holm, unpublished) and cytoplasmic modifications in the germinal vesicle stage oocyte during the growth and dominance phase of the follicle before the LH surge are probably key elements in this difference (Blondin *et al.*, 1997; Hyttel *et al.*, 1997; van de Leemput *et al.*, 1999; Hendriksen *et al.*, 2000). Niemann and Wrenzycki (2000) reported perturbations in the mRNA expression of certain growth factors in *in vitro*-derived compared with *in vivo*-derived zygotes. Furthermore, recent studies have shown a relationship between the developmental capacity of oocytes pre-matured/matured *in vivo* versus *in vitro*-matured oocytes from an abattoir and the presence of chromosomal aberrations in the respective embryos at day 3 after fertilization (Hendriksen *et al.*, 2001). Hence, it is likely that the observed differences in the duration of the first embryonic cell cycles of *in vivo*- versus *in vitro*-derived zygotes is a consequence of such cytoplasmic differences inherited from the mature oocyte and an increased ratio of chromosomal abnormalities.

The fourth cell cycle, which corresponds to the so-called lag phase (Grisart *et al.*, 1994; Holm *et al.*, 1998), differed between *in vivo*- and *in vitro*-derived zygotes matured in the absence of serum (45 ± 6 versus 49 ± 10 h, respectively; *P* < 0.01). The presence of serum during maturation eliminated this difference, which confirms the significance of the maturation conditions on later development and illustrates the multiplicity of maternally dependent events during the earliest cleavage stages.

The first cell cycle after the major genomic transition, this being the fifth cell cycle from the nine- to the 16-cell stage, was also shorter in *in vivo*-derived zygotes than in similarly cultured *in vitro*-derived zygotes. This finding indicates that

**Table 4.** Sizes of compact morulae and blastocysts and number of collapses in blastocysts developed *in vitro* from *in vivo* zygotes (*in vivo*) or *in vitro* in the presence of serum (standard IVP), in chemically defined medium without serum proteins (defined IVP) or in the presence of serum during *in vitro* maturation and fertilization (IVM–IVF), but in chemically defined *in vitro* culture (defined IVC)

Treatment	Presence of serum or serum proteins		Late morula area at maximal compaction ( $\mu\text{m}^2$ )*	Blastocyst diameter at hatching ( $\mu\text{m}$ )	Number of collapses of blastocyst cavity until hatching <sup>†</sup>
	IVM–IVF	IVC			
<i>In vivo</i> (n)	<i>In vivo</i> (+)	–	8978 $\pm$ 234 <sup>a</sup> (56)	201 $\pm$ 4 <sup>a</sup> (43)	5.9 $\pm$ 0.8 <sup>b</sup> (46)
Defined IVC (n)	+	–	8559 $\pm$ 262 <sup>a</sup> (47)	221 $\pm$ 4 <sup>b</sup> (13)	7.0 $\pm$ 0.9 <sup>b</sup> (24)
Defined IVP (n)	–	–	8764 $\pm$ 311 <sup>a</sup> (29)	222 $\pm$ 6 <sup>b</sup> (11)	10.0 $\pm$ 1.4 <sup>a</sup> (12)
Standard IVP (n)	+	+	9806 $\pm$ 378 <sup>b</sup> (18)	217 $\pm$ 6 <sup>b</sup> (14)	7.9 $\pm$ 1.5 <sup>ab</sup> (10)
P value			< 0.01	< 0.01	$\leq$ 0.03

Results are least squares means  $\pm$  SEM (n = number of embryos).

IVP: *in vitro* production.

\*The area of the late morula was calculated by Microvision™ software on the basis of the number of pixels within the circumference of its image. The areas of late morulae in which a larger part of the blastomeres (one-half, one-third or one-quarter) had degenerated during earlier cell cycles were adjusted before statistical analysis by dividing the measured area by 0.5, 0.67 or 0.75, respectively.

<sup>†</sup>A collapse was defined as any reduction in size (small or large) of the blastocoel.

Effect of treatment on cell cycle duration was calculated by Mixed Procedure, model  $y$  = treatment, random variable = trial.

<sup>ab</sup>Different superscripts within a column designate significant differences between individual treatments.

maternal cytoplasmic factors influencing the cell cycle may still be pertinent or that the genomic transition as such is perturbed in *in vitro*-derived zygotes and, thus, may influence the subsequent cell cycle. However, the times of compaction, blastulation or hatching did not differ between *in vivo*- and *in vitro*-derived zygotes cultured under similar conditions. This finding indicates that the parts of the embryonic genome that regulate these events are able to function normally under *in vitro* culture conditions. Recent studies of the expression of specific genes involved in compaction, blastulation and metabolism of bovine embryos have led to a similar suggestion, as no differences were found between blastocysts derived from *in vivo*- versus *in vitro*-matured oocytes (Knijn *et al.*, 2001).

No differences in the kinetics or time of compaction and blastulation between *in vivo*- and *in vitro*-derived zygotes cultured in defined, serum-free medium were observed. However, the average diameter of the zonae pellucidae of *in vivo*-derived zygotes was significantly smaller (201  $\mu\text{m}$  versus 217–221  $\mu\text{m}$ ). The zonae pellucidae of *in vivo*-derived zygotes differ from those of *in vitro*-derived zygotes in structure with respect to the exposure and coating of oviductal glycoproteins (Wegner and Killian, 1991) and resistance to proteases (Pollard and Leibo, 1993). Recently, Katska *et al.* (1999) demonstrated a relationship between exposure to oviductal glycoproteins and high protease resistance by exposing *in vitro*-matured oocytes to oviductal fluid and subsequently measuring the resistance of their zonae pellucidae to proteases. Even very short exposure of *in vitro*-matured oocytes to oviductal fluid produced high resistance similar to that observed in *in vivo*-derived zygotes. It is possible that the smaller hatching diameter of the *in vivo*-derived zygotes found in the present study is also a consequence of exposure to oviductal fluid causing a

more rigid or less elastic zona pellucida, which cracks at a smaller diameter. However, the possibility that the *in vivo*-derived bovine blastocysts secrete substances that directly affect the properties of the zona pellucida, as seen in hamsters, cannot be eliminated (Gonzales and Bavister, 1995; Mishra and Seshagiri, 1998). However, when *in vivo*-derived hamster blastocysts were cultured *in vitro*, the lysis of zonae pellucidae apparently correlated to the presence of albumin in the culture medium (Mishra and Seshagiri, 1998), whereas, in the present study, *in vivo*-derived zygotes were cultured in defined conditions.

The absence of serum during maturation and fertilization significantly increased the duration of the first and fourth cell cycles by 4–5 h. Holm *et al.* (1999) reported that maturation and fertilization of bovine oocytes under defined conditions resulted in lower cleavage, as well as lower developmental capacity of the zygotes, without affecting nuclear maturation. Eckert and Niemann (1995) reported that serum proteins were required for normal pronucleus formation but not for cleavage and subsequent embryo development, which could explain the prolonged first cell cycle of *in vitro*-derived zygotes matured in the absence of serum proteins. However, in a recent study looking at the kinetics of the second meiotic division (P. Holm, M. D. Madsen, P. J. Booth and H. Callesen, unpublished), sperm penetration in the absence of serum during both maturation and fertilization was delayed by about 4 h compared with fertilization in the presence of serum, thereby providing a likely explanation for the observed prolongation of the first cell cycle. However, the prolongation of the fourth cell cycle accommodating the major genomic transition indicates that there may be underlying cytoplasmic deficiencies related to suboptimal IVM–IVF under the defined, serum-free conditions.

Neither the kinetics of compaction nor the time of blastulation differed between *in vitro* zygotes matured and fertilized with or without serum. Blastocysts matured and fertilized without serum tended to collapse and re-expand more times before hatching, indicating a higher degree of dysfunction in the trophoblast cells of such embryos.

Serum accelerates preimplantation development of bovine embryos (Pinyopummintr and Bavister, 1994; van Langendonck *et al.*, 1997; Yoshioka *et al.*, 1997; Thompson *et al.*, 1998). It also can have a biphasic action during *in vitro* culture, suppressing precompaction embryo development but enhancing blastocyst development (Pinyopummintr and Bavister, 1994); thus, addition of serum to the culture medium after the eight-cell stage can increase blastocyst development and quality (Thompson *et al.*, 1998).

It is generally accepted that the ability of serum to support embryo development differs significantly between types and batches. In the present study, the specific batch of serum had been preselected for its overall ability to support blastocyst development.

No influence of serum on the duration of the cell cycle of the first cleavage was observed in the present study, but early blastocysts were generally observed about 16–18 h earlier under serum conditions compared with serum-free conditions. The duration of the fourth cell cycle, in which the maternal–embryonic genomic transition occurs, was shortened significantly, as was the interval from the early to compact morula stage, during which time the first trophoblast cell differentiation is observed (Ducibella *et al.*, 1975). Finally, the duration of the interval from the compact morula to the early blastocyst stage was significantly shortened as a result of premature blastulation. These observations are in full agreement with those of van Langendonck *et al.* (1997). Serum contains an abundance of potential peptide growth factors, of which several improve *in vitro* development to the blastocyst stage and are present as ligands or receptors in the genital tract and preimplantation embryos (Kane *et al.*, 1997). The culture medium in general and serum specifically exert a substantial influence on the expression of genes in early preimplantation embryos, including genes involved in compaction and blastulation (Niemann and Wrenzycki, 2000). Specific differences in mRNA expression have been observed from the fourth cell cycle onwards between embryos cultured in the presence and absence of serum (Wrenzycki *et al.*, 1999).

*In vitro*-produced compact morulae and blastocysts deviate ultrastructurally from those produced *in vivo*, irrespective of the presence or absence of serum in the culture medium (Crosier *et al.*, 2000, 2001), but the presence of serum during culture adds to the deviation with respect to some cellular ultrastructures (Gardner, 1994; Abe *et al.*, 1999; Crosier *et al.*, 2000, 2001). In the present study, the most striking morphological characteristic of premature blastulation was poor compaction of morulae; a feature that has also been noticed when comparing *in vivo* and *in vitro* morulae cultured in medium supplemented with serum (van Soom and de Kruif, 1992). In the present study, the accel-

erated blastocyst formation was accompanied by a 10% increase in the projected area of the cell mass of the morulae at maximal compaction. This may reflect an increased embryo volume due to early extracellular fluid accumulation between the relatively large blastomeres of the early morulae. Serum-induced premature blastulation is not accompanied by cell proliferation (Yoshioka *et al.*, 1997; Thompson *et al.*, 1998) and the present study showed that serum supplementation did not alter the speed or quality of blastocoel expansion. This finding indicates that the effect of serum may be linked to the cell biological processes initiating blastocoel formation within the fifth cell cycle of the bovine embryo rather than to the mechanisms involved in blastocoel expansion.

The present study has illustrated differences and similarities in the developmental kinetics of bovine *in vivo*- and *in vitro*-derived zygotes under different culture conditions. In general, the *in vitro* developmental capability of bovine zygotes was reflected in the duration of their first cell cycles. In addition, the time-lapse system permitted a description of the general patterns of embryo attrition and blastomere degeneration until the morula stage. From these analyses it can be concluded that development of most of the non-viable embryos was blocked in the second and fourth cell cycles, as two-cell and five- to eight-cell embryos, but also that a large proportion of activated *in vitro*-derived oocytes did not cleave at all. Moreover, blastomere degeneration was observed in all cell cycles until the morula stages, but most often at the third and fourth cell cycles of the viable embryos. Furthermore, it is concluded that, in general, viable *in vivo*-derived zygotes develop faster through the first cell cycles than do *in vitro*-derived zygotes. However, serum supplementation during both maturation and culture was able to modify developmental kinetics significantly, thus highlighting the need to be cautious when comparing *in vivo*- and *in vitro*-derived embryos. The absence of serum during IVM–IVF increased the duration of the first cell cycle, presumably as a result of compromised sperm penetration and, also, increased the duration of the fourth cell cycle, during which the major genome transition takes place. In contrast, serum supplementation during culture significantly decreased the duration of the fourth cell cycle and, furthermore, impaired compaction and induced premature blastulation by decreasing the time between the early morula, compact morula and early blastocyst stages without parallel cell proliferation.

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