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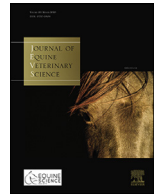
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Short Communication

Embryo Pulsing: Repeated Expansion and Contraction of *In Vivo* and *In Vitro* Equine Blastocysts

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A B S T R A C T

Morphokinetic evaluation of embryo development has allowed the discovery of events occurring during blastulation. Here, we describe equine embryo pulsing, determined as continued expansion and contraction of both *in vivo* and *in vitro* produced blastocysts. Using time-lapse imaging, we demonstrated that pulsing starts during early blastocyst development of *in vitro*-produced embryos in horses. The median time for a complete contraction was 0.22h (0.08h–2h; min-max) where embryos reduced their sizes around 12.0% (median; 2.3%–27.0%) and the median time for an expansion was 3.3h (0.75–9.0h) where embryo re-expanded around 16.9% (3.2%–42.8%). We also found that pulsing can be observed in *in vivo*-produced embryos obtained from mares 6.5 days after ovulation and continues during the expansion of the blastocysts. Even though its exact mechanism remains unknown, studies in human IVF suggest that the pulsing of embryos is associated with embryo quality and implantation rates. Thus, further investigations regarding this event in equine *in vitro* production procedures are warranted. Additionally, the pulsing in the *in vivo*-produced embryos could explain the diverse morphology occasionally observed in the collected or shipped embryos. Future studies are necessary to understand the underlying mechanism of pulsing and its association with embryo quality and embryo transfer outcome.

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1. Introduction

Time-lapse microscopic (TLM) technology has increased our understanding of embryo formation and allows us to observe morphological events during embryo development. One of the observed events during embryo development is repeated embryo expansion and contraction motion. This event was first reported for rabbit blastocysts in 1929 [1] and is defined as the separation of trophectoderm (TE) from zona pellucida (ZP) during contraction, followed by re-expansion. Embryo pulsing has also been described in mice [2], rats [3], and cattle [4], and it has been hypothesized that embryo pulsing is associated with blastocyst hatching

[4]. However, the complete hatching of a blastocyst without the pulsing has also been described [5]; thus, the exact purpose and underlying mechanism for embryo pulsing remains unknown. It has been suggested that the osmotic pressure, gap junctions in between TE and inner cell mass (ICM), ion and water channels and pumps, and contractile proteins are involved in this event [6–9].

Previously, our laboratory reported embryo pulsing being observed in *in vitro*-produced equine embryos [10]. Similarly, Lewis et al., reported the presence of pulsing in equine embryos using timelapse imaging [11]. These studies showed that the *in vitro* blastocysts start pulsing around 146.7h ± 3.9h and 176.6h ± 26.9h after the intracytoplasmic sperm injection (ICSI) [10,11]. Even though it seemed plausible, embryo pulsing has not been reported in *in vivo*-produced equine embryos. *in vitro*-produced embryos usually present distinctive differences from their *in vivo* counterparts, such as lower cell number and an altered ICM–TE ratio, and more importantly, in horses, the failure to produce the characteristic glycoprotein capsule, highlighting the differences between the two embryo production methods [12–16]. Therefore, we aimed to investigate the presence of embryo pulsing in the *in vivo*-produced

embryo. We postulated that these changes in the morphology of the embryos could be associated with the occasional findings of collapsed embryos after an embryo collection or shipment of embryos.

Several studies have investigated the association between pulsing and embryo quality, viability, and implantation rate. In humans, the degree of blastocyst pulsing was shown to be associated with implantation rate [7,17]. Similarly, the degree of contraction and expansion has been correlated with the quality of embryos in mice [9]. Additionally, the time-lapse observation of embryo pulsing has been suggested to be an indicator of embryo quality after the vitrification-warming step [18]. Before this event can be used for the assessment of embryo quality in horses, embryo pulsing needs to be fully characterized in the equine blastocyst. Therefore, the aim of this short communication was to detail the embryo pulsing event in *in vitro*-produced equine embryos. Additionally, we aimed to investigate the presence of pulsing in the *in vivo*-produced embryos. This information will serve as a foundation for implementing the use of time-lapse imaging to evaluate equine embryo quality.

2. Materials and Methods

2.1. In Vitro Embryo Production

Oocyte cumulus complexes (COC) were collected from client-owned mares, collected in our facility, or shipped to our laboratory at ambient temperature (21ºC–22ºC) in commercial holding medium as previously described [10]. After the holding period, the COCs were matured [10], and the oocytes that reached Metaphase II were selected for ICSI based on the presence of one polar body. Following ICSI, zygotes were transferred to a dish (CultureCoin, Esco Technologies) with 25 µl embryo culture media (54% DMEM/F-12, 40% Global, 6% FBS, 10 mL mL-1, ITS solution and 0.1 mM sodium pyruvate) under light mineral oil at 38.2°C, 5.8% CO_2, 5% O_2 and 89.2% N_2 and placed in the MiriTL imaging incubator [10]. Embryos were monitored continuously with noninvasive time-lapse imaging using the MiriTL incubator system with image-capture every 5 minutes for 7–9 days after ICSI, as previously described [10]. The individual time-lapse images were then assembled by the MiriTL software into AVI movies and archived for retrospective analysis. The initiation of embryo pulsing was calculated from the ICSI procedure (T0).

Additionally, to demonstrate that embryos resume the pulsing motion after they were vitrified, frozen embryos (n = 5) were warmed (thawed) and placed in *in vitro* culture conditions in the MiriTL incubator, as described above, for 48 hours to observe the continuation of their development.

2.2. In Vivo Embryo Production

Three reproductively healthy mares were scanned until the detection of a $\geq$ 35 mm preovulatory follicle accompanied by the presence of uterine edema. After induction of ovulation with 1,000 IU of hCG, artificial insemination was performed within 24 hours of induction of ovulation, using chilled semen from a stallion of known fertility. Mares were then scanned every 12 hours to confirm the ovulation. At day 6.5 after ovulation, their uterus was flushed using lactated Ringer's solution [18]. Recovered embryos were placed in equine holding medium (EquiHold, Minitube) at 22°C–24°C, and transported to the laboratory within 30 min of collection. Recovered embryos (n = 2) were rinsed in three droplets of a pre-equilibrated embryo culture media (described above) and individually placed into CultureCoin and incubated for 48 hours as described above. The presence of embryo pulsing was investigated by reviewing the TL recordings. After 48 hours of culture,

embryos were observed under the Axio Observer inverted microscope (Zeiss).

2.3. Morphokinetics Assessment

The presence of pulsing and changes in the blastocyst dimensions were evaluated by using the measurement tools incorporated in the MiriTL software. To determine changes in size over time, TL recordings were assessed frame by frame to determine the moment of the first full expansion, from that point, the video was advanced until the moment of the following full contraction occurred, and all consecutive full expansions and contractions were analyzed. Area (μm^2) was assessed using a circle measuring tool, drawing a circle around the blastocyst inside the ZP, and diameter (μm) was determined by tracing a measurement line from the outer edges of the ZP on opposite sides. This measurement allowed to determine the changes in overall size of the embryos on every expansion/contraction. Additionally, the measurement of the ZP thickness was analyzed to determine the degree of stretch/tension on every full expansion. To obtain an accurate measurement, the image was zoomed in to carefully determine the outer and inner edges of ZP.

3. Results

3.1. In Vitro Embryos

AVI movies from six embryos were analyzed. The *in vitro*-produced blastocysts showed pulsation which started at 148.8 $\pm$ 11.6 hours (mean $\pm$ SD) from the moment of ICSI (Video 1). During this period of development, there is an overall embryo growth which is characterized by the increase in diameter of the blastocoel (Fig. 1A) which causes the stretching and thinning of the ZP (Fig. 1B). The contraction and re-expansion of each embryo (n = 6) were detailed over 20 individual observation checkpoints (Fig. 1C). The smallest surface area was set as the base observation (contraction). The AVI movie was followed till the highest surface area for that embryo was measured (observation checkpoint 1, expansion), followed by the smallest surface area (observation checkpoint 2, contraction). Clear expansion and contraction cycles were observed for all the embryos (Fig. 1C). We also measured the changes in the surface area between each checkpoint (Fig. 1D). The median changes in the surface area were 12.0% and 16.4%, for contractions and expansions, respectively (2.3%–27.0%; min-max for contractions and 3.2%–42.8% for expansions). Based on the Wilcoxon rank-sum test, there was a significant difference between contractions and expansions for the changes in the surface areas ($P <.05$). We also assessed the time required for each complete contraction and re-expansion (Fig. 1E). The median time of contractions was 0.22h (0.08h–2h), while the embryos took significantly longer to re-expand ($P <.05$; Wilcoxon rank-sum test) with a median of 3.33h (0.75h-9h).

We also assessed the pulsing in the post-thaw/warming embryos. *In vitro-produced* embryos that were vitrified, then warmed and cultured for 48 hours, resumed the pulsing motion as soon as they were placed in the culture media. Additionally, they continued increasing in size and pulsing until the moment the culture was stopped after 48 hours. The characteristics of the pulsing were similar before and after vitrification.

3.2. In Vivo Embryos

The two embryos recovered on Day 6.5 after ovulation (n = 2) presented a similar appearance and morphology (Fig. 2A and Video 2) to those produced *in vitro* when they reach the blastocyst stage between day 7 and day 9 after ICSI. By analyzing the time-lapse
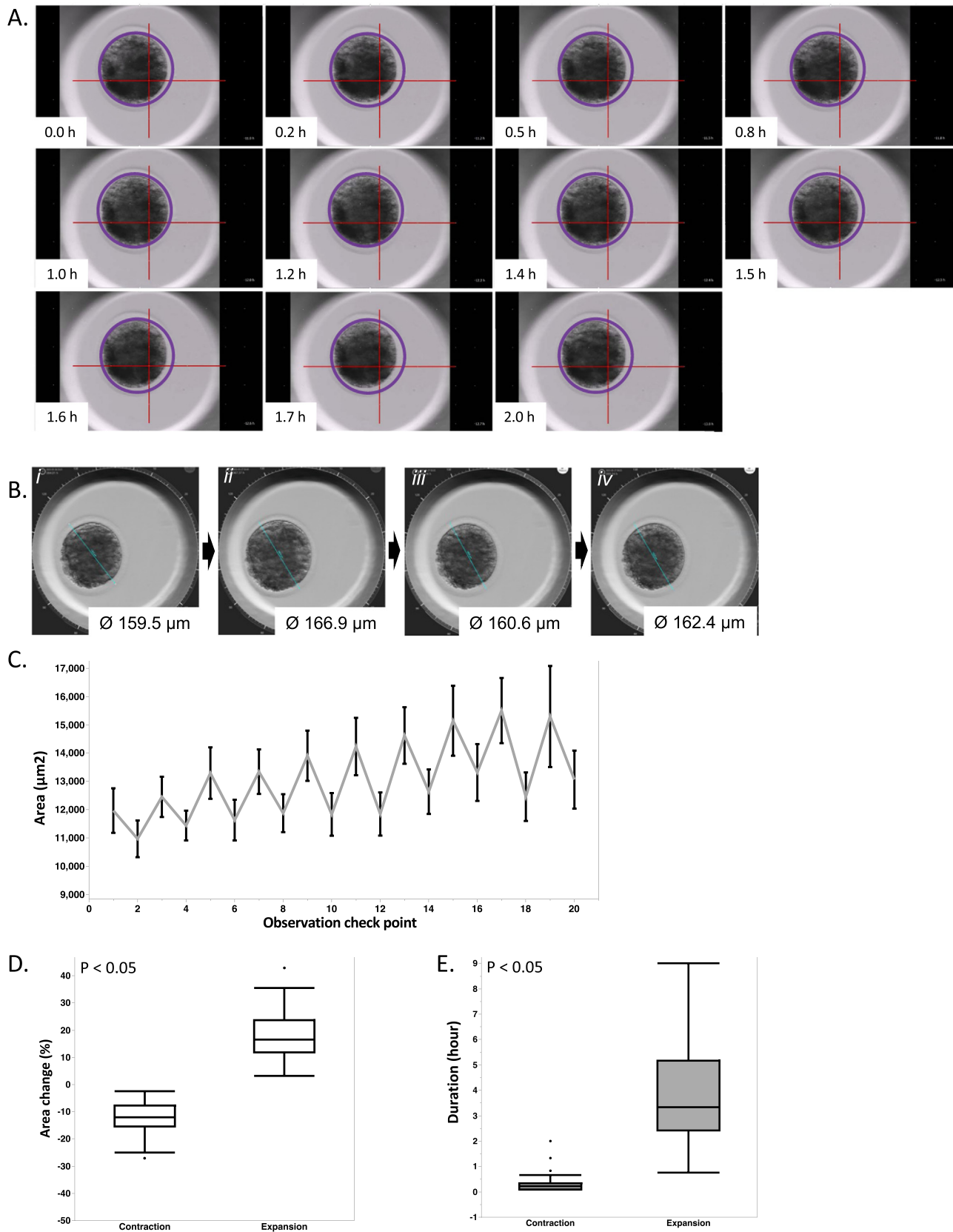
Fig. 1. *In vitro-produced* (IVP) embryos shows pulsing motion starting around 146 hours of culture. The cycle of contraction and expansion is represented by a decrease and increase in size over time accompanied by a progressive overall increase in size (A–C). We determined that IVP embryos increase their size by an average of 7 μm from the start of pulsing until the moment there is an observable formation of TE (n = 6). By using a circle drawing and the red cross as a reference, it can be observed the variation in size at different time points (A). Measurements of area (μm²) during consecutive full expansions and contractions show an overall increase in size over time (C) with a greater change in size during an expansion versus a contraction (D; n = 6). The time taken to fully expand is significantly longer than that taken for a full contraction (E; n = 6).
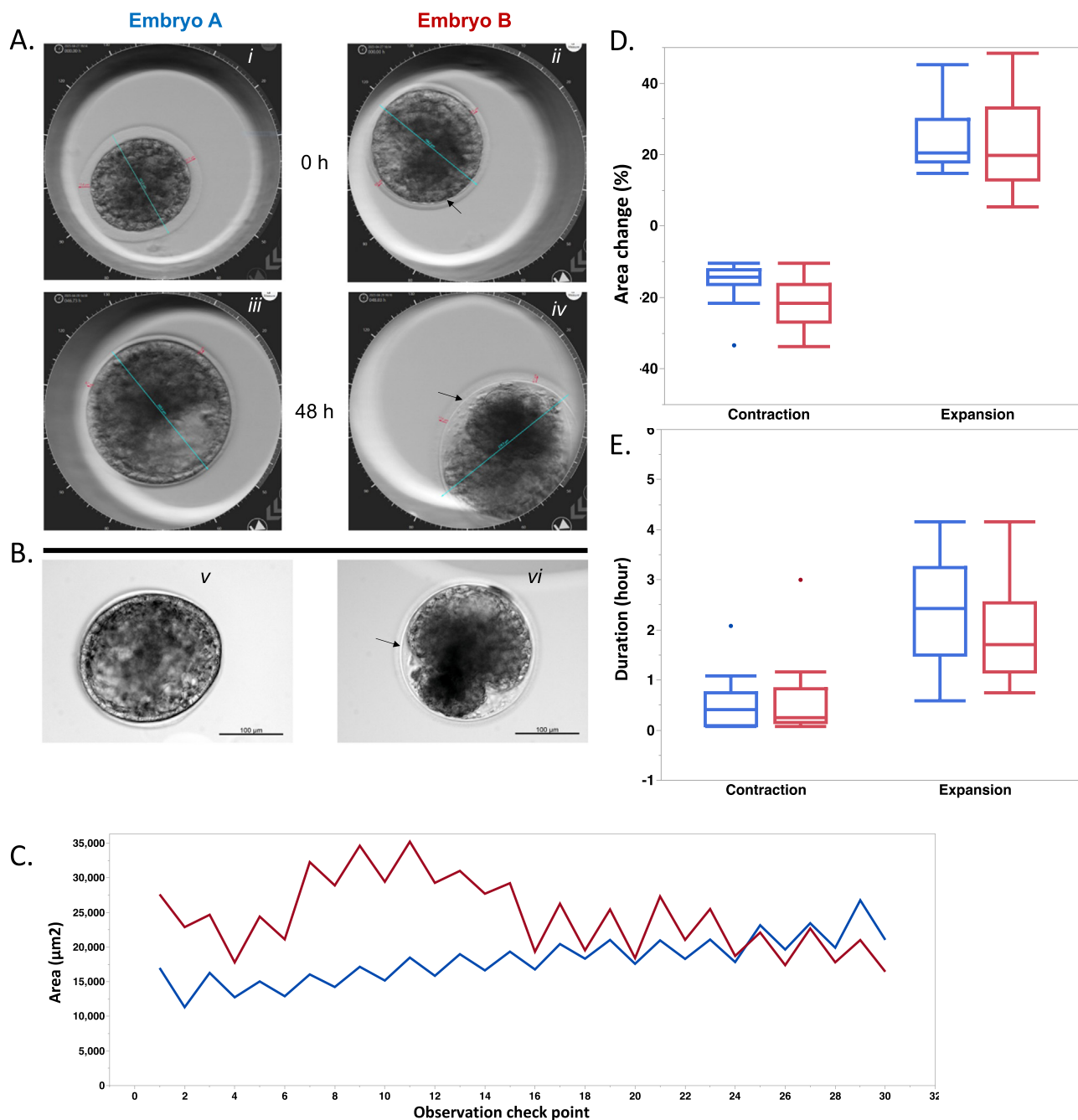
Fig. 2. Two *In vivo-produced* embryos were cultured *in vitro* in the MiriTL incubator for 48 hours. Measurements of the blastocyst diameter and ZP thickness at time 0 and at 48 hours (A) of culture are shown an overall increase in size and thinning of the ZP during the culture time. The images were also obtained from Embryo A and B (B) using the Axio Observer microscope (Zeiss). Black arrows denote the presence of the presumptive formation of a capsule (A–B). Measurements of area (μm²) during consecutive full expansions and contractions of the blastocoel revealed a pattern similar to that observed in IVP embryos (C), with a greater change in size during expansions compared to contractions (D) and a shorter time required for full contraction compared to the time required for a full expansion (E).

recordings, it was determined that *in vivo*-produced embryos also show a pulsing motion as soon as they were placed in the culture media (Video 2). It was observed that Embryo A kept expanding in size and its ZP reduced 60% of its thickness from the beginning of the culture (17 μm) until 48 hours after (6.95 μm) (Table 1, Fig. 2A–B). However, Embryo B showed the maximum expansion of the blastocoel around 14 hours after the beginning of culture, and at the 48 hours of culture, the blastocoel was "collapsed" into a smaller size. Interestingly, the full diameter of the embryo measured in the outer edge of ZP only slightly decreased in size after the maximum expansion (Table 1, Fig. 2A).

We also detailed the contractions and re-expansions of these two embryos (Fig. 1C). The median changes in the surface area during contraction were 14.2 % (10.3%–33.4%) and 21.6% (10.4%–33.8%) for Embryo A and B, respectively (Fig. 2D). The median changes in the surface area during expansion were 20.5% (14.7%–45.1%) and 19.8% (5.4%–48.3%) for Embryo A and B, respectively. The time required for each contraction and re-expansion was also measured (Fig. 2E). The time to contraction was 0.41h (0.08–2.08 hours) and 0.33h (0.08–3.00 hours) for Embryo A and B, respectively, while the time to re-expansion was 2.42h (0.58–4.16 hours) and 1.59 hours (0.75–3.17 hours).

Table 1

Morphological measurement of *in vivo*-produced embryos A and B during 48 hours of culture. The diameter was determined using the linear measurement tool (μm) and the area was calculated using the circle-area measurement tool (μm^2) of TL software. ZP: zona pellucida.

Time Point	Embryo A		Embryo B	
	Diameter (μm)	ZP Thickness (μm)	Diameter (μm)	ZP Thickness (μm)
T0	165.1	16.5	184.2	8.5
T12	168.5	16	222.0	7.3
T24	177.4	14.3	222.5.05	9.4
T48	203.4	6.6	218.5	10.65

It was determined that both *in vivo*-produced embryos failed to hatch from the ZP during the culture period. It was also observed that Embryo B presented a membrane-like structure (presumptive capsule) that could be observed adjacent to the inner side of the ZP (Figs. 2A and B, denoted with black arrows). Such structure was not observed in Embryo A. Embryo A was smaller in size at the time of collection and reached its maximum size by the end of the culture period with a diameter of 203.4 μm.

4. Discussion

We demonstrated the presence of embryo pulsing in both *in vitro*- and *in vivo*-produced equine embryos. We were able to identify the start of pulsing in the *in vitro*-produced embryos. However, for the *in vivo* embryos, the exact timepoint of start of pulsing remains unknown since the embryo pulsing was detected immediately after placing the blastocyst-stage embryos into the time-lapse incubator. Due to the restricted window of time during which *in vivo* embryos reach the uterus, identifying the time of the first pulsation could be challenging. Nevertheless, we were able to demonstrate that the *in vitro*-produced embryos pulse as early as the early blastocyst stage, and the *in vivo*-produced embryos show a very similar pattern of pulsing. We found that in both *in vivo* and *in vitro* embryos, the time embryos took to re-expand was longer than the time required for contractions. This could suggest that the mechanism responsible for expansion is a more passive process compared to the contraction mechanism which could be more active.

The time-lapse imaging system was set to capture an image every five minutes. Therefore, one limitation is the inability to fully analyze the frequency of pulsing since more than one cycle of contraction/expansion could be occurring in the interval between image capture. However, the intensity and morphodynamics of pulses can be measured and this information might be used to determine a correlation with embryo quality. It has been reported that excessive intensity of pulses or weak pulses are associated with altered hatching of embryos in mice [9]. This finding was not observed in human embryos and no significant correlation between lower/higher percent contraction and embryo hatching was observed [19]. However, in human embryos, the intensity of the pulses was shown to be associated with implantation rates, where the embryo with intense contractions (more than 50% of the total size) had lower implantation rates [7,20]. In our observation, among the *in vitro*-produced embryos, we observed less than 50% changes in the total surface area. An automated tool to measure the changes in the surface area could be of interest to assess the quality of embryos. In our laboratory, we have used the embryo pulsing event as an indicator for the blastocyst development stage and overall viability of embryos. In our setting, this parameter indicates the optimal time for the equine embryo vitrification and also could be used for the assessment of embryo quality postvitrification/warming. A future study on the morphokinetics of pulsing events and the pregnancy outcome is warranted.

One of the main limitations of this study is the low number of *in vivo*-embryos used for time-lapse evaluation. However, with two embryos we were able to demonstrate the embryo pulsing in the *in vivo*-produced equine embryo for the first time. This finding could explain those contracted (collapsed) embryos recover from uterine flushes and eventually the embryos become "normal" (expanded) after embryo washing or a brief holding step or explained the presence of a collapsed embryo after the shipment of a "normal" looking embryo. Another interesting observation in the study was documenting the formation of a membrane between the embryo TE and the ZP in one of the embryos, indicating that the capsule is created as the embryo further develops, and it seems like the time spent in the uterus is critical for this to occur. Similar finding was described by Tremoleda et al., showing the presence of a thin capsule on two 6.5D embryos between ZP and TE [13]. This finding shows that even a short period of contact between the embryo and endometrium could initiate the signal for the formation of the capsule. Finally, the hatching event where the formed embryo emerged from the zona pellucida was not observed within the 48 hours of culture of *in vivo* embryos or within 48 hours of culturing the vitrified embryos after warming, which suggests that the growth achieved during this time was insufficient or that the *in vitro* culture conditions lack the proper signaling molecules to trigger hatching even in embryos produced *in vivo*.

Overall, we demonstrated that both *in vitro* and *in vivo*-produced embryos show a pulsing motion during blastulation under *in vitro* culture conditions in the time-lapse imaging application. Further, we have also demonstrated that thawed embryos also show this very dynamic, and likely critical, pulsation event. Taken together, our findings indicate that equine blastocysts require this phenomenon of pulsation for optimal embryo survival.

Declaration of Competing Interest

The authors do not have any conflict of interest to declare.

CRediT authorship contribution statement

Alejandro de la Fuente: Conceptualization, Data curation, Formal analysis, Methodology, Writing – original draft. **Kornelia Omyla:** Methodology. **Peter Daels:** Conceptualization, Writing – review & editing. **Stuart Meyers:** Conceptualization, Resources, Writing – review & editing, Supervision. **Pouya Dini:** Conceptualization, Funding acquisition, Resources, Writing – original draft, Supervision.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.jevs.2023.104891.

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