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In vitro maturation in synthetic oviductal fluid increases gene expression associated with quality and lipid metabolism in bovine oocytes

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Research Article

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Summary

Traditionally, *in vitro* oocyte and embryo culture progresses through a series of varying culture medium. To investigate simplifying the *in vitro* production of bovine cumulus–oocyte complexes (COCs), this study used synthetic oviductal fluid (SOF) supplemented with conjugated linoleic acid (CLA). Special interest was placed on gene expression linked to lipid metabolism and oocyte maturation. COCs were matured in different media: Medium 199 (M199 group), M199 with 100 µM CLA (M199 + CLA group), SOF (SOF group), and SOF with 100 µM CLA (SOF + CLA group). COCs matured with SOF showed a higher relative abundance of mRNA of quality indicators gremlin 1 (*GREM1*) and prostaglandin-endoperoxide synthase 2 (*PTGS2*) in oocytes, and *GREM1* in cumulus cells compared with in the M199 group. SOF medium COCs had a higher relative abundance of fatty acid desaturase 2 (*FADS2*) compared with the M199 group, which is essential for lipid metabolism in oocytes. Furthermore, the abundance of stearoyl-coenzyme A desaturase 1 (*SCD1*) in oocytes matured with SOF was not influenced by the addition of CLA, whereas the relative abundance of *SCD1* was reduced in M199 medium with CLA. We concluded that maturation in SOF medium results in a greater abundance of genes linked to quality and lipidic metabolism in oocytes, regardless of the addition of CLA.

Introduction

In vitro embryo production (IVEP) includes oocyte maturation, fertilization, and embryo culture. A specific culture medium is used for each of these steps. Tissue culture medium 199 (TCM 199) is widely used as a base for *in vitro* maturation of bovine oocytes and can be modified to improve the results (Russell *et al*., 2006). Synthetic oviductal fluid medium (SOF) is commonly used in embryo culture, and is similar to TCM 199, medium modifications may improve performance with its use (Hosseini *et al*., 2008). When considering the development of an appropriate medium for all stages of IVEP, it is necessary that any added compound is capable of supporting both cumulus–oocyte complexes (COCs) and embryos, in addition to providing benefits such as increasing the quality of structures. The feasibility of using a suitable medium for all of these steps was evaluated by Gandhi *et al*. (2000). They showed that it is possible to achieve success using SOF in all stages of IVEP. One way to assess the quality of COCs is to evaluate the expression of genes that are markers of the quality of their structures. Different genes have been identified as important for oocyte competence including gremlin 1 (*GREM1*), prostaglandin-endoperoxide synthase 2 (*PTGS2*), and amphiregulin (*AREG*), which are closely related to the cumulus cell expansion process and acquisition of oocyte competence. It has been shown that embryonic cryotolerance is reduced in the presence of excessive lipid droplets and, to circumvent this problem, targeted modifications in the culture conditions can reduce lipid accumulation (Abe *et al*., 2002). The addition of conjugated linoleic acid (CLA), from the omega 6 family, to embryo culture medium containing fetal bovine serum (FBS), has been shown to reduce the accumulation of embryonic lipids and significantly improve post-cryopreservation survival (Pereira *et al*., 2007).

Matos *et al*. (2015) showed a direct effect of CLA on oocytes subjected to cryopreservation; however, further changes in maturation conditions capable of positively modifying the quality of the oocyte have the potential to generate embryos with greater cryoresistance. Therefore, embryonic lipid accumulation can be reduced by exposing these structures to CLA, and the use of CLA at an earlier stage can modulate the distribution of lipid droplets during IVEP, that is, in

oocytes. In this context, Lapa *et al.* (2011) demonstrated that the use of CLA during oocyte maturation interferes with lipid metabolism and improves the ability of oocytes to become higher quality blastocysts. Oocytes can be directed through the addition of molecules such as CLA or broader modifications to the culture medium to develop a lipid profile capable of providing greater cryoresistance once in the embryo stage.

Lipid metabolism can be evaluated by quantifying lipid droplets or evaluating the relative expression of associated genes. A range of genes regulates lipid metabolism, with fatty acid desaturase 2 (*FADS2*) and stearoyl-CoA desaturase 1 (*SCD1*) desaturases being responsible for catalyzing the synthesis of polyunsaturated fatty acids and reducing the lipotoxicity of saturated fatty acids, respectively (Aardema *et al.*, 2017; Warzych *et al.*, 2017), and sterol regulatory element-binding transcription factor 1 (*SREBP1*) being responsible for regulating the level of intracellular cholesterol (González-Serrano *et al.*, 2013).

In this study, SOF medium was used in the *in vitro* maturation of bovine COCs to develop a simplified and efficient IVEP system. Indeed, we hypothesized that SOF medium with or without CLA used *in vitro* maturation increases the expression of quality marker genes and lipid metabolism of bovine COCs. The aim of the present study was to investigate the use of SOF with CLA in the gene expression of quality markers (*GREM1*, *AREG* and *PTGS2*) and lipid metabolism (*SREBP1*, *FADS2* and *SCD1*) in matured COCs.

Materials and methods

Obtaining cumulus–oocyte complexes (COCs)

Immature COCs were collected from the ovaries of bovine females from a slaughterhouse located 20 km from the laboratory. Ovaries were harvested immediately after slaughter, immersed in saline solution (0.9% NaCl) at 37°C and taken to the laboratory for immediate use. After cleaning, the ovarian follicles with a diameter of 2–8 mm were aspirated with a needle (40 mm × 1.2 mm; 19 gauge) attached to a 10-ml syringe to obtain the COCs. The aspirated COC-containing follicular fluid was deposited in 15-ml sterile conical tubes and held at 37°C for ~20 min until pellet formation. After settling in follicular fluid, the COCs were recovered and classified into grades I, II, or III, according to their cellular characteristics as previously described (Khurana and Niemann, 2000) with the aid of a stereomicroscope (Nikon, SMZ800N, Tokyo, Japan). Only COCs with a homogeneous cytoplasm and three layers of compact cumulus cells were used (grades I and II).

In vitro maturation of COCs

COCs were distributed into the experimental groups: Medium 199 (M199, catalogue number 11150059; Gibco, USA) supplemented with 75 µg/ml amikacin, 4 mg/ml bovine serum albumin (BSA), 0.01 IU/ml follicle stimulating hormone receptor (FSHr), 0.001 µg/µl 17β-estradiol, and 1 µM cysteamine (M199 Group); Medium 199 supplemented with 75 µg/ml amikacin, 4 mg/ml BSA, 0.01 IU/ml FSHr, 0.001 µg/µl 17β-estradiol, 1 µM cysteamine, and 100 µM CLA (catalogue number O5507; Sigma-Aldrich, USA; mixture of *cis*- and *trans*-9,11- and 10-,12-octadecadienoic acid) diluted in dimethylsulfoxide (M199 + CLA group); SOF medium (107.63 mM NaCl, 7.16 mM KCl, 1.19 mM KH_2PO_4, 1.51 mM $MgSO_4$, 1.78 mM $CaCl.2H_2O$, 5.35 mM sodium lactate, 25.00 mM $NaHCO_3$, 7.27 mM Na-pyruvate, 0.20 mM L-glutamine, 45.0 µl/ml Basel Medium Eagle (BME) amino acids, 5.0 µl/ml Minimum Essential Medium (MEM) amino acids, 0.34 mM tri-sodium citrate 0.34 mM, 2.77 mM myo-inositol, 50 µg/ml gentamycin, 78.125 µg/ml amikacin, 4% bovine serum albumin) without hormones (SOF group); or SOF medium without hormones plus 100 µM CLA (SOF + CLA group). The media for all four treatments were supplemented with 10% FBS from the same lot. The selected COCs (20 COCs per treatment group) were matured in 100-µl drops of medium in sterile Petri dishes with a mineral oil overlay incubated at 38.5°C and 5% humidity for 22–24 h. Five repetitions were performed of the M199 and M199 + CLA groups and of the SOF and SOF + CLA groups, with 20 oocytes in each repetition, and each repetition producing cumulus cells.

Preparation of oocytes and cumulus cells for gene expression analysis

After the maturation period, oocytes were separated from the cumulus cells using Medium 199 with HEPES (M199H; catalogue number 12350039; Thermo Fisher Scientific, USA) supplemented with 75 µg/ml amikacin and 10% FBS by means of agitation with an automatic pipette. Oocytes and cumulus cells were placed in 1.5-ml tubes containing 30 µl of M199H and then agitated for ~5 min in a sample shaker (Marconi®, BR). After shaking and another round of M199H washing, oocytes were placed in 1.5-ml tubes and frozen at −80°C. The cumulus cells resulting from this process were centrifuged at 10,000 rpm for 5 min in tubes containing 100 µl of M199H. After centrifugation, the supernatant was discarded, and the cumulus cell pellet was frozen at −80°C.

Analysis of gene expression

Samples were subjected to RNA extraction using the PicoPure RNA Isolation Kit (KIT0204; Applied Biosystems, USA) according to the manufacturer's instructions. Total RNA concentrations and quality (ratio 260/280 nm) of cumulus cell samples were measured using spectrophotometry (NanoDrop, ND-2000®, Thermo Fisher Scientific, USA). All total RNA samples were treated with DNase before being subjected to reverse transcription (RT). RT of the samples was performed using a HighCapacity Kit (Applied Biosystems, USA) according to the manufacturer's instructions. Characteristics of the oligonucleotide primers used are listed in Table 1. RT-qPCR analysis of each target gene (*FADS2*, *SCD1*, *SREBP1*, *GREM1*, *AREG*, and *PTGS2*) was performed using the QuantStudio ™ 7 Flex equipment (Applied Biosystems, USA) and the SYBR® Green PCR Master Mix System (Applied Biosystems, USA). Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), cyclophilin A (*CYCA*) and ribosomal protein L15 (*RPL15*) were used as reference genes. The annealing temperature was 60°C for all primers used, and the final concentration of each primer used was 300 µM, except *GAPDH*, for which the concentration was 200 µM. To select the most stable reference gene for detailed analysis of oocytes and cumulus cells, the amplification profiles of *GAPDH*, *CYCA* and *RPL15* were compared using the NormFinder applet in Microsoft Excel (moma.dk/normfinder-software). The most stable reference gene for the cumulus cells was the combination of *CYCA* and *GAPDH* and for oocytes *GAPDH* was used. The efficiency-corrected ΔΔCt method was used to calculate the relative expression values for each target gene using a control sample as a calibrator, a relative quantification was performed with the Pfaffl method (Pfaffl, 2001). For the analysis of mRNA expression of the target genes, 1 µl of each sample and 24 µl of a mixture containing the probe and primer of interest were combined. The RT-qPCR

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Table 1. Gene symbol, protein name, and forward (F) and reverse (R) primer sequence of the genes quantified via qPCR in oocytes and cumulus cells

Gene	Protein name	Foward (F) and reverse (R) primer sequence	
AREG	amphiregulin	F	CTTTCGTCTCTGCCATGACCTT
		R	CGTTCTTCAGCGACACCTTCA
GREM1	gremlin 1	F	TGGTGCAAGGGCAAGAAGGATAG
		R	CACTGTGTTTGGAGGTTGGCCTTT
PTGS2	prostaglandin-endoperoxide synthase 2	F	AAGCCTAGCACTTTGGGTGGAGAA
		R	TCCAGAGTGGGAAGAGCTTGCATT
FADS2	fatty acid desaturase 2	F	GGGTGATGATGTGCTGGATT
		R	CCCTGGACATCTGAAGAGAAAG
SCD1	stearoyl-CoA desaturase 1	F	GACCCTGGGCAAGTCATTT
		R	AAACTGCCCTTTGAGGTAGG
SREBP1	sterol regulatory element-binding transcription factor 1	F	GACTACATCCGCTTCCTTCAG
		R	CCAGGTCCTTCAGCGATTT
PPIA	Peptidilpropil isomerase A (ciclofilina A) [G]	F	GCCATGGAGCGCTTTGG
		R	CCACAGTCAGCAATGGTGATCT
GAPDH	glyceraldehyde-3-phosphate dehydrogenase	F	GGCGTGAACCACGAGAAGTATAA
		R	CCCTCCACGATGCCAAAGT
CYCA	cyclin A	F	GCCATGGAGCGCTTTGG
		R	CCACAGTCAGCAATGGTGATCT
RPL15	ribosomal protein L15	F	CTCATCGTTGGTGCCAATGCAAGT
		R	TCACATCCACCCTGGGAAACAGAA

conditions were as follows: 95°C for 10 min, then 40 cycles of denaturation at 95°C for 10 s, followed by annealing and extension for 1 min.

Statistical analysis

The samples were distributed in a completely randomized 2 × 2 factorial scheme, totalling four treatments: six repetitions were performed of the M199 and M199 + CLA groups, and five repetitions of the SOF and SOF + CLA groups, with 20 oocytes in each repetition. Data were submitted to analysis of variance (ANOVA), and in case of significant difference, compared using Tukey's test. The results are presented as the mean ± standard error of the mean (SEM) values of the relative abundance of mRNA, with significance considered at $P \leq 0.05$.

Results

Considering genes linked to oocyte competence (*GREM1* and *PTGS2*) and to lipid metabolism (*FADS2*), there was a higher expression in oocytes matured in SOF compared with M199. However, a significant decrease in *SCD1* expression was observed in oocytes matured in SOF medium compared with those matured in M199 (Table 2).

With the exception of *SCD1*, there was no interaction between the medium (M199 or SOF) and the addition of CLA for the other genes, i.e. the use of CLA is not influenced by the medium used. The changes in *SCD1* expression in the different culture medium conditions are shown in Table 3. The expression of *SCD1* in oocytes matured in M199 medium was higher than that in oocytes matured in SOF without CLA and with CLA, whereas the addition of CLA to M199 medium decreased the expression of *SCD1* compared with M199 medium without CLA.

The expression levels of *FADS2*, *GREM1*, *SCD1*, *SREBP1*, *AREG*, and *PTGS2* in cumulus cells matured in M199 and SOF medium with or without CLA are presented in Table 4. In cumulus cells, *GREM1*, a gene linked to oocyte maturation, showed higher expression levels in SOF than in M199 medium. There was no interaction between the medium (M199 or SOF) and the addition of CLA for any of the genes studied in the cumulus cells, i.e. the use of CLA, was not influenced by the medium used.

Discussion

On the path to optimize bovine IVEP, this was the first study to evaluate the expression of genes related to oocyte competence (*GREM1*, *PTGS2*, and *AREG*) and lipid metabolism (*FADS2*, *SREBP1*, and *SCD1*) in COCs and cumulus cells matured in SOF medium with or without CLA. In this study, we observed an increased expression of *GREM1* and *PTGS2* in oocytes matured in SOF compared with those matured in M199. The expression of *GREM1*, a gene linked to oocyte maturation, was also increased in cumulus cells cultured in SOF compared with those in M199. These results indicated that the expression of these quality markers can be positively modulated by SOF culture medium. Although the relative abundance of mRNA does not mean that there is

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Table 2. Relative expression (mRNA) of gremlin 1 (*GREM1*), prostaglandin-endoperoxide synthase 2 (*PTGS2*), amphiregulin (*AREG*), fatty acid desaturase 2 (*FADS2*), sterol regulatory element-binding protein 1 (*SREBP1*), and stearoyl-coenzyme A desaturase 1 (*SCD1*) in oocytes matured under different conditions: medium (M199 or SOF) and CLA 100 μM (without or with)

Medium	FADS2	GREM1	SCD1	SREBP1	AREG	PTGS2
M199	0.24 ± 0.07[b]	1.26 ± 0.23[b]	0.72 ± 0.13	0.92 ± 0.12	1.30 ± 0.41	0.75 ± 0.25[b]
SOF	0.85 ± 0.14[a]	2.25 ± 0.30[a]	0.23 ± 0.05	1.60 ± 0.30	1.33 ± 0.39	1.86 ± 0.29[a]
CLA						
without	0.51 ± 0.18	1.45 ± 0.24	0.54 ± 0.17	1.36 ± 0.27	1.49 ± 0.38	1.24 ± 0.27
with	0.57 ± 0.12	2.06 ± 0.30	0.41 ± 0.09	1.15 ± 0.22	1.13 ± 0.43	1.37 ± 0.33
P-values						
Medium	0.0001*	0.0065*	<0.0001*	0.0541	0.9448	0.0040*
CLA	0.5940	0.0722	0.1105	0.5325	0.4698	0.6969
Interaction						
(medium × CLA)	0.2084	0.4095	0.0102*	0.5248	0.7176	0.8233

[a,b]Means ± standard error of the mean (SEM) followed by different letters in the same column differ according to Tukey's test at 5% significance ($P < 0.05$); *P*-values in bold and marked with an asterisk are statistically significant ($P < 0.05$).

Table 3. Main effect of interaction: medium (M199 or SOF) and CLA 100 μM (without or with) on the relative expression of stearoyl-coenzyme A desaturase 1 (*SCD1*) in oocytes

	CLA	
Medium	Without	With
M199	0.89^{Aa} ± 0.17	0.54^{Ab} ± 0.14
SOF	0.19^{Ba} ± 0.07	0.28^{Ba} ± 0.06
P-value	0.0102*	

Means ± SEM followed by different letters, uppercase in columns and lowercase in rows, differ by Tukey test at 5% significance ($P < 0.05$). M199: medium 199; SOF synthetic oviductal fluid; CLA: conjugated linoleic acid.

corresponding protein activity, it is a molecular indicator of its expression. *GREM1* and *PTGS2* in cumulus cells interact with factors secreted by oocytes and participate in the expansion process directly related to oocyte maturation and the resumption of oocyte meiosis (Shimada *et al*., 2006), and are therefore associated with greater oocyte competence and good quality embryos (Goldrat *et al*., 2019). *GREM1* and *PTGS2* have also been used as quality markers in buffalo cumulus cells (Pandey *et al*., 2018).

The expression of *GREM1* and *PTGS2* is signalled by the interaction of growth factors, such as *AREG*, with cumulus cell receptors (Pangas *et al*., 2004; Shimada *et al*., 2006). The upregulation of *PTGS2* has been linked to the transition of mouse oocytes from the germinal vesicle stage to metaphase II (Shao *et al*., 2015), the improvement of oocyte competence in pigs (Sugimura *et al*., 2015), and is associated with the development of superior quality embryos in human cumulus cells (McKenzie *et al*., 2004). These studies suggest that a higher expression of *PTGS2*, as observed here in oocytes matured in SOF medium, is desirable, and that SOF can be beneficial in other stages of IVEP. *AREG* is closely related to the cumulus cell expansion process and acquisition of oocyte competence (Shimada *et al*., 2006). In this study, we observed no difference in *AREG* expression between the SOF and M199 groups.

Lipid metabolism is regulated by some different genes: *FADS2* encodes an enzyme essential for lipid metabolism in oocytes, *SREBP1* encodes an enzyme critically involved in the regulation of intracellular cholesterol levels, and *SCD1* reduces the lipotoxicity of saturated fatty acids. In this study, *FADS2* expression was increased in oocytes matured in SOF medium compared with M199 medium, while there was no difference in *SREBP1* expression between groups. The expression of *SCD1* was significantly lower in oocytes matured in SOF medium compared with those matured in M199 medium. The observed increase of *FADS2* is notable as it is responsible for catalyzing the initial step of polyunsaturated fatty acid synthesis (Warzych *et al*., 2017). Only *FADS2* initiates the desaturation chain elongation cascade, in which essential fatty acids are transformed into polyunsaturated fatty acids (Stoffel *et al*., 2008). Lipid and energy metabolism, polyunsaturated fatty acid synthesis, cell membrane structures, and lipid signalling pathways depend on essential fatty acids (Cunnane, 2003), making *FADS2* essential for proper fatty acid metabolism.

While SOF medium increased the expression of *FADS2*, the expression of *SCD1*, also a desaturase, showed the opposite response. *SCD1* is responsible for reducing the lipotoxicity of saturated fatty acids through their conversion to monounsaturated fatty acids (Aardema *et al*., 2017). This conversion process is essential, as saturated fatty acids increase the levels of highly toxic reactive oxygen species, interfering with cellular metabolism (Brookheart *et al*., 2009). Inhibition of *SCD1* results in decreased triglyceride accumulation, lower aromatase gene expression, and lower estradiol production in human cumulus cells (Fayezi *et al*., 2018). Therefore, *SCD1* gene activity is required when lipotoxicity is present in the oocyte, as the addition of CLA to M199 medium decreased the expression of this gene in this study. It appears that SOF medium inhibits lipid toxicity, independent of CLA addition, and therefore expresses less *SCD1* than M199.

As observed in this study, M199 with added CLA resulted in a lower expression of *SCD1* in oocytes compared with those in M199 without CLA. The same did not occur with the SOF medium; the addition of CLA did not affect the expression of *SCD1*. The composition of M199 and SOF medium is different and, given the complexity of these solutions, it is difficult to confirm that components of the formula are linked to the response observed in this study.

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Table 4. Relative expression (mRNA) of gremlin 1 (*GREM1*), prostaglandin-endoperoxide synthase 2 (*PTGS2*), amphiregulin (*AREG*), fatty acid desaturase 2 (*FADS2*), sterol regulatory element-binding protein 1 (*SREBP1*), and stearoyl-coenzyme A desaturase 1 (*SCD1*) in cumulus cells matured under different conditions: medium (M199 or SOF) and CLA 100 μM (without or with)

Medium	FADS2	GREM1	SCD1	SREBP1	AREG	PTGS2
M199	0.52 ± 0.07	1.18 ± 0.30[a]	0.51 ± 0.11	0.56 ± 0.11	0.69 ± 0.14	0.27 ± 0.08
SOF	0.37 ± 0.03	2.14 ± 0.10[b]	0.32 ± 0.02	0.51 ± 0.04	0.54 ± 0.13	0.20 ± 0.04
CLA						
Without	0.49 ± 0.10	1.64 ± 0.27	0.48 ± 0.12	0.57 ± 0.11	0.76 ± 0.13	0.27 ± 0.08
With	0.40 ± 0.06	1.68 ± 0.23	0.35 ± 0.05	0.50 ± 0.04	0.47 ± 0.14	0.20 ± 0.05
P-values						
Medium	0.0731	0.0004*	0.0524	0.5668	0.3798	0.2224
CLA	0.3342	0.8521	0.1792	0.3903	0.0927	0.1979
Interaction (medium × CLA)	0.1132	0.1390	0.3052	0.1525	0.4396	0.8140

[a,b]Means ± standard error of the mean (SEM) followed by different letters in the same column differ according to Tukey's test at 5% significance ($P < 0.05$). *P*-values in bold with an asterisk are statistically significant ($P < 0.05$).

All experimental groups were supplemented with 10% FBS, which is linked to the undesirable accumulation of intracellular lipids (del Collado *et al.*, 2015). However, FBS contains substances that are beneficial for embryonic development (Leão *et al.*, 2015) and its total removal results in low rates of embryonic production (Duque *et al.*, 2003). Modifications of the culture medium and systems allows the use of FBS in culture medium, while mitigating its effects on the lipid composition of cultured structures. One possible modification is the addition of CLA.

While observing oocytes, we did not find any differences in the transcriptional profile linked to maturation with CLA.

Maintenance of membrane fluidity, control of the proportion of saturated and unsaturated fatty acids, and distribution of lipid droplets are essential to improving the structural quality of oocytes and embryos. Our findings show that SOF modulates structural gene expression. Therefore, it is possible to test strategies capable of generating modulations in favour of better COC competence for *in vitro* development.

There are indications that SOF can be used in oocyte maturation, but the present study has limitations, because it did not analyze the protein profile or enzymatic activity, the lipid content of the oocytes, and embryonic development and cryopreservation. More studies must be carried out to validate the use of SOF as a maturation medium to improve the lipid profile and embryonic quality.

In conclusion, this study shows that the use of SOF medium in COC maturation leads to higher expression of marker genes of quality oocytes (*GREM1* and *PTGS2*), lipid metabolism (*FADS2*), cumulus cells (*GREM1*), and modulation of genes linked to lipid metabolism independent of the addition of CLA. The use of SOF medium in COC maturation resulted in a lower expression of marker genes in lipid metabolism (*SCD1*) and CLA supplementation did not affect gene expression related to lipid metabolism or oocyte quality.

Supplementary material. The supplementary material for this article can be found at https://doi.org/10.1017/S0967199423000473

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Competing interests. The authors declare none.

Ethical standard. This project involved the use of abattoir-derived material only and did not require review by an Animal Welfare and Ethical Review Body.

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