Universidad de Oviedo

TESIS DOCTORAL

Programa de Doctorado en Biología Molecular y Celular

IDENTIFICACIÓN NO INVASIVA DE BIOMARCADORES DEL SEXO Y DE LA VIABILIDAD DE LA GESTACIÓN EN EMBRIONES BOVINOS PRODUCIDOS *IN VITRO* FRESCOS Y CRIOPRESERVADOS

ISABEL MARÍA GIMENO MIQUEL

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Oviedo, 2023

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RESUMEN DEL CONTENIDO DE TESIS DOCTORAL

1.- Título de la Tesis	
Español/Otro Idioma: Identificación no invasiva de biomarcadores del sexo y de la viabilidad de la gestación en embriones bovinos producidos *in vitro* frescos y criopreservados.	Inglés: Non-invasive identification of biomarkers for embryonic sex and pregnancy viability of bovine embryos produced in vitro fresh and cryopreserved.

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RESUMEN (en español)

El diagnóstico del sexo y la predicción de la capacidad del embrión para establecer la gestación y generar un ternero sano son objetivos de interés en vacuno. El objetivo principal de la presente tesis doctoral fue identificar biomarcadores del sexo del embrión y de la viabilidad de la gestación de manera no invasiva mediante el análisis metabolómico del medio de cultivo del embrión, y estudiar la salud perinatal de los terneros nacidos de embriones frescos y criopreservados.

El objetivo del **primer capítulo** fue identificar biomarcadores del sexo del embrión. Para ello, se analizaron mediante UHPLC-MS/MS N=127 muestras de medio de cultivo de embriones sexados producidos *in vitro* y N=40 de embriones transferidos a receptoras cuyo sexo se asignó a partir del ternero. A continuación, se realizó un análisis por bloques con factores fijos que incluían el tipo de cultivo (con o sin suero), raza de cada toro usado para producir los embriones, y estadios de desarrollo del embrión en día 6 y día 7, puesto que estos factores influyeron en el metaboloma del medio de cultivo del embrión, obstaculizando la identificación de metabolitos biomarcadores. De esta manera se obtuvieron 182 bloques y 31 metabolitos que permiten identificar el sexo del embrión. Las clases de metabolitos más relevantes para la identificación del sexo fueron lípidos y aminoácidos. Se demostró que no existe ningún biomarcador universal para diagnosticar el sexo en todas las condiciones del estudio. Sin embargo, cada metabolito fue específico para unas condiciones concretas (es decir, raza, medio de cultivo y estadio o sus combinaciones), lo que permite diagnosticar el sexo de todos los embriones en un cultivo utilizando 2 ó 3 metabolitos, pero con un solo metabolito para cada embrión.

En el **segundo capítulo** se identificaron biomarcadores para predecir la gestación, para lo cual se analizaron por UHPLC-MS/MS N=84 muestras de embriones producidos *in vitro* que fueron transferidos frescos o congelados a receptoras sincronizadas. También se realizó un análisis por bloques con factores fijos que incluían el tipo de cultivo, raza de toro, estadios de desarrollo del embrión antes y después del cultivo individual, el estado del embrión transferido (fresco o congelado), y el diagnóstico de la gestación (es

decir, si la receptora estaba preñada en día 40, día 62, y si la gestación llegó a término). Así, se obtuvieron 34 metabolitos implicados en 511 bloques, de los cuáles 198 bloques predijeron la gestación a término, 166 bloques la gestación en día 62, y 147 bloques en día 40. Determinados metabolitos mostraron una alta precisión en la predicción de la gestación en 95 bloques (ROC-AUC >0.7). En conjunto, según el análisis del metaboloma del medio de cultivo, los embriones que resultaron en gestación mostraron un consumo más elevado de aminoácidos y ácido cítrico, y menor de lípidos y ácido cis-aconítico que los embriones que no resultaron en gestación.

No obstante, el éxito de la gestación depende del embrión y de la receptora. Por ello, en el **tercer capítulo** usamos la información emparejada de 70 embriones y receptoras para desarrollar un modelo para predecir la gestación a término basado en aprendizaje e iteraciones. En primer lugar, identificamos biomarcadores de gestación mediante resonancia magnética nuclear ($1H^+$RMN) en muestras de plasma de las receptoras obtenido en día 0 (estro) y día 7 (pocas horas antes de realizar la transferencia). Se realizó un estudio por bloques con factores fijos que incluían la raza de la receptora (Holstein o Asturiana de los Valles), el día de recogida de la sangre, y el estado del embrión transferido (fresco o congelado). Se identificaron 28 biomarcadores candidatos con representación en 190 bloques que predijeron la gestación. El metabolito más representativo entre bloques fue la creatina. Los metabolitos más eficaces para predecir la gestación a día 40, día 62 y a término a partir de plasma de día 0 fueron creatina, acetona y fenilalanina, mientras que glutamina, lisina y ornitina lo fueron con plasma de día 7. En general, se identificaron más biomarcadores en plasma de día 7 que en día 0, y su capacidad de predicción fue más alta en los días 40 y 62 de gestación que a término. Un mayor número de receptoras de embriones congelados fueron inicialmente mal clasificadas en comparación con las receptoras de embriones frescos, probablemente debido a las mayores pérdidas gestacionales. No obstante, la capacidad de estas receptoras para gestar se identificó correctamente cuando se contrastó con la información procedente del metaboloma analizado en el medio de cultivo del embrión. De esta manera, mediante aprendizaje e iteraciones, 12 biomarcadores mejoraron su ROC-AUC (>0.65) a término, y se identificaron 5 nuevos biomarcadores. Este enfoque mejoró sustancialmente la predicción de la gestación y la precisión de los biomarcadores.

Finalmente, en el **cuarto capítulo** se examinó la influencia de la criopreservación del embrión en la salud perinatal de terneros nacidos de embriones transferidos frescos (N=13), congelados (N=24) y vitrificados (N=14) en día 0 (antes y después de la ingesta de calostro), día 15 y día 30 de vida, con examen clínico (N=13 características) y análisis bioquímico hemático básico (N=18 analitos). Los terneros nacidos de embriones congelados, vitrificados y frescos no se distinguieron por su peso al nacimiento ni por la duración de la gestación. En día 0, antes de la ingesta del calostro, el tiempo de llenado capilar y la concentración de creatinina fueron más altos en los terneros nacidos de embriones vitrificados y congelados que en los frescos. La sangre de los terneros nacidos de embriones congelados mostró menor presión parcial de CO_2 (PCO_2) que los terneros procedentes de embriones frescos. Además, la ingesta de calostro no disminuyó la PCO_2 en los terneros de embriones vitrificados, a diferencia de los congelados y frescos. El valor del hematocrito y la concentración de hemoglobina se redujeron en los terneros nacidos de embriones congelados con respecto a los nacidos

de embriones vitrificados. En cambio, los terneros nacidos de embriones vitrificados mostraron niveles más altos de Na^+ que los terneros procedentes de embriones frescos y congelados. En día 15, no se observaron efectos de la criopreservación del embrión en el ternero. Sin embargo, la aparición de diarrea, principalmente entre los días 15 y 30, redujo los valores de TCO_2, HCO_3^- y BE en día 30, y aumentó los valores de anión gap, con un efecto más marcado en terneros nacidos de embriones vitrificados. Otros parámetros afectados por la diarrea fueron la temperatura, PCO_2 y Na^+, aunque sin diferencias entre grupos de terneros. No obstante, casi todos los valores obtenidos se encontraron comprendidos en rangos descritos como saludables en la literatura. Por tanto, se concluyó que la criopreservación de embriones afecta a los terneros sanos, con cambios más notables en día 0 que en los siguientes días de vida. Sin embargo, el calostro tuvo efectos comparables entre los tres grupos de terneros, indicando una capacidad adaptativa en los primeros días de vida similar entre todos ellos.

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RESUMEN (en Inglés)

Diagnosis of embryonic sex and prediction of embryonic viability for pregnancy and birth are major objectives in cattle farming. The main objective of this thesis was to identify biomarkers of embryonic sex and for pregnancy prediction noninvasively by metabolomic analysis of the embryo culture medium and to study the fitness of the calves born from fresh and cryopreserved embryos during the perinatal period.

The objective of the **first chapter** was to identify biomarkers of embryonic sex. For this, we analyzed by UHPLC-MS/MS N=127 culture medium samples from in vitro-produced and sexed embryos, and N=40 samples from embryos transferred to recipients whose sex was identified in calves at birth. A study by sample subsets (blocks) was performed with fixed factors including culture medium (with or without serum), breed of each bull used to produce the embryos, and day 6 and day 7 embryonic stages. These factors influenced the metabolome analyzed in the spent culture medium and can obscure the identification of biomarker metabolites. We obtained 31 differentially accumulated metabolites through 182 subsets that identified the embryonic sex. The most relevant metabolite classes for sex diagnosis were lipids and amino acids. None of the metabolites obtained could accurately diagnose the embryonic sex in all the conditions of the study. However, each metabolite was found to be effective under specific conditions (i.e., breed, culture medium, and developmental stage or combinations thereof). This allowed for the identification of the sex of all embryos in culture using two or three metabolites, with only one metabolite required per embryo.

In the **second chapter**, we aimed to identify biomarkers for pregnancy prediction on Day-40, Day-62, and birth. In this study, we analyzed N=84 culture medium samples spent by in vitro-produced embryos that were transferred fresh or frozen to estrus-synchronized recipients using UHPLC-MS/MS. We performed an analysis by sample subsets with fixed factors including culture medium, bull breed, embryonic developmental stages before and after individual culture, the status of the transferred embryo (fresh or frozen), and the pregnancy diagnosis (i.e., pregnant status on Day-40, Day-62, and birth). Our findings revealed 34 accumulated metabolites through 511 blocks, 198 for birth, 166 for Day-62, and 147 for Day-40. Certain metabolites predicted pregnancy with high accuracy in 95 blocks (ROC-AUC >0.7). Overall, according to the metabolome analysis of the spent culture medium, embryos resulting in pregnancy consumed more amino acids and citric acid, and depleted fewer lipids and cis-aconitic acid than embryos that did not reach pregnancy.

However, successful pregnancy depends on the embryo and the recipient. Therefore, in the **third chapter**, we used the paired metabolic information from 70 embryos and recipients to develop a model based on machine learning and iterations to predict pregnancy. First, we identified pregnancy biomarkers using nuclear magnetic resonance ($1H^{+}$NMR) in plasma samples from recipients obtained on Day-0 (estrus) and on Day-7 (4 to 6 hours before embryo transfer). We performed a block study with fixed factors that included the recipient's breed (Holstein or Asturiana de los Valles), the day of blood collection, and the status of the embryo transferred (fresh or frozen). Twenty-eight

candidate biomarkers were identified with representation in 190 blocks that predicted pregnancy. The most representative metabolite between blocks was creatine. The most relevant metabolites for pregnancy prediction on Day- 40, Day-62, and to term in Day-0 plasma were creatine, acetone, and phenylalanine and glutamine, lysine, and ornithine in Day-7 plasma. In general, more biomarkers were identified on Day-7 plasma than on Day-0, and their predictive ability was higher on Day-40 and Day-62 of pregnancy than at birth. Within frozen embryos, more recipients were initially misclassified compared to recipients of fresh embryos, probably due to higher pregnancy losses. However, the pregnancy capability of these recipients was correctly identified when combined with information from the metabolome analyzed in the spent culture medium of the embryo. Thus, with machine learning and iterations, 12 biomarkers improved their ROC-AUC (>0.65) at birth, and 5 new biomarkers were identified. This approach improved the pregnancy prediction and accuracy of single biomarkers.

Finally, the **fourth chapter** examined the influence of embryo cryopreservation on the fitness of calves born from fresh (N=13), frozen (N=24), and vitrified (N=14) transferred embryos during the thirty-first days of life. Thirteen clinical and morphological signs, and N=18 analytes (basic blood biochemical analysis) were measured in calves at birth (Day 0, before and after colostrum intake), Day 15 and Day 30 of life. Gestation length and birth weight did not differ between calves born from frozen, vitrified, and fresh embryos. On Day-0, before colostrum intake, capillary refill time and creatinine concentration were higher in calves born from vitrified and frozen embryos than from fresh embryos. The blood of calves born from frozen embryos showed lower partial pressure of CO_2 (PCO_2) than calves from fresh embryos. In addition, colostrum intake did not decrease PCO_2 among calves of vitrified embryos, contrary to in calves from frozen and fresh embryos. Hematocrit value and hemoglobin concentration were reduced in calves born from frozen embryos in comparison to those from vitrified embryos. In contrast, calves from vitrified embryos showed higher Na^+ levels than calves from fresh and frozen embryos. On Day-15, the effects of embryo cryopreservation disappeared., and, the appearance of diarrhea, mostly between Day-15 and Day-30, reduced TCO_2, HCO_3^- and BE values on Day-30, and increased gap anion values, with a more pronounced effect in calves born from vitrified embryos. Other parameters affected by diarrhea were temperature, PCO_2, and Na^+, but without differences between calf groups. Nevertheless, almost all the values obtained were within reference values described as normal in the literature. We concluded that embryo cryopreservation affects healthy calves, with more pronounced changes on Day-0 than afterward. However, colostrum had comparable effects among the three groups of calves, indicating a similar adaptation to extrauterine life among all of them.

SR. PRESIDENTE DE LA COMISIÓN ACADÉMICA DEL PROGRAMA DE DOCTORADO EN BIOLOGÍA MOLECULAR Y CELULAR

LISTADO DE ABREVIATURAS

Ac-CoA	Acetil Coenzima A
AOS	Síndrome del ternero anormal (A*bnormal Offspring Síndrome*)
BE	Exceso de base (*Base Excess*)
BSA	Albúmina sérica bovina (B*ovine Serum Albumin*)
CIV	Cultivo I*n Vitro*
COC	Complejo cúmulo-ovocito (*Cumulus-Oocyte Complex*)
CRT	Tiempo de llenado capilar (*Capillary Refill Time*)
DMSO	Dimetilsulfóxido
DOHaD	Orígenes de la salud y enfermedad en el desarrollo (*Developmental Origins of Health and Disease*)
EG	Etilenglicol
EGA	Activación del genoma embrionario (*Embryonic Genome Activation*)
EM	Espectrometría de Masas
ESI	Ionización por electrospray (*Electrospray Ionization*)
ET	Transferencia de embriones (*Embryo Transfer*)
FCS	Suero bovino fetal (*Fetal Calf Serum*)
FIV	Fecundación I*n Vitro* (*In vitro fertilization*)
FSH	Hormona folículo estimulante (*Follicle-Stimulating Hormone*)
FTIR	Espectroscopia infrarroja por transformada de Fourier (*Fourier Transform Infrared Spectroscopy*)
GC	Cromatografía de gases (*Gas chromatography*)
GnRH	Hormona liberadora de gonadotropinas (G*onadotropin-Releasing Hormone*)
IA	Inseminación Artificial
ICM	Masa celular interna (*Inner Cell Mass*)
IVD	Producidos *in vivo* (*In Vivo Derived*)
IVP	Producidos *in vitro* (*In Vitro Produced*)
LC	Cromatografía líquida (*Liquid Chromatography*)
LH	Hormona luteinizante (*Luteinizing Hormone*)
LOS	Síndrome del ternero grande (*Large Offspring Syndrome*)
MC	Medio de Cultivo

MIV	Maduración *In Vitro*
MOET	Ovulación múltiple y transferencia de embriones (*Multiple Ovulation and Embryo Transfer*)
OPU	Aspiración folicular guiada por ultrasonido (*Ovum Pick-Up*)
PCO_2	Presión parcial de CO_2
PCR	Reacción en cadena de la polimerasa (*Polymerase Chain Reaction*)
PFK	Fosfofructokinasa (*Phosphofructokinase*)
PGD	Diagnóstico genético preimplantacional (*Preimplantation Genetic Diagnosis*)
PGF2α	Prostaglandina F2 alfa
PHE	Penicilamina, Hipotaurina y Epinefrina
PO_2	Presión parcial de O_2
PPP	Ruta de las pentosas fosfato (*Pentose Phosphate Pathway*)
PVA	Alcohol de polivinilo (*Polyvinyl Alcohol*)
PVP	Polivinilpirrolidona
RMN	Resonancia Magnética Nuclear
ROS	Especies reactivas del oxígeno (*Reactive Oxygen Species*)
SCNT	Transferencia nuclear de células somáticas (*Somatic Cell Nuclear Transfer*)
sO_2	Saturación de Oxígeno
SOF	Fluido sintético de oviduct (*Synthetic Oviductal Fluid*)
TALP	Tyrode-Albúmina-Lactato-Piruvato
TCA	Ácido tricarboxílico (*Tricarboxylic Acid*)
TCM199	*Tissue Culture Medium* 199
TCO_2	CO_2 total
TE	Trofoblasto (*Trophectoderm*)
UHPLC-MS/MS	Cromatografía líquida de alta resolución acoplada a espectrometría de masas en tándem (*Ultra-High Performance Liquid Chromatography Tandem Mass Spectrometry*)
VM	Medio de vitrificación (*Vitrification Medium*)
VS	Solución de vitrificación (*Vitrification Solution*)
ZP	Zona Pelúcida

ÍNDICE

1| INTRODUCCIÓN GENERAL

1. INTRODUCCIÓN GENERAL

1.1. Antecedentes

En los últimos años, las biotecnologías reproductivas se han convertido en herramientas indispensables para incrementar la eficiencia de la producción y rentabilidad de las explotaciones de ganado vacuno (Hansen, 2014). Entre las técnicas reproductivas más empleadas destacan la superovulación, la inseminación artificial (IA), la ovulación múltiple y transferencia de embriones (MOET, del inglés *Multiple Ovulation and Embryo Transfer*), y la producción de embriones *in vitro*. La producción de embriones *in vitro* tiene múltiples aplicaciones, como la mejora de la producción animal, al aumentar el potencial reproductivo de individuos genéticamente superiores. Además, es posible obtener embriones tomando ovocitos de hembras con ciertas alteraciones del tracto reproductivo o hembras de alto mérito genético terminales (por enfermedad, accidente, o edad) o que van a ser sacrificadas. En el caso de vacas repetidoras, que son aquellas que a pesar de presentar útero y ovarios sanos no consiguen gestar tras tres o más montas o inseminaciones, la transferencia de embriones producidos *in vitro* (IVP, del inglés *In Vitro Produced*) se utiliza como una solución terapéutica para problemas de fertilidad (Hansen, 2014; Sirard, 2018; Sanches *et al.*, 2019).

A finales de 1980 comenzó a estar disponible la técnica de aspiración folicular guiada por ultrasonido (*Oocyte puncture ultrasonography*, también conocida como *Ovum Pick-Up*; OPU), la cual permite obtener ovocitos de vacas vivas. La asociación de OPU con la producción *in vitro* de embriones (OPU-IVP) se estableció a escala comercial en Estados Unidos a finales de 1990 (Ferré *et al.*, 2020a). La mejora de los medios de cultivo *in vitro* (CIV), la introducción del semen sexado y la selección genómica propiciaron un incremento en la aplicación comercial de la producción *in vitro* de embriones (Blondin, 2015; Sanches *et al.*., 2019; Ferré *et al.*, 2020a). Desde entonces la producción de embriones *in vitro* ha aumentado considerablemente en el mundo, y lo continúa haciendo a expensas de la producción de embriones *in vivo* convencional, que sigue decreciendo (Figura 1; Viana, 2022). La posibilidad de obtener un número elevado de embriones a un coste relativamente bajo gracias a la recuperación de ovarios de matadero ha hecho que las técnicas reproductivas no sólo sean utilizadas con una aplicación comercial, sino que

también son empleadas en investigación para optimizar las condiciones y los medios de CIV, para mejorar la eficiencia de las propias técnicas, o para estudiar los mecanismos fisiológicos del desarrollo embrionario temprano en mamíferos (Lonergan & Fair, 2014). De esta manera, los embriones bovinos se han convertido en un modelo para la investigación de otras especies, incluyendo la humana.

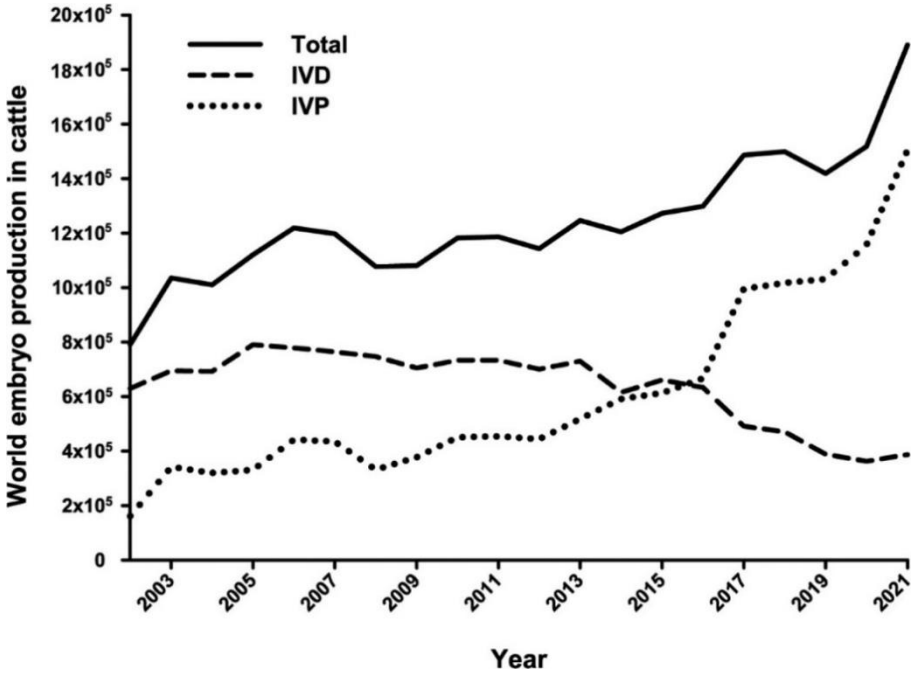

Figura 1. Número de embriones bovinos producidos *in vivo* (IVD) por MOET, embriones producidos *in vitro* (IVP) a partir de ovocitos recuperados de ovarios de matadero y de OPU (*Ovum Pick-Up*), y la suma de ambos entre el periodo de 2002 a 2021 (Viana *et al.*, 2022).

Sin embargo, a pesar de las ventajas y el potencial de la producción de embriones *in vitro*, su rendimiento es relativamente bajo, ya que sólo entre el 20-40% de los cigotos cultivados *in vitro* alcanza el estadio de blastocisto, en comparación con el 80% obtenido con ovocitos madurados *in vivo* (Ferré *et al.*, 2020a). La transferencia de embriones IVP también produce índices de gestación inferiores a los obtenidos con embriones producidos *in vivo* (IVD, del inglés *in vivo derived*) (Numabe *et al.*, 2000a; Numabe 2000b; Pontes *et al.*, 2009; Siqueira *et al.*, 2009). Según un metaanálisis que incluye estudios de los últimos 25 años, los índices de gestación obtenidos con embriones IVP son un 25% inferiores a los obtenidos con embriones IVD, y el rendimiento de la transferencia de embriones IVP es bajo, ya que tan sólo el 27% de las receptoras transferidas llegan a término (Ealy *et al.*, 2019). No obstante, los datos más actuales de

nuestro grupo muestran índices de gestación más elevados con embriones IVP, logrando el 47%, 45% y 53% de nacimientos tras la transferencia de embriones cultivados sin suero frescos, congelados y vitrificados, respectivamente (Gómez *et al.*, 2020a).

Las proporciones de pérdidas durante la gestación también son mayores, alcanzando el 16% con embriones IVP frente al 7% con embriones IVD (Hasler, 2010), al igual que se dan más terneros nacidos con alteraciones, incluyendo aumento del peso al nacimiento, y una incidencia de anormalidades congénitas superior al 4% (Farin *et al.*, 2006; Bonilla *et al.*, 2014; Ealy *et al.*, 2019; Lopes *et al.*, 2020; Beilby *et al.*, 2023). Tales diferencias pueden ser debidas a que las condiciones de CIV no logran reproducir bien el ambiente *in vivo*, lo cual induce alteraciones en el desarrollo de los embriones, entre las que se incluyen cambios en el patrón de metilación (Salilew-Wondim *et al.*, 2015), en el perfil transcriptómico (Driver *et al.*, 2012; Heras *et al.*, 2016) y en el perfil metabólico (Sturmey *et al.*, 2010), y una mayor sensibilidad de los embriones IVP a la criopreservación (Sudano *et al.*, 2014). Por tanto, son necesarias técnicas que permitan seleccionar los embriones con mayor potencial reproductivo para asegurar un correcto establecimiento de la gestación, alcanzar el parto y obtener descendencia sana.

Actualmente, la selección de embriones previa a la transferencia o la criopreservación se realiza mediante evaluación morfológica. No obstante, este procedimiento presenta limitaciones por ser altamente subjetivo y por la variabilidad entre evaluadores (Farin *et al.*, 1995; Borman *et al.*, 2020). La mejora de la predicción de la viabilidad contribuiría a optimizar la eficiencia de los programas de transferencia de embriones IVP, los cuales presentan una peor supervivencia, especialmente cuando son sometidos a criopreservación. En los últimos años ha aumentado el uso de técnicas "ómicas" de alto rendimiento, como la genómica, proteómica, transcriptómica o metabolómica, las cuales permiten estudiar de forma global un conjunto de moléculas de una muestra. De esta manera, las ómicas contribuyen a dilucidar mecanismos moleculares implicados en el desarrollo embrionario y en la comunicación materno-embrionaria, y permiten descubrir biomarcadores asociados a la viabilidad del embrión (Hernández-Vargas *et al.*, 2020). El medio de cultivo (MC) donde se ha desarrollado un embrión representa una vía de acceso al embrión valiosa y no invasiva que contiene moléculas como proteínas, metabolitos, y RNA no codificante (Rødgaard *et al.*, 2015). Por tanto, el MC se puede utilizar para analizar el estado fisiológico del embrión sin alterar sustancialmente las características de este.

La selección del sexo de la descendencia es deseable en ganadería, por ejemplo, en la industria láctea, donde es preferible el nacimiento de hembras, mientras que en la industria cárnica se prefiere el nacimiento de machos (Umehara *et al.*, 2020). La práctica habitual para conseguir descendencia de un sexo determinado es la utilización de semen sexado en IA, el cual permite optimizar la producción y rentabilidad de los rebaños (Hansen, 2014). Esta selección del sexo fue posible gracias al desarrollo de la citometría de flujo para sexar o separar los espermatozoides portadores del cromosoma X e Y, basándose en la tinción del DNA con el fluoróforo Hoechst 33342 para medir la diferencia de tamaño entre ambos cromosomas (Garner *et al.*, 1983). No obstante, el uso de semen sexado cuenta también con desventajas. Entre ellas, para sexar los espermatozoides se requiere de un citómetro de flujo especialmente adaptado, un equipo caro y de elevados costes de mantenimiento y operatividad. El sexado requiere mucho tiempo, puesto que se separa un único espermatozoide por unidad de tiempo, lo que se refleja en un coste mayor de las pajuelas de semen sexado. Por otro lado, los índices de gestación obtenidos tras la IA con semen sexado son inferiores a los obtenidos con semen no sexado (47.3% *vs.* 56.9%, respectivamente), así como los porcentajes de nacimientos (41.3% *vs.* 54.6%, respectivamente) (Oikawa *et al.*, 2019; Reese *et al.*, 2021). Por ello, el uso de semen sexado resulta más apropiado en ganaderías que presentan buena fertilidad (Cottle *et al.*, 2018). Por otro lado, el uso semen sexado para producir embriones *in vitro* aumenta el porcentaje de ovocitos no fecundados y embriones con interrupción del desarrollo, además de disminuir el porcentaje de blastocistos totales, blastocistos expandidos y eclosionados (Palma *et al.*, 2008; Blondin *et al.*, 2009; Bermejo-Álvarez *et al.*, 2010a; Trigal *et al.*, 2012a; Mikkola & Taponen, 2017; Steele *et al.*, 2020). Si bien la causa de estas menores tasas de desarrollo y gestación no se conocen completamente, se especula con que, durante el proceso de separación -*sorting*- los espermatozoides están sometidos a estrés causado por el tiempo de exposición y la temperatura, así como estrés mecánico y químico, lo cual altera su morfocinética (Palma *et al.*, 2008; Steele *et al.*, 2020; Reese *et al.*, 2021). Los embriones producidos con semen sexado también pueden presentar alteraciones en la estructura y número de orgánulos como las mitocondrias, retículo endoplasmático rugoso y membrana nuclear (Palma *et al.*, 2008). En este contexto, las técnicas ómicas también pueden utilizarse para determinar la actividad metabólica propia de embriones machos y hembras, con el fin de obtener biomarcadores no invasivos del sexo del embrión y mejorar los medios de cultivo.

1.2. Producción *in vitro* en embriones bovinos

La producción *in vitro* de embriones en bovino se puede dividir en tres grandes procesos secuenciales, que son la maduración *in vitro* (MIV) de ovocitos, la fecundación *in vitro* (FIV) de los ovocitos madurados, y el CIV de los ovocitos fecundados o cigotos.

1.2.1. Maduración *in vitro*

Los ovocitos para la producción *in vitro* de embriones se pueden obtener mediante aspiración folicular de novillas y vacas vivas (OPU) o bien a partir de ovarios obtenidos de animales sacrificados en el matadero (usualmente de folículos antrales de 3-8 mm de diámetro). Los complejos cúmulo-ovocito (COCs, del inglés *cumulus-oocyte complex*) más aptos para la MIV son aquellos en los que el ovocito está rodeado por tres o más capas de células del cúmulo y muestran un citoplasma homogéneo.

La maduración ovocitaria incluye tanto la maduración nuclear como la citoplasmática, y ambas son imprescindibles para que se produzcan la fecundación y el ulterior desarrollo embrionario (Ferré *et al.*, 2020a). La maduración nuclear implica la progresión de la meiosis desde la profase de la primera división meiótica a la metafase de la segunda división meiótica, mientras que la maduración citoplasmática es apreciable en virtud de un incremento en el número de orgánulos, como mitocondrias o aparato de Golgi, y en su reorganización (Racedo *et al.*, 2012; Lonergan & Fair, 2016). Al mismo tiempo, se acumulan mRNAs, proteínas y lípidos en el ovocito (Ferreira *et al.*, 2009; Gegenfurtner *et al.*, 2020; Jiménez *et al.*, 2022). El proceso de la MIV generalmente se completa al cabo de 20 ó 24 h. No obstante, a pesar de que aproximadamente el 90% de los ovocitos alcanza la MII al final de la MIV (Lonergan & Fair, 2016), el porcentaje de blastocistos que se obtienen con ovocitos madurados *in vitro* es inferior al obtenido con ovocitos madurados *in vivo* (Rizos *et al.*, 2002). Por tanto, el éxito de la maduración depende de las diferencias entre el medio de cultivo y las condiciones *in vivo*. Existen diversos medios de cultivo comerciales que se utilizan como sustrato para elaborar los medios de maduración. El Tissue Culture Medium 199 (TCM199) es probablemente el más utilizado, y contiene sales inorgánicas, bicarbonato sódico (tampón), aminoácidos y vitaminas, entre otros componentes. Generalmente, estos medios son suplementados con hormonas como la hormona folículo estimulante (FSH), la hormona luteinizante (LH) y

17-β-estradiol para favorecer la expansión del cúmulo y la maduración nuclear y citoplasmática, y una fuente de proteína como el suero fetal bovino (FCS, del inglés *fetal calf serum*) o la albúmina sérica bovina (BSA, del inglés *bovine serum albumin*).

1.2.2. Fecundación *in vitro*

La fecundación es el proceso por el cual el ovocito maduro y el espermatozoide interactúan y se fusionan para formar el cigoto. A tal efecto, los espermatozoides tienen que modificarse previamente mediante la capacitación, que se observa básicamente por una serie de cambios bioquímicos y biofísicos, que incluyen alteraciones de las membranas, cambios en la concentración de iones y del pH intracelular, en el metabolismo celular, y en su composición proteica, a la vez que por un notable aumento de la motilidad con un movimiento característico (hiperactivación), lo cual permite adquirir al espermatozoide la capacidad de fecundar al ovocito (Sutovsky, 2018; Delgado-Bermúdez *et al.*, 2022). *In vivo*, la capacitación espermática ocurre fisiológicamente en el tracto genital femenino. Para que se produzca la fecundación, los espermatozoides deben atravesar las células del cúmulo que protegen al ovocito hasta llegar a la zona pelúcida (ZP). La ZP es una membrana compuesta por glicoproteínas (ZPA/ZP2, ZPB/ZP4 y ZPC/ZP3 en bovino) que actúa como mecanismo de protección del ovocito y barrera contra la polispermia. El reconocimiento entre los dos gametos se produce por el contacto entre los receptores de superficie del espermatozoide y las cadenas de oligosacáridos de la ZP, lo que induce la reacción acrosómica en el espermatozoide, típicamente un proceso de exocitosis por el que se liberan enzimas hidrolíticas necesarias para atravesar la ZP y llegar al espacio perivitelino (Tumova *et al.*, 2021). Tras esta exocitosis se produce la fusión entre la membrana del espermatozoide y la membrana del ovocito, denominada oolema, y a continuación la fusión entre el espermatozoide y el citoplasma del ovocito u ooplasma, activándose así el ovocito para iniciar la formación de los pronúcleos (Sutovsky, 2018). La unión de ligandos del espermatozoide a ZPC produce una cascada de señalización que finaliza con la entrada de calcio extracelular en el citoplasma del ovocito. El aumento del calcio intracelular induce la liberación de enzimas contenidas en los gránulos corticales del ovocito que alteran la conformación de la ZP, evento conocido como reacción zonal, disminuyendo así la afinidad de los espermatozoides por el ovocito y bloqueándose la polispermia (Abbott & Ducibella,

2001). Estos gránulos corticales son vesículas secretoras producidas por el aparato de Golgi durante el desarrollo folicular, y se distribuyen periféricamente cerca de la membrana cuando el ovocito madura. Otros eventos como la proteólisis y diferentes modificaciones de ZP2 y ZP3, como la glicosilación, también contribuyen al bloqueo de la polispermia para evitar la unión de nuevos espermatozoides (Yonezawa, 2014). Es en este momento cuando la meiosis, que estaba detenida en metafase II, prosigue y se completa con la segregación del segundo corpúsculo polar. Finalmente, tras la activación del ovocito, se forman los pronúcleos masculino y femenino (Sutovsky, 2018).

En el laboratorio, las técnicas más comunes para capacitar los espermatozoides *in vitro* son el *swim-up* y los gradientes de densidad (Mermillod *et al.*, 2006; Ferré *et al.*, 2020a). Ambos procedimientos permiten eliminar también el medio de congelación, el plasma seminal, restos celulares y detritus, y espermatozoides muertos o inmóviles. El *swim-up* selecciona los espermatozoides en función de su motilidad, para lo cual se deposita el semen en la parte inferior de un tubo que contiene medio de capacitación, de manera que los espermatozoides móviles se mueven a través del medio. Transcurrido un periodo de incubación, generalmente una hora, se recoge la fracción superior de medio que contiene los espermatozoides móviles, y se centrifuga para eliminar el plasma seminal y concentrar los espermatozoides. En cambio, en el gradiente de densidad se separan los espermatozoides por centrifugación a través de un gradiente con dos o tres medios de diferente densidad, y se recoge la fracción del fondo del tubo que contiene los espermatozoides móviles y de mejor calidad que han conseguido atravesar todos los gradientes. Los gradientes de densidad utilizan el Percoll, compuesto por partículas de sílice coloidal recubiertas de polivinilpirrolidona (PVP), aunque actualmente hay disponibles otros gradientes formados por una suspensión coloidal de partículas de sílice con silano (Morrell *et al.*, 2017; Baldi *et al.*, 2020). En los últimos años han surgido nuevas técnicas de capacitación basadas en microfluidos (como el mini-Percoll) y microfiltros, las cuáles reducen la fragmentación del DNA y mejoran los parámetros cinéticos de los espermatozoides y la producción de embriones (Weng, 2019; Vega-Hidalgo *et al.*, 2022).

Existen diferentes medios de fecundación, siendo el más utilizado en bovino el medio TALP (Tyrode's-Albúmina-Lactato-Piruvato). Estos medios son suplementados con agentes que inducen la capacitación espermática, como la heparina, el más común (Parrish, 2014; Sirard, 2018). En ocasiones también se añaden otros agentes para

estimular y mantener la motilidad espermática, como son la penicilamina, hipotaurina y epinefrina (PHE) (Gonçalves *et al.*, 2014). Generalmente, la fecundación se realiza con una concentración de espermatozoides de entre 1 y 2 millones/ml (Ferré *et al.*, 2020a), coincubando los ovocitos y espermatozoides durante 18 a 24 h, aunque 10 h se estima suficiente para alcanzar la máxima producción de blastocistos (Ward *et al.*, 2002). *In vitro*, aproximadamente el 80% de los ovocitos resultan correctamente fecundados y se dividen al menos una vez (Lonergan *et al.*, 2003).

1.2.3. Cultivo *in vitro*

Una vez que se produce la fecundación, comienza el desarrollo embrionario con la formación del cigoto. A través de sucesivas divisiones mitóticas, el número de células del embrión, llamadas blastómeras, va aumentando sin que se produzca crecimiento celular (Figura 2). Durante las primeras fases del desarrollo (*early cleavage stages*), los transcritos de RNA y las proteínas que se almacenaron en el ovocito durante el desarrollo intrafolicular son esenciales hasta que se produce la activación del genoma embrionario (EGA, del inglés *embryonic genome activation*). Esta activación ocurre de forma gradual en el embrión de 4 a 16 células, aunque el grueso de los cambios (*major* EGA) ocurre en la transición de 8 a 16 células (Graf *et al.*, 2014). Conforme el desarrollo continúa, los transcritos y proteínas de origen materno se sustituyen por otros sintetizados por el propio embrión, en virtud de la activación de su genoma, en un proceso conocido como transición materno-embrionaria. La adhesión intercelular también aumenta entre blastómeras debido al establecimiento de uniones GAP, y se crea una unión más estrecha entre células, produciéndose así la compactación, que continúa hasta que el embrión toma el aspecto de una masa de células uniforme conocida como mórula, de aproximadamente 32 blastómeras (Boni *et al.*, 1999). Durante este proceso, en el interior del embrión comienza a formarse una cavidad -el blastocele- que contiene un líquido, y al embrión se le denomina entonces blastocisto. En este estadio se pueden distinguir dos grupos celulares: los blastómeros externos o trofoblasto (TE), que formarán la placenta, y los blastómeros internos o masa celular interna (ICM), que originarán el feto. Las bombas Na^+/K^+ ATPasas en el trofoblasto aseguran el transporte activo de iones de Na^+ hacia el espacio extracelular, creando un gradiente osmótico que favorece el transporte pasivo de agua gracias a la presencia de acuaporinas (Płusa & Piliszek, 2020). Mientras que el

blastocele va aumentando de tamaño, merced al continuo aporte de líquido producido en las células del TE, el embrión se expande y la ZP reduce su grosor. Finalmente, el embrión se libera de la ZP en un proceso que se denomina eclosión en el que interviene la fuerza mecánica ejercida tanto por la presión hidrostática sobre la ZP por la expansión del blastocisto, como por las contracciones y reexpansiones del embrión (Seshagiri *et al.*, 2016). La degradación enzimática y las penetraciones de proyecciones de células del TE en la ZP también contribuyen al debilitamiento de la misma. En el animal vivo, el embrión entraría en el útero en estadio de mórula (día 5) y, tras alcanzar el estadio de blastocisto y eclosionar, comenzaría la elongación, que implica una serie de cambios morfológicos. El embrión pasa de tener una morfología esférica (día 7), a ovoide (día 12-13), tubular (día 14-15) y finalmente filamentosa (día 16-17) (Lonergan *et al.*, 2016). A partir del día 19, el embrión totalmente elongado se une al epitelio luminal del endometrio para iniciar la implantación.

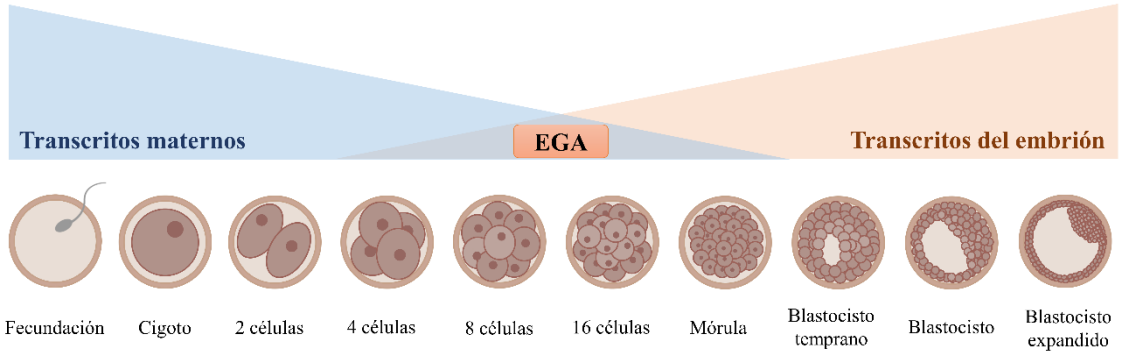

Figura 2. Representación del desarrollo embrionario bovino. El número de transcritos maternos disminuye desde la fecundación hasta la activación del genoma embrionario (EGA, del inglés *embryonic genome activation*), mientras que el número de transcritos producidos por el embrión incrementa a partir de la EGA.

Generalmente los embriones se mantienen en CIV durante 7-8 días, hasta que los más avanzados alcanzan el estadio de blastocisto o blastocisto expandido. El CIV puede tratar de reproducir condiciones fisiológicas características del tracto genital durante el desarrollo *in vivo*, aunque no es infrecuente que el desarrollo *in vitro* se produzca satisfactoriamente en condiciones muy distintas. Actualmente existe una amplia variedad de sistemas y medios de CIV. El medio más común es el fluido sintético de oviducto (SOF, del inglés *synthetic oviductal fluid*), el cual generalmente es suplementado con

aminoácidos esenciales y no esenciales, citrato sódico, mioinositol y una fuente de proteínas como el FCS o la BSA (Holm *et al.*, 1999). No obstante, el uso de FCS y BSA es controvertido, pues, aunque el CIV con suero favorece el desarrollo embrionario, incrementando el porcentaje de blastocistos (Pinyopummintr & Bavister, 1994; Thompson *et al.*, 1998; Leivas *et al.*, 2011; Murillo *et al.*, 2017), altas concentraciones de FCS disminuyen la calidad del embrión (Rizos *et al.*, 2003; Sudano *et al.*, 2011). Además, el cultivo de embriones con suero altera la expresión génica, incrementa la acumulación de lípidos en los blastocistos y reduce la supervivencia a la criopreservación (Abe *et al.*, 2002; Heras *et al.*, 2016; Murillo *et al.*, 2017). Otro inconveniente es que la composición exacta del suero es desconocida, contiene ácidos grasos, aminoácidos y factores de crecimiento, y la concentración de dichos componentes varía entre lotes (van der Valk *et al.*, 2018), ya que está influida por factores como la dieta, época del año, localización geográfica, o la edad gestacional del feto del que se obtiene. Por tanto, el uso de suero puede alterar la reproducibilidad de los experimentos (Baker, 2016). La transferencia de embriones producidos en un medio suplementado con suero también se ha relacionado con alteraciones fetales, como el síndrome del ternero grande (LOS, del inglés *large offspring syndrome*) o síndrome del ternero anormal (AOS, del inglés *abnormal offspring síndrome*) (Farin *et al.*, 2001; Lazzari *et al.*, 2002). Este síndrome se ha relacionado con mayor incidencia de abortos, gestaciones más prolongadas, aumento del peso del feto o del peso al nacimiento, organomegalia, y menor supervivencia durante los primeros meses de vida (Rivera *et al.*, 2021). El LOS/AOS puede ocurrir de forma natural, pero el uso de técnicas de reproducción asistida, como la transferencia de núcleos procedentes de células somáticas (SCNT), el CIV de embriones en presencia de suero y el cocultivo con células somáticas, incrementa el número de terneros afectados (Rivera *et al.*, 2021). La incidencia del LOS/AOS es desconocida, varía entre especies, y la severidad del fenotipo depende de la técnica de reproducción utilizada y de las condiciones de cultivo, viéndose agravada cuanto mayor es la manipulación del embrión, como en el caso de la SCNT (Farin *et al.*, 2006; Hill, 2014; Li *et al.*, 2019; Zander-Fox *et al.*, 2021).

La albúmina, proteína más abundante en el plasma de los vertebrados, ha reemplazado el suero como componente macromolecular en el medio de cultivo. No obstante, al igual que ocurre con el suero, la composición exacta de la BSA es desconocida, y contiene otros componentes que pueden influir en el desarrollo del embrión, como el citrato,

factores de crecimiento, ácidos grasos y otros metabolitos, cuya concentración además varía entre lotes (Rorie *et al.*, 1994; Wale & Gardner, 2016). Por ello, la tendencia actual pasa por disminuir o eliminar completamente el suero y la BSA en el medio de cultivo (Murillo *et al.*, 2017), y utilizar medios químicamente definidos que no contengan productos de origen animal (Moreno *et al.*, 2015). Sin embargo, de esta manera se priva al embrión de la exposición a un gran número de proteínas como las que se encontraría *in vivo* en el fluido uterino (Forde *et al.*, 2014). El fluido de oviducto y fluido uterino se han propuesto como alternativas al uso del suero y la BSA (Hamdi *et al.*, 2018), pero, igual que estos, es un suplemento de origen animal y su composición entre lotes es variable, ya que depende de factores como el ciclo estral, la edad o el estado nutricional del animal (Hugentobler *et al.*, 2007; Lamy *et al.*, 2018).

El alcohol de polivinilo (PVA) es una macromolécula sintética propuesta como alternativa al uso de suplementos de origen animal por sus propiedades surfactantes. El cultivo con PVA permite el desarrollo de los embriones y la obtención de terneros saludables (Holm *et al.*, 1999; Lim *et al.*, 2007). Sin embargo, el PVA reduce los porcentajes de blastocistos (Orsi & Leese, 2004), y altera la expresión de genes importantes en el desarrollo (Wrenzycki *et al.*, 1999), y el metabolismo de aminoácidos (Orsi & Leese, 2004).

El análisis del MC por técnicas metabolómicas requiere la eliminación de las proteínas del medio, las cuales pueden producir efecto matriz, interferir en la detección de los metabolitos, disminuyendo la precisión y exactitud del análisis, y disminuir la vida útil de los componentes de los instrumentos (Raterink *et al.*, 2014). Por ese motivo, en un estudio anterior se propuso comenzar en día 6 de desarrollo del embrión un cultivo individual de duración limitada a 24h, de manera que hasta el día 6 el embrión se encuentra en cultivo en grupo en un medio con BSA, evitando los efectos negativos que tendría la sustitución de proteína por PVA durante todo el periodo de CIV (Murillo-Ríos *et al.*, 2017). El cultivo durante 24 h con PVA, aunque disminuye el desarrollo de los embriones, mejora la supervivencia a la vitrificación, disminuye el número de abortos y produce terneros sanos (Murillo-Ríos *et al.*, 2017; Gómez *et al.*, 2020a). Este sistema de cultivo tiene las ventajas de la ausencia de proteína, resultando en un medio químicamente definido, importante desde el punto de vista comercial.

1.2.4. Criopreservación

La criopreservación es el proceso de conservación de los embriones a muy bajas temperaturas, ralentizando así el metabolismo celular hasta conseguir prácticamente su detención, pero manteniendo la viabilidad del embrión. Esta técnica es imprescindible en reproducción asistida, ya que permite el almacenaje de embriones vivos en estado quiescente durante largos periodos de tiempo. En bovino, además, la criopreservación es lo que hace posible el intercambio de embriones a nivel mundial y optimizar recursos cuando no se dispone de receptoras para transferir o bien existe un excedente de embriones susceptibles de ser criopreservados.

Las células animales están constituidas por más de un 80% de agua que se encuentra principalmente libre (agua osmótica), y que puede convertirse en hielo durante la criopreservación. El principio de la criopreservación radica en la eliminación de la mayor cantidad de agua posible del interior de las células antes de la congelación para evitar la formación de grandes cristales de hielo, tanto intracelulares como extracelulares, que causarían un daño celular irreversible (destruyendo o dañando las células por una acción mecánica), comprometiendo así la viabilidad del embrión (Lehn-Jensen & Rall, 1983). La presencia de crioprotectores incrementa la concentración de solutos, reduce el punto de congelación de la solución, permitiendo una mayor deshidratación, y reduce la tendencia a cristalizar, disminuyendo la formación de hielo (Pegg, 2007; Bakhach, 2009; Wolkers & Oldenhof, 2021). La pérdida de agua intracelular depende de la permeabilidad de la propia membrana celular al agua, característica que es específica de cada tipo de célula. En los embriones depende también del estadio del embrión, el cual determina cambios en la composición de la membrana y el volumen de las blastómeras que alteran la permeabilidad al agua (Leibo, 2008).

Los crioprotectores se pueden dividir en dos grupos en función de su capacidad para penetrar en la célula a través de la membrana: crioprotectores permeables, que son pequeñas moléculas polares que atraviesan la membrana por difusión pasiva como el glicerol, etilenglicol (EG), dimetilsulfóxido (DMSO), o propilenglicol, y crioprotectores no permeables, que incluyen azúcares como la sacarosa, glucosa, galactosa, y polímeros como el polietilenglicol y PVP (Vatja & Nagy, 2006). Los crioprotectores permeables reemplazan el agua intracelular, y no forman cristales cuando se congelan, lo que disminuye el daño intracelular. Los crioprotectores no permeables actúan produciendo un

gradiente de presión osmótica que favorece la deshidratación celular. Estos últimos no se utilizan solos, sino en combinación con agentes permeables (Wolkers & Oldenhof, 2021).

Existen dos procedimientos de criopreservación de embriones, la congelación lenta y la vitrificación. Ambas técnicas tienen ventajas y desventajas (Woods *et al.*, 2004; Fahy & Wowk, 2015; Ferré *et al.*, 2020b). La vitrificación es un proceso sencillo, rápido (si el número de embriones no es muy alto), no requiere equipos costosos, y minimiza la formación de cristales de hielo en el interior de las células del embrión. Sin embargo, para vitrificar hace falta personal entrenado, ya que requiere un manejo preciso de los embriones y de la solución puesto que se trabaja con volúmenes mínimos y con tiempos muy cortos (25-30 s) cuando se transfiere el embrión a la solución de vitrificación. Este punto es crítico puesto que una exposición más prolongada en este medio con elevadas concentraciones de crioprotector resulta tóxica para el embrión. La congelación lenta requiere equipos costosos, pero este proceso precisa menos dedicación y tiempo que la vitrificación cuando el número de embriones a criopreservar es alto. Además, la congelación lenta emplea concentraciones de crioprotector mucho más bajas que la vitrificación, lo que potencialmente supone menor toxicidad para los embriones, y permite la transferencia directa a receptoras, por lo que es una técnica de elección en ganadería.

Congelación lenta

La congelación lenta facilita el paso del estado líquido a sólido como consecuencia de la disminución progresiva de la temperatura (a un ritmo inferior a -1°C/min). El procedimiento favorece la deshidratación celular, minimizando la formación de cristales de hielo intracelular, pero sin conseguir evitarla totalmente. Durante la congelación se forma hielo en la solución extracelular debido a que ésta mantiene una temperatura inferior al punto de congelación de la solución intracelular. La formación de hielo extracelular convierte la solución en hipertónica, provocando deshidratación en las células para restablecer el equilibrio (Gosden, 2014). El tamaño de los cristales de hielo depende directamente de la tasa de enfriamiento. Si ésta es demasiado rápida, la deshidratación podría ser insuficiente, ralentizando la salida del agua intracelular, que estaría disponible para formar un mayor número de cristales de hielo y de mayor tamaño,

lo cual podría provocar lisis celular. En cambio, si el proceso es demasiado lento, la célula podría deshidratarse en exceso pudiendo llegar a un colapso irreversible que le impediría recuperar su estructura y función normales tras la descongelación. El exceso de deshidratación también supone una exposición a concentraciones elevadas de sales intracelulares que podrían desnaturalizar las proteínas del citoplasma (el llamado "efecto solución") y dar lugar a concentraciones elevadas de crioprotectores que podrían resultar tóxicas (Vladimirov *et al.*, 2017). Por tanto, para causar el mínimo daño posible es necesario encontrar la tasa de enfriamiento óptima (Gosden, 2014; Fahy & Wowk, 2015). En términos de criopreservación, la deshidratación se considera alcanzada cuando la concentración intracelular de solutos es suficientemente elevada como para evitar la formación de cristales de hielo dañinos (Woods *et al.*, 2004).

En la congelación lenta generalmente se utiliza glicerol o EG como crioprotectores. Los embriones son expuestos al crioprotector a una temperatura entre 25-35°C, y, sin abandonar ya este medio, se disponen en pajuelas (Figura 3) que se colocan en dispositivos de congelación programables. En las condiciones de nuestros estudios (Gómez *et al.*, 2020a), una vez colocadas las pajuelas con los embriones en el dispositivo de congelación, se hace disminuir la temperatura hasta los -6°C. En este momento se induce la cristalización por contacto con un dispositivo previamente enfriado a temperaturas notablemente inferiores a la actual (generalmente pinzas o bastones de algodón superenfriados en nitrógeno líquido). Los embriones se mantienen a esta temperatura durante 8 min para favorecer el equilibrio, y, a continuación, la temperatura desciende a una velocidad de -0.5°C/min hasta alcanzar entre los -35°C. Es en este momento cuando las pajuelas se sumergirán y serán almacenadas en nitrógeno líquido (- 196°C).

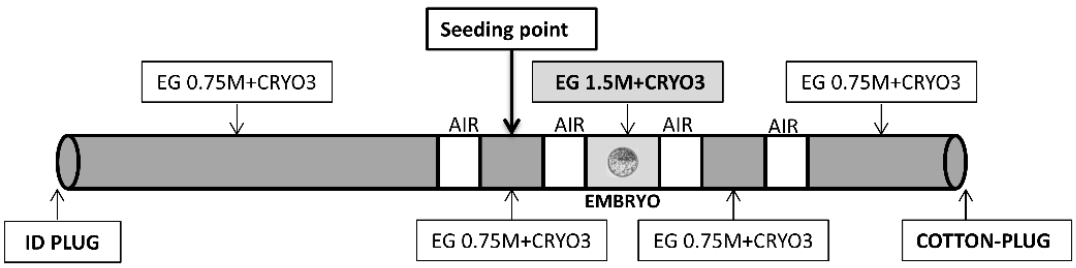

Figura 3. Distribución de la solución de crioprotector y del embrión para la congelación y transferencia directa. EG: etilenglicol. CRYO3: reemplazo macromolecular (Gómez *et al.*, 2020a).

La supervivencia a la descongelación depende de la velocidad de enfriamiento y del crioprotector empleado. En caso de que la temperatura de congelación haya alcanzado entre -30 y -40°C, es necesaria una descongelación rápida para evitar la recristalización de los pequeños cristales de hielo y su conversión en grandes cristales que podrían causar graves lesiones en las células (Woods *et al.*, 2004). En cambio, si la temperatura es inferior, una descongelación muy rápida podría provocar estrés osmótico y lisis celular, por lo que la descongelación debe ser más lenta (10°C/min).

El éxito de la supervivencia de un embrión a la descongelación depende en gran medida del comportamiento y tipo de crioprotector empleado durante la congelación. Determinados crioprotectores de baja permeabilidad celular como el glicerol o el DMSO requieren sucesivos pasos del embrión en medios con concentraciones decrecientes de crioprotector. Estos cambios sucesivos permiten eliminar el crioprotector y evitar el choque osmótico provocado por la entrada brusca de agua en la célula. El número de pasos y tiempo entre cada paso, pueden variar con las concentraciones de crioprotector empleadas en las soluciones de dilución. A diferencia del DMSO, otros crioprotectores como el EG no necesitan ser diluidos tras la descongelación debido a su elevada permeabilidad celular y a su rápida difusión (Voelkel & Hu, 1992), lo cual permite la transferencia directa de embriones sin necesidad de extraer el embrión de la pajuela ni evaluar su morfología al microscopio (Leibo, 1984; Sanches *et al.*, 2016; Gómez *et al.*, 2020a). De esta manera, la descongelación se realiza de manera muy rápida y sencilla, en nuestro caso manteniendo la pajuela 10 s al aire, y sumergiendo a continuación en un baño de agua a 35° durante 30 s (Gómez *et al.*, 2020a).

Vitrificación

La vitrificación es la solidificación directa de un líquido a un estado amorfo, sin estructura, evitando la cristalización. De esta manera se elimina el daño causado por la formación de cristales de hielo intracelulares. A diferencia de la congelación, la vitrificación no requiere el equilibrio osmótico entre el medio extra e intracelular a medida que desciende la temperatura, sino un incremento radical de la velocidad de enfriamiento (>20.000°C/min). Tal incremento se consigue mediante inmersión directa en nitrógeno líquido (Fahy & Wowk, 2015). La vitrificación emplea concentraciones más

elevadas de crioprotectores que la congelación lenta para producir la deshidratación celular. No obstante, a elevada concentración los crioprotectores son más tóxicos y provocan daño osmótico. Para evitar la toxicidad se suelen combinar diferentes crioprotectores (al menos uno debe ser permeable) o bien se exponen los embriones a crioprotectores a bajas temperaturas, con lo que el efecto tóxico es menor. El crioprotector suele añadirse en dos fases, aumentando en la segunda fase su concentración. Aunque el tipo de crioprotector, la concentración, y el tiempo de exposición al mismo pueden variar en los protocolos de vitrificación (Vatja & Nagy, 2006), es necesario alcanzar un equilibrio óptimo entre obtener un alto nivel de deshidratación y elevada viscosidad, evitando la toxicidad (Woods *et al.*, 2004). Por ello, para asegurar una velocidad de enfriamiento rápida se utiliza el mínimo volumen de medio posible.

En las condiciones de nuestra investigación (Caamaño *et al.*, 2015; Gómez *et al.*, 2020a), la vitrificación se realiza individualmente con blastocistos expandidos en una superficie calefactada (41°C) y utilizando como base del medio de vitrificación (VM) TCM 199-HEPES + 20% (v/v) FCS. Primero los blastocistos se mantienen en la solución de vitrificación-1 (VS1: VM con 7.5% EG, 7.5% DMSO) durante 3 min, y, a continuación, 20-25 s en la solución de vitrificación-2 (VS2: VM con 16.5% EG, 16.5% DMSO y 0.5M sacarosa). En este momento los embriones se colocan en un soporte adecuado y se vitrifican por contacto directo con un bloque superenfriado en nitrógeno líquido. Existen diferentes soportes para manejar los embriones durante la vitrificación, como Open Pulled Straw, Cryoloop, Cryotop o Fibreplug (este último usado en nuestro caso). La mayoría de estos soportes son sistemas abiertos en los que el embrión entra en contacto directo con el nitrógeno líquido para facilitar el aumento de la velocidad de enfriamiento y, con ello, la eficiencia del procedimiento. Y es en estos soportes donde los embriones son almacenados hasta su uso (Vatja & Nagy, 2006), por lo que existe un riesgo de contaminación de las muestras a través del nitrógeno (Joaquim *et al.*, 2017).

El calentamiento es la parte del procedimiento de vitrificación que devuelve al embrión a su estado original. Este proceso requiere de una velocidad de calentamiento muy elevada, lo cual se consigue mediante el paso directo de la muestra desde el nitrógeno líquido a un medio de cultivo mantenido a temperatura de incubación. A continuación, los embriones pasan por medios con concentraciones de crioprotector no permeable cada vez más reducidas, a fin de diluir el crioprotector del interior de las células y permitir su rehidratación. En nuestros estudios (Caamaño *et al.*., 2015; Gómez *et al.*, 2020a;), la

desvitrificación se realiza sumergiendo el fibreplug en 1.2 mL de VM con 0.25 M sacarosa y dejando el embrión 5 min en el medio. Luego se realizan dos lavados en VM y otros dos en mSOF con 6 mg/mL BSA y 10% FCS. Aprovechando que el embrión se ha manipulado bajo el microscopio, los blastocistos se pueden cultivar por un tiempo para valorar su reexpansión como indicador de supervivencia a la vitrificación. Algunos estudios proponen sistemas con un protocolo de desvitrificación *in-straw* compatibles con la transferencia directa, ya que evitan los gradientes de desvitrificación (Inaba *et al.* 2011; Morató & Mogas, 2014; Caamaño *et al.*, 2015). Sin embargo, la falta de estudios que realicen transferencia de embriones a gran escala con estos sistemas para demostrar que son manejables y resultan en índices de gestación aceptables, limitan su aplicación práctica.

Supervivencia y gestación en embriones criopreservados

El éxito de la criopreservación está influenciado por numerosas variables, no sólo por el origen de los embriones (*in vitro* o *in vivo*), sino también por el medio de cultivo utilizado, el estadio en el que se encuentra el embrión en el momento de la criopreservación, la calidad del embrión, el contenido en lípidos, la permeabilidad de la membrana, el tamaño y forma de las células, la raza, e incluso el propio toro empleado en la fecundación (Massip, 2001; Vajta & Kuwayama, 2006; Marsico *et al.*, 2019). Las mejoras en los sistemas de producción de embriones a menudo resultan en mejoras de la calidad del embrión y, por ende, de su capacidad de supervivencia a la criopreservación. Entre las estrategias utilizadas destaca la eliminación del suero del medio de cultivo, la utilización de una atmósfera baja en oxígeno para reducir el estrés oxidativo, o la adición de suplementos al medio de cultivo que producen diversos efectos beneficiosos para el embrión. Entre todos ellos, podemos destacar los antioxidantes (β-mercaptoetanol o resveratrol), los inhibidores de la apoptosis, los agentes delipidantes o lipolíticos para reducir el contenido lipídico (L-carnitina), los factores de crecimiento [insulin-like growth factor 1 (IGF1) o leukaemia inhibitory factor (LIF)], o cualquier otra molécula que reduzca el daño provocado por la criopreservación (Salzano *et al.*, 2014; Pero *et al.*, 2018; Do *et al.*, 2019; Vendrell-Flotats *et al.*, 2020).

En general, los embriones IVP sobreviven peor a la criopreservación que los embriones IVD. La congelación lenta es el método de elección para la criopreservación de embriones IVD, mientras que la vitrificación surgió como alternativa para los embriones IVP, toda vez que la supervivencia a la vitrificación de este tipo de embriones se considera superior a la obtenida tras congelación (Marsico *et al.*, 2019). No obstante, en determinadas condiciones, los índices de gestación obtenidos con embriones IVP congelados son equiparables a los obtenidos con embriones vitrificados (Sanches *et al.*, 2016) y frescos (Gómez *et al.*, 2020a). De hecho, un metaanálisis demostró que, en los experimentos *in vitro*, los embriones vitrificados (tanto IVP como IVD) presentan mayor ratio de reexpansión, eclosión y supervivencia, y un mayor número de células tras la criopreservación que los embriones congelados. En cambio, tras la transferencia, los evidentes signos de calidad que mostraban los embriones supervivientes a la vitrificación no guardaron relación con mayores índices de gestación, sino que fueron comparables a los obtenidos con embriones congelados (Arshad *et al.*, 2021).

1.3. Metabolismo embrionario y dimorfismo sexual

1.3.1. Generalidades del metabolismo del embrión temprano

El desarrollo embrionario temprano es un proceso muy dinámico durante el cual el embrión experimenta cambios en el metabolismo. El embrión obtiene el ATP a través de la vía aeróbica (fosforilación oxidativa) y la vía anaeróbica (glicólisis). La ruta oxidativa tiene lugar en la mitocondria, requiere oxígeno, y es la vía por la que se obtiene la mayor parte de energía, ya que puede producir entre 30 y 32 moléculas de ATP, CO_2, H_2O y acetil coenzima A (Ac-CoA) por la oxidación completa del piruvato obtenido a partir de la glucosa. En cambio, la glicólisis ocurre en el citoplasma, no requiere oxígeno, y genera lactato y únicamente 4 moléculas de ATP junto con 2 moléculas de NADH por cada molécula de glucosa oxidada (de Souza *et al.*, 2015; Gardner & Harvey, 2015).

Durante las etapas iniciales del desarrollo, antes de la compactación y hasta que se produce la EGA, la actividad transcripcional y metabólica de los embriones es muy limitada. El ovocito fecundado consume muy poco oxígeno, y obtiene energía principalmente de la oxidación del piruvato y del lactato, mostrando un consumo estable durante las primeras fases del desarrollo (Figura 4). El piruvato es transportado a la

mitocondria donde será metabolizado a través del ciclo del ácido tricarboxílico (TCA). En cambio, el lactato es convertido a piruvato en el citosol con la conversión de NAD^+ a NADH (Takahashi & First, 1992; Thompson *et al.*, 1996; Khurana & Niemann, 2000; Lane & Gardner, 2005). En este momento la capacidad de metabolizar la glucosa para obtener energía es muy limitada, ya que el embrión temprano se caracteriza por un elevado ratio ATP:ADP, lo que inhibe alostéricamente la enzima fosfofructokinasa (PFK), que es la encargada de transformar la glucosa-6-fosfato, limitando el flujo de glucosa hacia la ruta glicolítica antes de la compactación (Leese *et al.*, 1984; Gardner *et al.*, 2000). De hecho, la adición de glucosa al medio de cultivo durante las primeras fases del desarrollo es perjudicial para el embrión (Takahashi & First, 1992). En cambio, las células del cúmulo que rodean al ovocito son capaces de utilizar la glucosa para producir piruvato y lactato, asegurando la disponibilidad de estos metabolitos en el entorno del ovocito y el cigoto (Sutton *et al.*, 2003; Thompson *et al.*, 2007). La glucosa puede ser metabolizada a través de tres rutas principales: la vía glicolítica, que produce piruvato y lactato; la ruta de las pentosas fosfato (PPP, del inglés *pentose phosphate pathway*), la cual produce NADPH y ribosa 5-fosfato, un precursor para la biosíntesis de nucleótidos; y la ruta de la biosíntesis de hexosaminas. Durante las primeras fases del desarrollo, el metabolismo de la glucosa puede estar limitado a la biosíntesis de fosfolípidos, triacilglicéridos u otros precursores en lugar de a la obtención de energía (Gardner *et al.*, 2000). El embrión en esta fase temprana también puede utilizar aminoácidos como la glutamina y aspartato para producir energía a través del transportador (*shuttle*) malato-aspartato (Lane & Gardner, 2005). Una vez que se activa el genoma embrionario, la demanda de ATP se incrementa para mantener la proliferación celular, la formación del blastocisto y la expansión del blastocele. Es entonces cuando disminuye la ratio ATP:ADP, pues el ATP se consume rápidamente, activándose la PFK y la vía glicolítica para metabolizar la glucosa (Leese *et al.*, 1984; Gardner & Harvey, 2015).

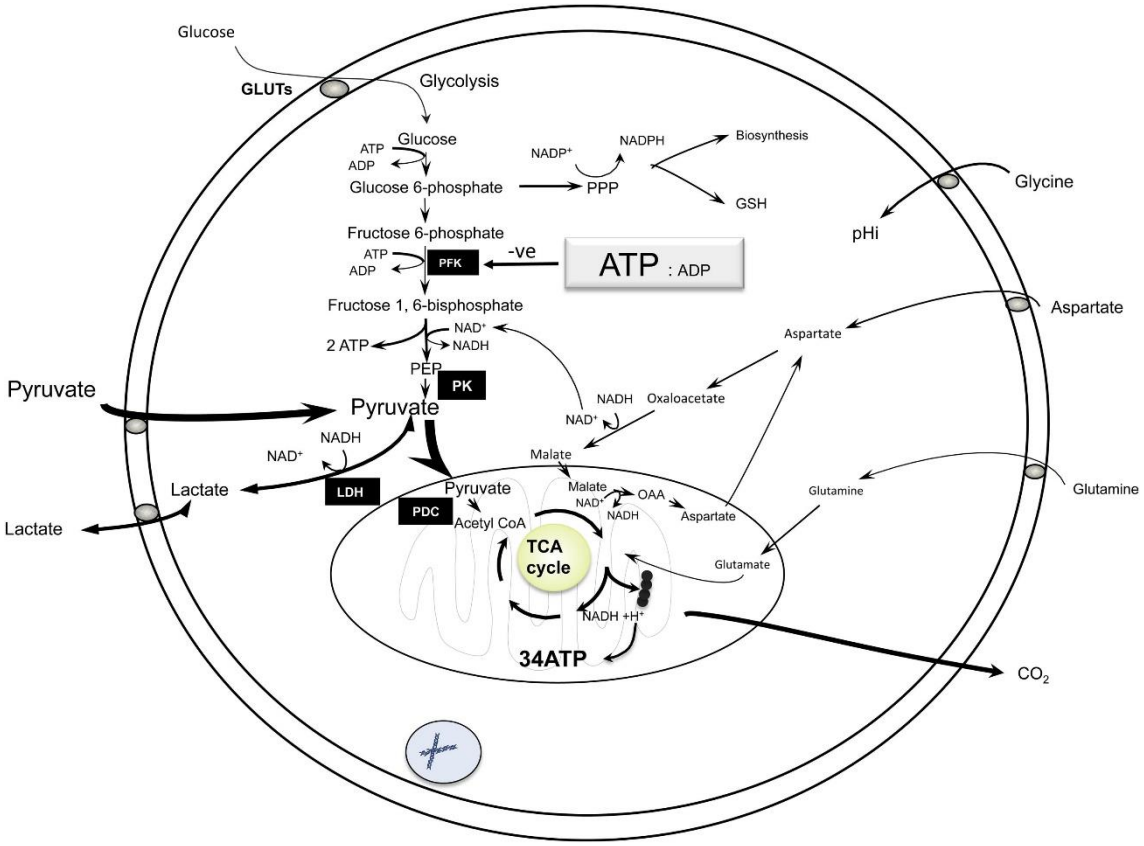

Figura 4. Metabolismo del embrión antes de la compactación (Gardner & Harvey, 2015).

Durante el desarrollo desde el estadio de mórula a blastocisto eclosionado aumentan drásticamente los consumos de glucosa, piruvato y oxígeno, y se produce mucho más lactato y ATP (Thompson *et al.*, 1996; Khurana & Niemann, 2000; Guerif *et al.*, 2013; Obeidat *et al.*, 2019). Los blastocistos convierten aproximadamente la mitad de la glucosa consumida en lactato a través del ciclo del TCA produciendo NAD^+. Este proceso, que sucede aún en presencia de suficiente oxígeno como para oxidar por completo la glucosa, se conoce como glicólisis aeróbica o efecto *Warburg*, y es característico de las células indiferenciadas (tumorales y *stem cells*) (Warburg *et al.*, 1956; Krisher & Prather, 2012). El lactato producido se libera al medio (Gardner & Harvey, 2015), creando un ambiente extracelular alto en lactato y bajo en pH, probablemente favorecedor de la invasión del TE y reductor de la respuesta inmune local, contribuyendo así a evitar el rechazo materno (Gardner, 2015). El Ac-CoA producido por la fosforilación oxidativa sirve de sustrato para el ciclo del TCA, produciendo NADH y $FADH_2$. Ambos cofactores redox serán reducidos para producir ATP utilizando el O_2 como aceptor de electrones, generando especies reactivas del oxígeno (ROS, del inglés *reactive oxygen species*), muy dañinas

para diversas estructuras celulares. Las células poseen diversos mecanismos para neutralizar las ROS, como la reducción de H_2O_2, que resulta en la oxidación de glutatión, destacando el papel de los aminoácidos como antioxidantes (Harvey *et al.*, 2002).

Además de glucosa, los embriones tienen la capacidad de utilizar aminoácidos y ácidos grasos como fuente de energía. Los aminoácidos también actúan como precursores biosintéticos, tampones de pH intracelular, antioxidantes, quelatos, moléculas señalizadoras, y participan en procesos como la regulación del metabolismo de carbohidratos. La adición de aminoácidos al medio de cultivo ha demostrado ser beneficiosa para el desarrollo embrionario. Antes de la compactación, los aminoácidos no esenciales y la glutamina mejoran el desarrollo (Lane & Gardner, 1997; Steeves & Gardner, 1999). Sin embargo, tras la compactación, los aminoácidos no esenciales promueven el desarrollo de los blastocistos y la eclosión, y los aminoácidos esenciales son necesarios para el correcto desarrollo de la ICM. Los ácidos grasos también pueden actuar como sustratos para producir energía, entrando en la mitocondria a través de transportadores específicos, donde son metabolizados a unidades de Ac-CoA, las cuáles alimentan el ciclo del TCA (Sutton-McDowall *et al.*, 2012).

1.3.2. Dimorfismo sexual en el metabolismo

Los embriones machos y hembras no se diferencian únicamente en los cromosomas sexuales, sino también en la expresión de genes autosómicos (Bermejo-Alvarez *et al.*, 2010b; Forde *et al.*, 2016), en las marcas epigenéticas (Bermejo-Alvarez *et al.*, 2008), e incluso en el perfil metabólico (Sturmey *et al.*, 2010; Gómez *et al.*, 2016; Rubessa *et al.*, 2018). Durante el desarrollo embrionario temprano, y hasta la etapa de blastocisto, los dos cromosomas X del embrión femenino mantienen su transcripción en funcionamiento hasta que se produce la inactivación de una de las dos copias. La transcripción duplicada hace que durante un periodo de tiempo estos embriones expresen mayores cantidades de mRNA y proteínas asociados al cromosoma X (Gardner *et al.*, 2010), muchas de ellas involucradas en el metabolismo energético. Una de estas proteínas es la enzima glucosa 6-fosfato deshidrogenasa, implicada en el metabolismo de la glucosa a través de la PPP. De esta manera, el desarrollo de los embriones de uno u otro sexo puede verse afectado

por factores ambientales relacionados con el metabolismo, que pueden incluir la dieta materna o la composición del medio de cultivo de los embriones.

Uno de los metabolitos más estudiados en relación con el efecto en la proporción de los sexos en los embriones es la glucosa. Las concentraciones de glucosa entre 2 y 5.6 mM producen un aumento del sex ratio (Kimura *et al.*, 2005; Rubessa *et al.*, 2011). Aunque se desconoce la causa, una posible hipótesis es que se produce un incremento en algún metabolito o en ROS por una mayor expresión de un gen ligado al cromosoma X, lo cual inhibe el desarrollo de las hembras (Kimura *et al.*, 2005). Por el contrario, concentraciones más elevadas de glucosa (20-30 mM), favorecen el desarrollo de los embriones hembras (Jiménez *et al.*, 2003). La mayor supervivencia de las hembras en estas condiciones puede ser debido a una mayor expresión del gen XIAP, también ligado al cromosoma X, que produce una proteína inhibidora de la apoptosis. Otros suplementos como el citrato y mioinositol también producen cambios en la proporción de sexos a favor de las hembras (Rubessa *et al.*, 2011). En cambio, la presencia de suero promueve el desarrollo de embriones macho (Gutiérrez-Adán *et al.*, 2001). Los ácidos grasos poliinsaturados presentes tanto como suplemento en la dieta de las vacas como en el CIV de embriones también sesgan la proporción de sexos a favor de los machos (Marei *et al.*, 2018), aunque el mecanismo molecular involucrado se desconoce.

El consumo de aminoácidos cambia entre embriones de distinto sexo, lo cual podría deberse a una expresión diferente de genes ligados al cromosoma X o ser propio de un dimorfismo sexual sensible a condiciones estresantes, como el CIV. Esto se infiere de que los embriones *in vivo* no presentan diferencias tan pronunciadas entre sexos (Sturmey *et al.*, 2010). Sin embargo, la presencia de un embrión macho o hembra en el endometrio induce cambios en la composición de aminoácidos (Forde *et al.*, 2016) y en la expresión de proteínas (Gómez *et al.*, 2013) del fluido uterino. Además, el análisis de células del tejido extraembrionario reveló un total de 5132 genes diferencialmente expresados entre embriones de distinto sexo, que corresponden a 23 rutas diferenciales implicadas en el metabolismo de aminoácidos (Forde *et al.*, 2016), lo cual confirma que existe un metabolismo diferente entre sexos.

1.4. Selección de embriones y receptoras para transferencia

Hasta el momento no existe ningún parámetro que prediga el potencial de implantación de un embrión con total certeza. Esta carencia es en parte debida a que para lograr una gestación a término es necesario disponer tanto de una receptora fértil como de un embrión viable o competente, e identificar correctamente a ambos. Los métodos actuales de evaluación y selección de embriones se centran básicamente en la morfología, por lo que no se dispone de la información necesaria para conocer la viabilidad del embrión. Por lo tanto, el desarrollo de un método eficaz de selección de receptoras y embriones es un objetivo innovador en el ganado vacuno, especialmente en embriones criopreservados. La selección más precisa de embriones y receptoras permitiría optimizar el rendimiento de las transferencias de embriones y aumentar la probabilidad de conseguir gestación a término.

1.4.1. Selección de receptoras

La infertilidad o subfertilidad son problemas importantes en ganadería. El establecimiento y el mantenimiento de la gestación dependen tanto del embrión como de la receptora. Por lo tanto, es importante seleccionar las receptoras competentes antes de realizar la transferencia de embriones, y asegurar una correcta sincronía entre el ciclo estral de la receptora y la edad y el estadio de desarrollo del embrión que se va a transferir (Hasler & Barfield, 2021). En embriones IVP, el día 0 se considera el día de la fecundación, mientras que en las receptoras el día 0 es el día que comienza el estro. Existen varios sistemas de sincronización de receptoras, basados en la utilización de prostaglandina F2α (PGF2α) como agente luteolítico o su combinación con análogos de la hormona liberadora de gonadotropinas (GnRH), conocido como Ovsynch (Bó *et al.*, 2011). Uno de los protocolos más comunes consiste en insertar un dispositivo intravaginal de liberación de progesterona durante 7-11 días, seguido de la administración de PGF2α 48h antes o al momento de retirar la progesterona (Lucy *et al.*, 2001). Otros protocolos administran dos dosis de PGF2α con un intervalo de 11-14 días (Bó *et al.*, 2011).

Las receptoras se seleccionan para transferencia de embriones generalmente por ultrasonografía o palpación rectal, asociada a una correcta detección del momento del celo y a la concentración de progesterona en sangre (Gómez *et al.*, 2020b). La

ultrasonografía persigue determinar el correcto estado del tracto genital y, en particular, la presencia de un cuerpo lúteo funcional y de buena calidad antes de la transferencia. El cuerpo lúteo es una glándula endocrina que se forma en el ovario a partir de las células de la pared folicular que quedan tras la ovulación, y cuya función principal es producir la progesterona necesaria para el establecimiento y mantenimiento de la gestación (Thomson *et al.*, 2021). Existe controversia acerca de los niveles de progesterona mínimos necesarios para establecer correctamente la gestación. En general, las concentraciones elevadas de progesterona el día de la transferencia se correlacionan con mayores índices de gestación (Siqueira *et al.*, 2009), aunque concentraciones muy elevadas (entre 7.50 y 10.90 ng/ml) dos días antes de la transferencia conducen a peores índices de gestación (Rabaglino *et al.*, 2023). En un estudio se vio que el mínimo incremento en la concentración de progesterona entre día 0 y día 7 en receptoras gestantes es de 2.71 veces, y de 1.48 veces entre día 7 y día 14, y que la probabilidad de perder la gestación entre día 28 y día 42 es mayor cuando la concentración de progesterona en día 14 es inferior a 5 ng/mL (Kenyon *et al.*, 2013). Sin embargo, estos procedimientos pueden excluir animales fértiles (Siqueira *et al.*, 2009), por lo que es deseable un método más objetivo de selección de receptoras.

Está ampliamente documentado que el estado nutricional y metabólico de las receptoras influye en el éxito de la gestación (Leroy *et al.*, 2015; Robles & Chavatte-Palmer, 2017). La composición del fluido del oviducto y del útero puede estar influenciada por la composición de la sangre, que a su vez refleja los cambios metabólicos de la receptora (Leese *et al.*, 2008), por lo que los componentes de la sangre pueden reflejar la capacidad del animal para gestar. El perfil metabólico de la receptora en plasma en día 0 (estro) y día 7 (antes de realizar la transferencia) analizado por espectroscopia infrarroja por transformada de Fourier (FTIR) permite distinguir entre receptoras con capacidad o no de alcanzar la gestación a término (Muñoz *et al.*, 2014a; Muñoz *et al.*, 2020b). Estudios posteriores basados en Resonancia Magnética Nuclear (RMN) identificaron metabolitos en el plasma de la receptora capaces de predecir la gestación en receptoras de embriones frescos y vitrificados (Gómez *et al.*, 2020b; Gómez *et al.*, 2020c). Por lo tanto, la capacidad de gestar de una receptora puede ser analizada en el plasma, siendo una técnica poco invasiva y de fácil acceso. Además, el interés de identificar biomarcadores de gestación es mayor en receptoras de embriones criopreservados debido a su peor supervivencia a largo plazo (Mogas, 2019).

1.4.2. Selección de embriones según la calidad del embrión

Las técnicas de selección de embriones se pueden clasificar en invasivas, como la biopsia embrionaria, y no invasivas, como la evaluación morfológica y morfocinética o el análisis del MC.

La biopsia embrionaria consiste en obtener una o más blastómeras del embrión para obtener información genética o cromosómica (Park *et al.*, 2001). Para extraer tales células es necesario atravesar la ZP, bien utilizando un método químico (ácido Tyrode's), o mecánicamente con pipetas o láser. Sin embargo, la biopsia no es comúnmente utilizada en especies domésticas, a diferencia de en embriones humanos, ya que es una técnica compleja que requiere personal entrenado y equipos caros. Además, es una técnica invasiva que puede comprometer la viabilidad del embrión (Hasler *et al.*, 2002; Korhonen *et al.*, 2012), aunque algunos estudios no han encontrado diferencias en los índices de gestación tras la transferencia de embriones biopsiados en fresco (Lopes *et al.*, 2001; de Sousa *et al.*, 2017). No obstante, la manipulación asociada a la biopsia aumenta la sensibilidad del embrión a la criopreservación (Najafzadeh *et al.*, 2021), reduciendo los índices de gestación tras la transferencia de embriones biopsiados congelados (Gustafsson *et al.*, 1994). Por lo general, el tiempo necesario para obtener el resultado de la biopsia y la disponibilidad de receptoras o el exceso de embriones obliga a recurrir a la congelación de embriones. Por tanto, una técnica ideal para determinar la viabilidad de un embrión debería ser no invasiva, rápida y sencilla.

En cuanto a las técnicas no invasivas, actualmente, la evaluación de la calidad del embrión se realiza basándose en criterios morfológicos. Los parámetros de morfología en embriones bovinos, según los criterios establecido por la IETS (Bó & Mapletoft, 2013) incluyen el estadio del embrión, la forma, el color, el número y tamaño de las blastómeras, la homogeneidad del citoplasma, la presencia de residuos celulares en el espacio perivitelino y la integridad de la ZP. No obstante, la principal limitación de la morfología es la subjetividad, ya que depende del criterio del propio observador, puesto que la evaluación morfológica devuelve un parámetro estático que no tiene en cuenta lo que ocurre antes y después. Además, supone una disrupción del cultivo del embrión al implicar su extracción del incubador, donde las condiciones de pH, temperatura, humedad y tensión de oxígeno están controladas. Por otro lado, la morfología no siempre guarda relación con otros parámetros, como la dotación cromosómica o la presencia de

mutaciones. Por tanto, un embrión morfológicamente normal no es necesariamente un embrión cromosómicamente normal (Jakobsen *et al.*, 2006; Capalbo *et al.*, 2014). Por ello es necesario desarrollar métodos más exactos y objetivos para seleccionar el mejor embrión para transferir.

Como alternativa no invasiva a la habitual evaluación morfológica se encuentra la combinación de los criterios morfológicos y la velocidad de división celular, utilizando para ello la tecnología *time-lapse*. Los sistemas *time-lapse* capturan imágenes del embrión en cultivo en un determinado intervalo de tiempo, y, además, permiten visualizar el embrión en tiempo real, minimizando de esta manera la manipulación y disminuyendo el estrés asociado a extraer el embrión del incubador por los cambios de temperatura y gases o la exposición a la luz. De esta manera se genera una grabación del desarrollo del embrión que permite analizar retrospectivamente la cinética del desarrollo, el número de blastómeras, la simetría de la división celular, y la fragmentación del citoplasma. También se registra el momento en el que ocurren los diferentes eventos del desarrollo del embrión (extrusión del segundo corpúsculo polar, primera división mitótica, segunda división, etc.). Hay estudios que han demostrado la relación de estos parámetros con la tasa de gestación (Sugimura *et al.*, 2017), proponiendo así nuevos marcadores de gestación para seleccionar el mejor embrión a transferir.

El consumo de oxígeno refleja la actividad mitocondrial, la cual es imprescindible para la fecundación y el desarrollo temprano del embrión. Las mitocondrias, además de generar la energía en forma de ATP, tienen otras funciones importantes como la homeostasis de calcio. El ATP se produce a través de la fosforilación oxidativa y la glicólisis, por lo que su producción puede estimarse a través de la medida del consumo de oxígeno y de glucosa, o de la producción de lactato. Durante los primeros días de desarrollo del embrión, el consumo de oxígeno se mantiene constante, pero se incrementa durante la compactación y continúa haciéndolo durante la formación y expansión del blastocele (Thompson *et al.*, 1996). El consumo de oxígeno muestra una correlación positiva con la valoración morfológica de la calidad y con el estadio de desarrollo del embrión (Lopes *et al.*, 2005; Lopes *et al.*, 2007; Sakagami *et al.*, 2007). También con el número total de células, de la ICM y del TE, y con la capacidad para eclosionar (Sugimura *et al.*, 2012). Así, los embriones con una mayor actividad respiratoria muestran mayores índices de gestación (Moriyasu *et al.*, 2007; Sakagami *et al.*, 2007). El análisis del consumo de oxígeno en el MC del embrión en combinación con parámetros analizados

con la tecnología *time-lapse* se ha sugerido como alternativa para tratar de mejorar la predicción de la gestación (Sugimura *et al.*, 2012).

También se ha propuesto el estrés oxidativo como biomarcador no invasivo de la calidad del embrión, ya que los altos niveles de ROS son indicativos de un peor desarrollo. Las ROS participan en importantes procesos biológicos, pero su exceso puede causar daños irreversibles en las células, incluyendo daños en las membranas y en el DNA (fragmentación), afectando negativamente al desarrollo del embrión. Las ROS también pueden producir alteraciones epigenéticas en el embrión con efectos transgeneracionales (Hardy *et al.*, 2021). No obstante, en embriones humanos hay estudios que encontraron una correlación entre altos niveles de ROS y una peor calidad del embrión (Bedaiwy *et al.*, 2004), y otros que no (Lan *et al.*, 2019).

Aunque se ha encontrado DNA en el MC que podría utilizarse para diagnóstico genético preimplantacional (PGD, del inglés *preimplantation genetic diagnosis*) o como marcador de implantación del embrión, de modo no invasivo (Stigliani *et al.*, 2014; Xu *et al.*, 2016), se sabe que durante el desarrollo embrionario temprano las células aneuploides son eliminadas mediante apoptosis. Por tanto, el DNA presente en el medio podría proceder de estas células apoptóticas, limitando el uso de esta estrategia para valorar la calidad del embrión (Ramos-Ibeas *et al.*, 2020). Además, el MC puede contener DNA que no proviene del embrión, sino de otros orígenes como las células del cúmulo o incluso puede estar presente en el propio medio comercial antes de ser expuesto al embrión, procedente probablemente de la fuente de suplementación proteica del medio. De esta manera se estaría alterando el resultado del PGD (Hammond *et al.*, 2017). El PGD no invasivo es un método potencial para el diagnóstico genético, aunque los estudios publicados adolecen de bajo número de muestras y heterogeneidad entre ellos, y faltan trabajos a gran escala, lo que hace su aplicación muy limitada (Huang *et al.*, 2023). Además, estos estudios analizan únicamente la euploidía de los embriones, pero no la capacidad de implantación del embrión.

El MC del embrión también contiene metabolitos que pueden relacionarse con el éxito de la gestación, como se ha visto en humano (Brison *et al.*, 2004; Seli *et al.*, 2008; Boyama *et al.*, 2016) y en bovino (Muñoz *et al.*, 2014a; Muñoz *et al.*, 2014b; de Oliveira Fernandes 2021). En particular, el balance entre el consumo y la producción de aminoácidos

(*turnover*) se ha propuesto como opción para predecir la viabilidad del embrión (Picton *et al.*, 2010; Sturmey *et al.*, 2010).

1.4.3. Selección de embriones según el sexo

Uno de los objetivos más perseguidos por los ganaderos es conseguir descendencia de un sexo predeterminado, especialmente en la industria láctea, donde las hembras tienen un mayor valor económico y se asocian con un menor riesgo de distocia (Norman *et al.*, 2010). La selección del sexo puede realizarse en tres momentos diferentes: antes de la concepción o fecundación, donde la selección se hace sobre los espermatozoides (selección primaria del sexo o pre-selección del sexo); tras la fecundación, que consiste en realizar una transferencia selectiva de un embrión del sexo deseado (selección secundaria del sexo); y durante la gestación, donde se decidiría si continuar o terminar la gestación en función del sexo del feto (selección terciaria del sexo) (de Wert & Dondorp, 2010).

La selección primaria del sexo es la más deseable puesto que se optimizan los ovocitos. Esta se consigue a través del uso de semen sexado, el cual presenta una precisión en la selección del sexo de más del 90%, por lo que es el método de elección en ganadería para asegurar el sexo deseado de la descendencia. No obstante, se han reportado menores índices de gestación con semen sexado en comparación con el semen convencional (Norman *et al.*, 2010; Healy *et al.*, 2013), y mayores tasas de mortalidad en los teneros (Siqueira *et al.*, 2017), aunque existe controversia en este punto (Healy *et al.*, 2013; Maicas *et al.*, 2020).

La técnica más habitual para determinar el sexo del embrión es la biopsia, en la que se realiza un PGD utilizando técnicas como la reacción en cadena de la polimerasa (PCR) (Park *et al.*, 2001). Entre los genes más utilizados para determinar el sexo se encuentra el gen de la amelogenina, que codifica para una proteína presente en el esmalte dental, y cuya secuencia génica del cromosoma X es diferente al cromosoma Y. La amplificación del gen de la amelogenina es una técnica de control integrada (*built-in control*) que utiliza un único par de cebadores o *primers* para amplificar la secuencia, con la ventaja de utilizar el cromosoma X como control interno. De esta manera, aunque la técnica falle, no genera diagnósticos erróneos. La presencia de una única banda de 270 pares de bases en el gel

de electroforesis se asocia a un embrión hembra, mientras que, si aparecen dos bandas, una de 270 pares de bases y otra de 214, el embrión se diagnostica como macho (Trigal *et al.*, 2012b). Sin embargo, dada la complejidad de la técnica y la posible alteración de la viabilidad del embrión causa por la biopsia, en la práctica son preferibles otras técnicas.

Las estrategias no invasivas de selección del sexo están basadas en el distinto metabolismo entre embriones machos y hembras, que permite identificar en el MC metabolitos secretados o consumidos relacionados con el sexo del embrión. Uno de ellos, quizás el primero que se identificó, fue la glucosa, cuyo consumo muestra dimorfismo sexual tanto en bovino (Tiffin *et al.*, 1991) como en humano (Gardner *et al.*, 2011; Nasiri *et al.*, 2019). También se propuso el análisis del consumo de oxígeno, pero no se encontró correlación alguna con el sexo del embrión (Agung *et al.*, 2005; Lopes *et al.*, 2005). En bovino, los embriones de diferente sexo muestran un consumo de aminoácidos diferente (Sturmey *et al.*, 2010; Rubessa *et al.*, 2018). Este consumo, además, cambia con el desarrollo del embrión, por lo que los metabolitos que pueden actuar como biomarcadores del sexo varían en función del estadio en el que se encuentre el embrión (Rubessa *et al.*, 2018).

1.4.4. Técnicas ómicas y selección de embriones

Las disciplinas conocidas genéricamente como ómicas, entre las que cabe citar la genómica, la transcriptómica, la proteómica, la metabolómica y la epigenómica, estudian de un modo amplio las bases moleculares de los procesos biológicos y sus interacciones (Figura 5). Tales estudios hacen posible comprender los procesos moleculares que tienen lugar en un sistema biológico, desde los genes hasta los metabolitos. Las ómicas han dado lugar a nuevas estrategias de identificación de marcadores, ya que permiten obtener un análisis global de la fisiología del embrión. De esta manera, con el perfil genético, transcriptómico, proteómico y metabolómico del embrión se busca caracterizar fenotipos, como pueden ser la viabilidad o el sexo del embrión.

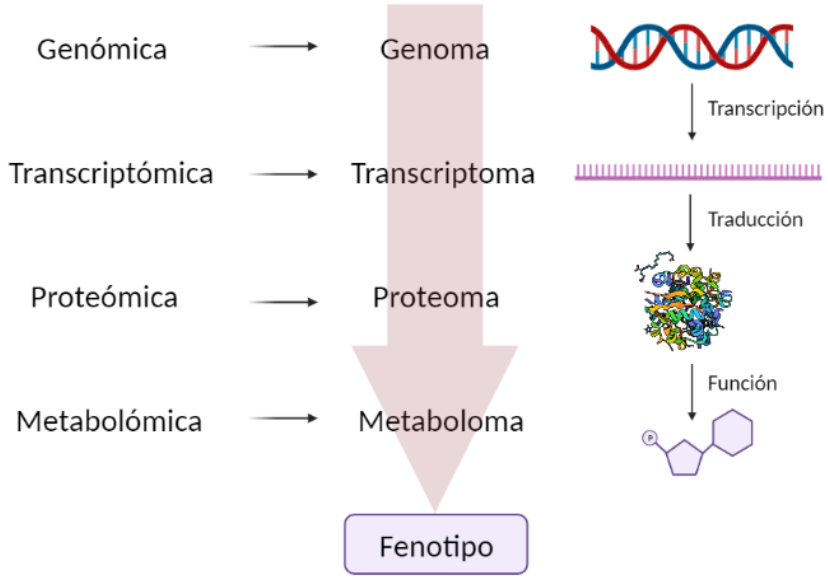

Figura 5. Ómicas comúnmente utilizadas en investigación para analizar de forma global el conjunto de moléculas presentes en una muestra biológica, desde el DNA, pasando por los transcritos de RNA y proteínas, hasta los metabolitos, que son el producto final de las reacciones bioquímicas.

Genómica y transcriptómica

En ganadería, la genómica ha permitido acelerar el progreso genético, seleccionar animales con características de interés, como son la mayor producción de leche o la resistencia a enfermedades, y reducir el intervalo intergeneracional (Sanches *et al.*, 2019). El análisis genómico también puede ser empleado para seleccionar embriones portadores de dichas características de interés y para evaluar la estabilidad cromosómica (Moore & Hasler, 2017), pero, hasta el momento, no se ha encontrado ninguna secuencia de DNA asociada a una mayor viabilidad (Hernández-Vargas *et al.*, 2020). La presencia de mosaicismo cromosómico, alteración en la que existen poblaciones celulares con distinto contenido cromosómico en los embriones, aumenta el riesgo de obtener falsos positivos y negativos en el PGD (Hernández-Vargas *et al.*, 2020). Además, los embriones son capaces de eliminar las células aneuploides a través de apoptosis o autofagia, o bien derivarlas al trofoectodermo, corrigiendo así las alteraciones cromosómicas (Ramos-Ibeas *et al.*, 2020). Según un metaanálisis, el 59.6% de los embriones bovinos diagnosticados como euploides son capaces de establecer la gestación, y el 46.7% resultan en nacimientos (Silvestri *et al.*, 2021). La incidencia de aneuploidías disminuye a lo largo del desarrollo del embrión, desde el 9.1% en embriones de día 7 hasta el 1.9% en

embriones de día 8 y 9 (Silvestri *et al.*, 2021). Por tanto, la genómica no es suficiente para determinar el potencial de implantación del embrión, el cual depende de la interacción entre el genotipo y el ambiente.

La transcriptómica, en cambio, ha asociado el perfil de expresión genético de los embriones con el establecimiento y mantenimiento de la gestación y el nacimiento de los terneros (El Sayed *et al.*, 2006; Salilew-Wondim *et al.*, 2010; Ghanem *et al.*, 2011; Zolini *et al.*, 2020). Algunos genes cuya expresión está sobrerregulada en embriones que dieron lugar a terneros nacidos vivos, como PLAC8, TXN, HSPD1, COX2 o BMP15, entre otros, podrían ser candidatos para determinar la viabilidad a término de los embriones, puesto que se encontraron tanto en biopsias de embriones IVD (Ghanem *et al.*, 2011) como en embriones IVP (El-Sayed *et al.*, 2006). No obstante, aunque son necesarios más estudios para validar el análisis transcriptómico en la selección de embriones, la principal limitación de la genómica y la transcriptómica es que requieren hacer una biopsia del embrión, con el consecuente riesgo de disminuir su calidad, creando falsos negativos.

Proteómica

Las proteínas producidas y secretadas por los embriones pueden ser analizadas en el MC. La ventaja de la proteómica frente a la transcriptómica es que la transcriptómica no considera los mecanismos de degradación de transcritos de mRNA antes de ser traducidos, por lo que la transcriptómica no siempre predice el proteoma. Sin embargo, la presencia de albúmina y otras proteínas abundantes del suero en el MC, como las globulinas, dificultan la detección de proteínas de baja expresión producidas por el embrión, lo que junto con la falta de sensibilidad de las plataformas proteómicas y la imposibilidad de analizar muestras de embriones individuales suponen la principal limitación de esta ómica (Katz-Jaffe & McReynolds, 2013; Rødgaard *et al.*, 2015). Aunque algunos estudios en embriones humanos han encontrado un perfil proteómico diferente entre grupos de embriones que implantaron y embriones que fallaron en la implantación (Katz-Jaffe *et al.*, 2006; Domínguez *et al.*, 2008), la diversidad de protocolos y medios de cultivo utilizados en la producción de embriones, y los distintos diseños experimentales hacen que hasta el momento no se haya obtenido ningún biomarcador proteómico.

Metabolómica

La metabolómica permite analizar el conjunto de moléculas de bajo peso molecular, por lo general metabolitos, de una muestra biológica. El metabolismo está regulado tanto por la genética y expresión génica, como por los estímulos ambientales a los que el embrión está sometido. En consecuencia, el metaboloma está más cerca del fenotipo que el genoma o el transcriptoma, tanto que a veces, como producto final de los procesos celulares, es el propio metabolito el que determina un fenotipo. La medición de los metabolitos, por tanto, refleja mejor el nivel funcional del embrión (Hernández-Vargas *et al.*, 2020).

El metabolismo del embrión experimenta cambios durante el desarrollo preimplantatorio, y como consecuencia varía la demanda y el consumo de nutrientes, los cuáles se reflejan en el MC (Gardner & Harvey, 2015; Obeidat *et al.*, 2019). Así, las variaciones en los metabolitos del MC pueden actuar como indicadores de la actividad metabólica durante el CIV (Uyar & Seli, 2014).

Existen diferentes técnicas de análisis metabolómico. La espectroscopía vibracional permite obtener perfiles metabólicos a partir del MC de embriones. En bovino, la espectroscopia infrarroja por transformada de Fourier (FTIR) se ha utilizado como método no invasivo para predecir la gestación (Muñoz *et al.*, 2014a; Muñoz *et al.*, 2014b) y el sexo del embrión (Muñoz *et al.*, 2014c). En humano, el perfil metabólico del MC del embrión también se ha correlacionado con la gestación mediante espectroscopía Raman y con espectroscopia de infrarrojo cercano (Seli *et al.*, 2007; Scott *et al.*, 2008). No obstante, las técnicas más utilizadas son la espectrometría de masas (EM), y la RMN por ser técnicas de alto rendimiento que permiten obtener información espectroscópica muy precisa sobre una amplia variedad de metabolitos. En los últimos años se ha incrementado la tendencia a utilizar la EM frente a la RMN. Mientras que la RMN identifica una media de 37 metabolitos por análisis, la EM alcanza los 197 (Miggiels *et al.*, 2019). Sin embargo, como no es posible analizar la totalidad de metabolitos con una única plataforma, no son infrecuentes los análisis combinados con varias técnicas para identificar un mayor número de metabolitos.

La RMN permite reproducir los resultados mejor que la EM, a la vez que requiere de una preparación mínima de las muestras, y los compuestos son fácilmente cuantificables. Por

el contrario, la sensibilidad de la RMN es menor que la EM, por lo que es necesario más cantidad de muestra de partida y mayor concentración de metabolitos para poder detectarlos (Emwas, 2015; Miggiels *et al.*, 2019). Mediante RMN se han relacionado concentraciones más altas de glutamato y una mayor ratio alanina/lactato en el MC de embriones humanos con mayores índices de gestación (Seli *et al.*, 2008). Determinadas regiones del espectro de RMN correspondientes a lípidos también distinguen entre embriones que consiguen gestar y embriones que no (Marhuenda-Egea *et al.*, 2011). En bovino, la RMN también permitió encontrar diferencias metabólicas entre embriones según su velocidad de desarrollo (Perkel & Madan, 2017). Así, los embriones de 4 y 16 células con mayor velocidad de desarrollo presentan un mayor consumo de leucina/isoleucina, mientras que los embriones con menor velocidad de desarrollo de 4 células consumen más piruvato, los de 8 células más histidina, y los embriones de 16 células menos acetato, triptófano y valina. De igual manera, la RMN reveló diferencias en el consumo de aminoácidos entre embriones de diferente sexo, el cual cambia con el desarrollo del embrión. Así, el consumo de la isoleucina y valina en cultivo se asocia al sexo del embrión durante las etapas más tempranas del desarrollo (hasta día 3 del desarrollo), y el consumo de piruvato en las etapas más tardías (día 3 hasta día 7) (Rubessa *et al.*, 2018). La RMN también identificó metabolitos en el MC de células de endometrio bovino que experimentaban cambios dependientes del sexo del embrión cocultivado (Muñoz *et al.*, 2020).

La EM separa los iones de una muestra en función de su relación masa/carga (*m/z*). La principal ventaja de esta técnica es su alta sensibilidad (permite detectar metabolitos a concentraciones muy bajas) y precisa unos pocos microlitros de muestra, lo que la hace especialmente apropiada para el MC de embriones. No obstante, la reproducibilidad de la EM es baja, ya que los resultados dependen del instrumento y del protocolo utilizado. Los equipos de EM están compuestos principalmente por cuatro elementos, un sistema de introducción o separación de la muestra [como la cromatografía de gases (GC), cromatografía líquida (LC) o electroforesis capilar], una fuente de ionización [como impacto electrónico, ionización por electrospray (ESI) o ionización/desorción de matriz (MALDI), entre otras], un analizador de masas [como el cuadrupolo, triple cuadrupolo (MS/MS), o tiempo de vuelo (TOF)], y un detector.

La cromatografía líquida de alta resolución acoplada a espectrometría de masas en tándem (UHPLC-MS/MS) es una técnica muy potente por combinar el poder de separación de la

LC con elevadas selectividad, sensibilidad y precisión en la determinación de la masa molecular (Emwas, 2015; Miggiels *et al.*, 2019). En embriones humanos se utilizó HPLC para analizar el *turnover* de aminoácidos en el MC y distinguir entre embriones que se desarrollaron hasta la fase de blastocisto y embriones que detuvieron su desarrollo (Houghton *et al.*, 2002). Se observó que los embriones que se desarrollaban correctamente presentaban en general un menor *turnover* de aminoácidos, y un mayor consumo de leucina. Dos años más tarde, el mismo equipo detectó la presencia de altos niveles de asparagina y bajos de glicina y leucina en el MC de embriones humanos con mayores índices de gestación (Brison *et al.*, 2004). El *turnover* de estos aminoácidos también difiere entre embriones humanos cromosómicamente normales y embriones con aneuploidías (Picton *et al.*, 2010). En bovino, se comparó el perfil de aminoácidos entre embriones IVP y embriones IVD (observando en general un mayor consumo de aminoácidos por parte de los embriones IVP) y entre embriones machos y hembras (Sturmey *et al.*, 2010). Sin embargo, con los avances en el desarrollo de la metabolómica, el enfoque ha cambiado, y ya no se analizan aminoácidos de manera individual, sino que se considera más valioso e informativo analizar el metabolismo global del embrión. De acuerdo con esta idea, un estudio más reciente observó una mayor intensidad de tres iones que no fueron identificados en las muestras de cultivo de embriones que dieron lugar a gestación, y una mayor concentración de piruvato y menor de lactato en el caso de blastocistos expandidos que gestaron (de Oliveira *et al.*, 2021).

El análisis metabolómico del MC de embriones puede ayudar a comprender los procesos metabólicos implicados en el desarrollo embrionario temprano y a desarrollar estrategias para mejorar la calidad y viabilidad del embrión. También se puede utilizar para identificar biomarcadores específicos de gestación en el MC, los cuáles puede mejorar la selección de embriones y aumentar el éxito de la transferencia. Respecto al sexo del embrión, un mejor conocimiento del metabolismo de embriones machos y hembras medido a través del consumo y aparición de metabolitos en el MC, puede conducir a identificar metabolitos capaces de actuar como marcadores del sexo del embrión. Además, la mejor comprensión de las necesidades metabólicas de los embriones machos y hembras permitiría diseñar medios de cultivo adaptados para embriones de cada sexo, optimizando así el desarrollo de los embriones producidos con semen sexado.

1.5. Gestación y nacimiento de terneros de embriones producidos *in vitro*

Además de evaluar la viabilidad *in vitro* de los embriones producidos mediante diferentes técnicas de reproducción asistida y los índices de gestación, es necesario estudiar el impacto de estas técnicas en la descendencia. La adaptación de los embriones a condiciones subóptimas durante la producción *in vitro* puede producir cambios en el fenotipo que persisten hasta la edad adulta. Este concepto se conoce como "*Developmental Origins of Health and Disease* (DOHaD)" (Fleming *et al.*, 2015), o traducido, orígenes de la salud y enfermedad en el desarrollo. Fue descrito por David Barker en la década de los ochenta a partir de estudios epidemiológicos en humano, y más tarde extendido a otras especies de mamíferos, incluido el bovino (Robles & Chavatte-Palmer, 2017; Duranthon & Chavatte-Palmer, 2018). Los embriones IVP presentan un desarrollo anormal de la placenta (Bertolini *et al.*, 2002), mayor dificultad de parto (Bonilla *et al.*, 2014), mayor incidencia de individuos muertos al nacimiento, y mayor frecuencia de mortalidad perinatal (30 primeros días de vida) (Numabe *et al.*, 2000b; Bonilla *et al.*, 2014; Siqueira *et al.*, 2017). La transferencia de embriones IVP da lugar a fetos de menor tamaño al comienzo de la gestación (concretamente, entre los días 37 y 58 de gestación) (Bertolini *et al.*, 2002; Vailes *et al.*, 2019). Sin embargo, los terneros procedentes de embriones IVP, en particular los cultivados con suero, presentan mayor peso al nacimiento que los nacidos por IA y por transferencia de embriones IVD (Jacobsen *et al.*, 2000; Numabe *et al.*, 2000b; Bonilla *et al.*, 2014; Siqueira *et al.*, 2017).

Una de las alteraciones más comunes inducidas por la transferencia de embriones IVP es el LOS/AOS. Este síndrome no siempre se caracteriza por un crecimiento excesivo y extremidades más largas, sino que incluye otros fenotipos como malformaciones musculares y esqueléticas, organomegalia, mayor incidencia de abortos, de tasas de muerte fetal y neonatal, y de hernias umbilicales, además de vascularización placentaria anormal (Rivera *et al.*, 2021; Li *et al.*, 2022). La etiología de estos síntomas se atribuye a alteraciones epigenéticas y transcriptómicas que se remontan al embrión (Rivera *et al.*, 2021). Así, el transcriptoma del trofoblasto de embriones de 14 días de desarrollo es distinto entre embriones IVP y embriones IVD, mostrando 20 genes expresados exclusivamente en los embriones IVD, 27 genes exclusivos de los embriones IVP, y un total de 29 genes diferencialmente expresados entre ambos tipos de embrión (Leme *et al.*, 2021). Aun cuando los terneros producidos a partir de embriones IVP se muestren fenotípicamente normales, pueden aparecer cambios en la metilación y expresión de

determinados genes que no se dan en terneros obtenidos con la técnica MOET (Rabaglino *et al.*, 2021).

Generalmente, los estudios sobre la descendencia obtenida por técnicas de reproducción asistida se han centrado en analizar la morfometría de los terneros nacidos y su supervivencia. Sin embargo, tan sólo unos pocos estudios han mostrado el perfil bioquímico, hematológico y hormonal de terneros nacidos a partir de embriones IVP, en el marco de la transferencia somática nuclear y la producción *in vitro* (Sangild *et al.*, 2000; Chavatte-Palmer *et al.*, 2002; Rérat *et al.*, 2005).

La criopreservación de embriones se utiliza comúnmente en programas de reproducción asistida en animales para facilitar la gestión y la distribución de animales de alto valor genético. La congelación y la vitrificación alteran la fisiología de los embriones (de Oliveira Leme *et al.*, 2016; Hayashi *et al.*, 2019; Estudillo *et al.*, 2021; Gutierrez-Castillo *et al.*, 2021; Fryc *et al.*, 2023), pudiendo afectar a la calidad y viabilidad de los mismos. Estas alteraciones podrían tener consecuencias negativas en la salud y el rendimiento de la descendencia. Por lo tanto, es importante comprender los efectos de la criopreservación en la descendencia para garantizar la seguridad y efectividad de los programas de reproducción asistida. En conejo, la descendencia obtenida tras la transferencia de embriones vitrificados, aunque presenta mayor peso al nacimiento, exhibe un patrón de crecimiento más lento que sus homólogos nacidos por monta natural (García-Dominguez *et al.*, 2020a). En la edad adulta, dicha descendencia presenta menor peso corporal y de órganos como el hígado y el corazón, mayor concentración de albúmina y disminución del colesterol, aunque con valores dentro de los rangos considerados normales. Los individuos adultos procedentes de embriones vitrificados también muestran disminución de la expresión de proteínas en el hígado con respecto a los animales de monta natural; algunas de estas proteínas participan en la fosforilación oxidativa y en el metabolismo del zinc y de los lípidos (García-Dominguez *et al.*, 2020a).

En bovino es difícil realizar un seguimiento a largo plazo de los animales, puesto que a menudo se venden a edades muy tempranas, se usan durante un tiempo muy limitado, y se sacrifican a los pocos años (Duranthon & Chavatte-Palmer, 2018). Quizás de ahí que, en bovino, los efectos de la criopreservación de embriones en la descendencia se circunscriban al estudio de unos pocos parámetros fenotípicos (Bonilla *et al.*, 2014). Para estos autores, el peso al nacimiento, la edad y el porcentaje de preñez al primer servicio,

y la producción de leche no varían entre hembras procedentes de IA y de transferencia de embriones IVP en fresco y vitrificados. En cambio, en conejo, especie con un tiempo de generación más corto, los efectos de la vitrificación de embriones mostraron un impacto transgeneracional, incluyendo reducción en la velocidad de crecimiento y en el peso corporal en el adulto hasta la tercera generación (García-Dominguez *et al.*, 2020b). El transcriptoma del hígado mostró 642 transcritos diferencialmente expresados en la primera generación, de los cuales 133 se mantuvieron en la segunda generación y 120 en la tercera generación, y el metaboloma reveló 151, 190 y 159 metabolitos diferencialmente acumulados en la primera, segunda y tercera generación, respectivamente. Las rutas metabólicas implicadas comprendieron el metabolismo de ácidos grasos, entre otras. Por tanto, la criopreservación, aunque genera individuos aparentemente normales y sanos, puede producir alteraciones fenotípicas y moleculares en la descendencia directa que pueden tener un efecto transgeneracional.

2| OBJETIVOS

2. OBJETIVOS

El objetivo general de la tesis es identificar metabolitos biomarcadores en el medio de cultivo de embriones producidos *in vitro* para diagnosticar el sexo y predecir la viabilidad del embrión de manera no invasiva. Con este propósito, los objetivos específicos del presente trabajo fueron los siguientes:

1- Examinar el metaboloma de embriones machos y hembras reflejado en el medio de cultivo mediante UHPLC-MS/MS para identificar biomarcadores del sexo. A tal fin, se analizaron muestras de medio de cultivo de orígenes muy variables para realizar una aproximación más real a los laboratorios de producción *in vitro* de embriones, incluyendo dos razas de toro, dos condiciones de cultivo, y distintas transiciones embrionarias (**Capítulo I**).

2- Identificar metabolitos biomarcadores para predecir el éxito de la gestación en tres momentos diferentes (día 40, día 62, y gestación a término), mediante el análisis por UHPLC-MS/MS de muestras de medio de cultivo de embriones frescos y congelados transferidos a receptoras. En este estudio también se incluyeron muestras de embriones producidos con dos razas de toro, dos medios de cultivo diferentes, y diferentes transiciones embrionarias (**Capítulo II**).

3- Buscar metabolitos biomarcadores de gestación en el plasma de receptoras de embriones frescos y congelados en día 0 (estro) y día 7 (horas antes de realizar la transferencia), mediante $1H^{+}$NMR, y mejorar la capacidad predictiva de la información contenida en el metaboloma de la receptora a partir de una estimación precisa de la viabilidad del embrión (**Capítulo III**).

4- Analizar los efectos de la criopreservación de embriones en la descendencia durante el primer mes de vida, a través de un examen clínico y hemático en los terneros (**Capítulo IV**).

3| CAPÍTULO I

NON-INVASIVE IDENTIFICATION OF SEX IN CULTURED BOVINE EMBRYOS BY UHPLC-MS/MS METABOLOMICS

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ORIGINAL ARTICLE

Non-Invasive Identification of Sex in Cultured Bovine Embryos by UHPLC-MS/MS Metabolomics

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Abstract

Introduction Different gene expression between male and female bovine embryos leads to metabolic differences.
Objective We used UHPLC-MS/MS to identify sex metabolite biomarkers in embryo culture medium (CM).
Methods Embryos were produced in vitro under highly variable conditions, i.e., fertilized with 7 bulls, two breeds, and cultured with BSA or BSA + serum until Day-6. On Day-6, embryos were cultured individually for 24 h. CM of Day-7 embryos (86 female and 81 male) was collected, and Day-6 and Day-7 embryonic stages recorded.
Results A study by sample subsets with fixed factors (culture, bull breed, and Day-6 and Day-7 stages) tentatively identified 31 differentially accumulated metabolites through 182 subsets. Day-6 and Day-7 stage together affected 13 and 11 metabolites respectively, while 19 metabolites were affected by one or another stage and/or day. Culture supplements and individual bull changed 19 and 15 metabolites, respectively. Single bull exerted the highest influence (20 metabolites with the significantly highest p values). Lipid (93 subsets; 11 metabolites) and amino acid (55 subsets; 13 metabolites) were the most relevant classes for sex identification.
Conclusions Single biomarker led to inefficient sex diagnosis, while metabolite combinations accurately identified sex. Our study is a first in non-invasive sex identification in cattle by overcoming factors that induce metabolic variation.

Keywords Metabolomics · Bovine · Embryo · Mass-spectrometry · Liquid-chromatography · Sex

1 Introduction

Within farm animals, sex selection is a major inseparable trait for dairy (females) and is preferred for beef production (where males have a higher price than females) (Umehara et al., 2020). Sexing can be done using different technologies (Xie et al., 2020), although sex-sorted semen is indispensable in cattle (89% births of the selected sex in dairy (DeJarnette et al., 2009)). In vitro embryo production followed by embryo transfer (ET), which steadily yield greater numbers of calves worldwide (Viana, 2020), can also be performed with sex-sorted semen. However, one of the limitations of sex-sorted sperm is the reduced fertility rates both in vivo (when follicle-stimulated donors are inseminated with sex-sorted semen) (Monteiro et al., 2016), and in vitro (Palma et al., 2008) in comparison with conventional semen.

Once the primary sex ratio of the embryo is decided after in vitro fertilization (IVF), metabolic differences appear between cultured male and female embryos (Gómez et al., 2018a, b; Rubessa et al., 2018). Sex differences between embryos are quite well known, but not yet exploited to: (1) efficiently diagnose sex; (2) to identify metabolic and nutritional requirements for embryos of each sex; (3) to develop culture systems tailored for embryos of each sex, and (4) to study endometrial receptivity towards a particular embryonic sex (Gómez et al., 2013; Gómez, Sánchez-Calabuig, Gómez et al., 2018a, b; Muñoz et al., 2020). Embryo

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culture medium (CM) can be interrogated to achieve such objectives, as it reflects embryonic metabolism. Hence, non-invasive embryonic sex diagnosis is possible using metabolomics in CM. However, embryo CM metabolomics is limited by drawbacks both technical –due to the sample traits- and intrinsic –due to embryonic phenotypes and/or culture conditions.

The technical limitations in embryo CM metabolomics are low sample volume and low abundance of metabolites; the time of the exposure to the embryo to CM, so as to provide sufficient metabolite information and turnover; and the need to minimize analytical and operational shifts (Segers et al., 2019). Such deviations could be induced by sample processing and extraction, a required step for metabolomics when macromolecular concentration is above a threshold, as occurs with serum and BSA concentrations frequently used in embryo CM. BSA -or serum- bind unknown compounds such as cytokines, growth factors, steroids, amino-acids, and a variety of contaminants (Francis, 2010) such as citrate, which is present in modified-synthetic oviduct fluid (mSOF) (Holm et al., 1999), probably the most widely used embryo CM in cattle. Together with the intrinsic variability of extractive techniques (Engskog et al., 2016), extraction sweeps along small molecules (Balcerczyk et al., 2020; Liu et al., 2017) that could lead to altered analytical results and/or loss of biomarkers. To overcome these limitations, we have used a single culture step without protein that allows for direct chromatographic analysis without protein extraction. Embryos produced with such a system yield reliable pregnancy rates and calves born from fresh, vitrified and frozen transferred embryos (Gomez et al., 2021; Gómez et al., 2020; Murillo-Ríos et al., 2017).

Intrinsic phenotype variability in embryos includes metabolic changes due to developmental progress, embryonic breed and culture systems. The morula to blastocyst is recognized as the most metabolically dynamic period within the early development in vitro (Guerif et al., 2013; Obeidat et al., 2019). During blastulation, the embryo metabolism evolves by changing glycolytic flux and mitochondrial respiration (de Lima et al., 2020; Smith, 2017) because of strict epigenetic control (Ehnes et al., 2020). Regarding breed, dairy, beef and crossbred cattle markedly differ in metabolism (Gamarra et al., 2018; Gómez et al., 2020d; Huang et al., 2017), and differences in gene expression and amino-acid depletion and/or appearance (specifically in serine, asparagine, methionine and tryptophan) between purebred and crossbred embryos have also been reported (Lazzari et al., 2011). Culture systems are also real sources of metabolic variation within embryos, such as oxygen tension or culture composition (e.g., glucose concentration or culture with fetal calf serum (FCS) and BSA), which can in turn induce changes in carbohydrate, lipid and amino-acid metabolism in embryos (de Lima et al., 2020; Murillo et al., 2017). Thus, we identified accumulated metabolites in pregnancy-prone embryos (i.e., embryos transferred and diagnosed as pregnant on Day-40, Day-62 and/or birth) that are breed-specific and CM-specific (Gimeno et al., 2021), which suggest that those same factors may impact on the metabolomic profile of each embryo sex differently.

However, although testing the great assortment of laboratory culture conditions is unfeasible and impractical, the variability induced by the fixed factors bull breed, embryonic stage and culture system is controllable in the laboratory. In contrast, random variability due to individual bulls and oocytes from different donors needs to be approached by testing enough individuals to reach experimental randomness. Moreover, the use of a single 24 h, Day-6 to Day-7 common culture step permits direct comparisons between samples produced with different previous culture conditions (e.g., culture with or without serum supplementation until Day-6) and laboratories, and overcomes the matrix effect.

In previous studies, we used Fourier Transform Infrared Spectroscopy (FTIR) and Ultra-High Performance Liquid Chromatography (UHPLC) coupled with Mass Spectrometry (MS) to non-invasively diagnose embryonic sex in embryo CM (Gómez et al., 2016; Muñoz et al., 2014). Such metabolomic techniques have advantages and disadvantages (Segers et al., 2019), however, UHPLC works well with the low sample volumes used for single embryo culture. Furthermore, UHPLC shows a high sensitivity, and provides a wider range of potential metabolites to be discovered, making it more appropriate for untargeted studies with not frequently studied matrixes, such as CM.

The objective of this study was to examine the embryonic metabolome of male and female embryos in CM by UHPLC-MS/MS through specific developmental transitions, culture conditions, and bull breeds. We notably increased the number of samples analyzed over previous studies and their variability, in this way approached real scenarios from in vitro embryo production laboratories. We hypothesized that efficient sex identification needs to rely on discriminating factors responsible for metabolic shifts for each metabolite. At the same time, we hypothesized that the embryonic metabolism is better defined between embryonic stages than as single stage snapshots. Therefore, breaking down culture conditions, bull breeds and development between two defined embryonic stages can contribute to a better identification of metabolites, and non-invasive identification of embryonic sex. To our knowledge, the coverage of this metabolomic study is unprecedented in cattle.

2 Materials and methods

Experimental procedures were approved by the Animal Research Ethics Committee of SERIDA (PROAE 26-2016; Resolución de 25 de Julio de 2016 de la Consejería de Medio Rural y Recursos Naturales), in accordance with European Community Directive 86/609/EC. Unless stated otherwise, all chemicals and reagents were purchased from SIGMA (Madrid, Spain). Experimental procedures are graphically described in Fig. 1.

2.1 In vitro embryo production

Embryos were in vitro produced (IVP) by previously described procedures (Gómez et al., 2020a), with minor modifications. Briefly, antral follicles (3–8 mm diameter) from slaughtered cow ovaries (Matadero de Guarnizo, Spain; Matadero Municipal de Leon, Spain) were obtained by aspiration, and good-quality oocytes (>3 cumulus cell layers and homogenous cytoplasm) were transferred to holding medium (HM) TCM199 (Invitrogen, Barcelona, Spain), 25 mM HEPES and 0.4 mg/mL BSA. For in vitro maturation (IVM), cumulus-oocyte complexes (COCs) were washed three times in maturation medium (MM) consisting of TCM199 $NaHCO_3$ (2.2 mg/mL) supplemented with 10% FCS (v/v) (F4135), 1.5 µg/mL of porcine FSH-LH (Stimufol; ULg FMV, Liège, Belgium) and 1 µg/mL 17 β-estradiol. COCs (N = 30–50) were matured in a 4-well dish with 500 µL of MM for 22–24 h at 38.7 °C, 5% CO_2 and saturated humidity.

For in vitro fertilization (IVF; Day 0), motile sperm were obtained by a swim-up procedure, using frozen sperm from Asturiana de los Valles (AV; N = 4) and Holstein (N = 3) bulls with proven fertility. Briefly, sperm were incubated for 1 h with pre-equilibrated Sperm-TALP (Tyrode's albumin lactate pyruvate). Then the upper layer with motile sperm was centrifuged 7 min at 200 x g, and the resultant supernatant was removed. COCs were washed twice in HM and placed in 4-well dishes with 500 µL of pre-equilibrated fertilization medium (Fert-TALP) with heparin (10 µg/mL; Calbiochem, La Jolla, CA, USA). COCs and sperm cells (2×10^6 cells/mL) were co-incubated for 18–20 h at 38.7 °C in a 5% CO_2 humidified atmosphere.

Thereafter, presumptive zygotes were denuded by vortexing and cultured in modified synthetic oviduct fluid (mSOF) containing 45 µL/mL BME amino acids solution (B6766), 5 µL/mL MEM non-essential amino acids solution (M7145), citrate (0.1 µg/mL), myo-inositol (0.5 µg/mL), and BSA (A3311) (6 mg/mL), with or without 0.1% (v/v) FCS (SIGMA F4135), under mineral oil, as previously described (Murillo-Ríos et al., 2017). In vitro culture (IVC) was performed in groups (N = 35–50) at 38.7 °C, 5% CO_2, 5% O_2, 90% N_2, and saturated humidity until Day-6. On Day-6 (143 h post-insemination -PI-), good quality morulae (M), early blastocyst (EB) and blastocyst (B) were selected and cultured individually in 12 µL mSOF with 0.5 mg/mL polyvinyl-alcohol PVA (P8136) under mineral oil for 24 h. This CM without protein allows a direct chromatographic analysis without sample pre-processing (i.e., extraction), and leads to high pregnancy and birth rates with vitrified/warmed (V/W), frozen/thawed (F/T) and fresh embryos (Gómez et al., 2020a; Murillo-Ríos et al., 2017). On Day-7 (168 h PI) blastocyst development was assessed and sex was identified in expanding (ExB) and fully expanded blastocysts (FEB), the preferred stage for cryopreservation and commercial exchanges. At the time of embryo collection, 10 µL of spent CM of each embryo and blank samples (i.e.,

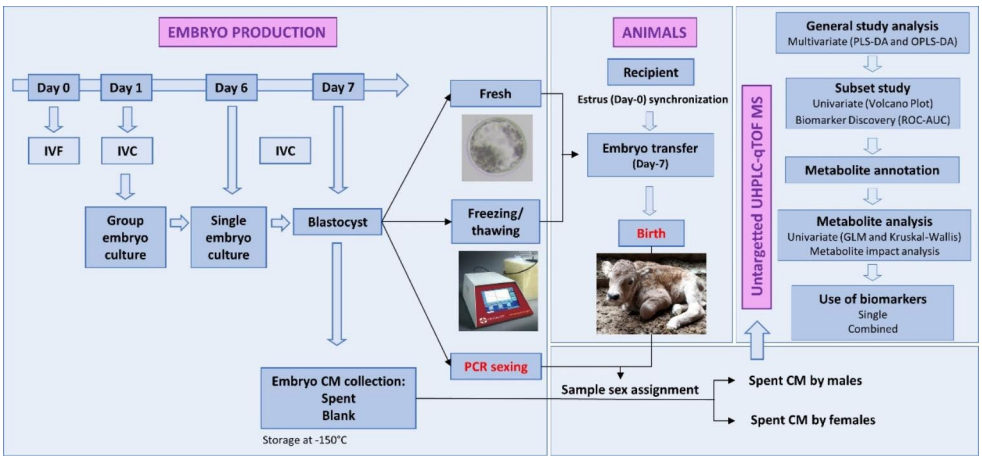

Fig. 1 Experimental workflow. Embryos were in vitro produced from slaughtered cow ovaries and cultured in group until Day-6. On Day-6, good quality embryos were selected for a 24 h single culture. On Day-7, blastocysts were transferred to recipient (fresh or after freezing/thawing) or sexed by PCR. Spent culture medium (CM) of embryos was collected and stored until metabolomic analysis by UHPLC-MS/MS. At birth, calf sex was checked and assigned to their corresponding CM sample. IVF: In vitro fertilization; IVC: In vitro culture

CM incubated without embryo) were also collected and stored at -150 °C until metabolomics analysis.

2.2 Analysis of embryonic sex

Sexing was performed in ExB and FEB by a built-in control PCR technique based on amelogenin gene amplification (Trigal et al., 2012) or by checking calf sex at birth after embryo transfer (ET) with recipients of embryos used in another study. Briefly, the PCR reaction was performed in a total volume of 20 µL containing 6 µL of heated water, 2x QIAGEN Multiplex master mix (containing HotStar Taq DNA Polymerase, Multiplex PCR Buffer, dNTP mix), Q-solution 5x, and 3.0 µM amelogenin forward (5′-CAGCCAAACCTCCCTCTGC) reverse (5′-CCCGCTTGGTCTTGTCTGTTGC). PCR rounds with each set of primers were performed in a Bio-Rad thermocycler with one cycle of 95° C for 15 min, followed by 35 cycles of 94° C for 20 s, primer annealing temperatures of 60 °C for 40 s and 72 °C for 20 s, and a final extension step of 72 °C for 10 min. Amplification products were analyzed on a SYBR® Safe stained 2% agarose gel. The gel was visualized under ultraviolet illumination. Single fragments of 270 bp were assigned as female, while two fragments of 270 bp and 214 bp were assigned as male.

2.3 Untargeted metabolomic analysis

All samples were diluted 1:3 (v/v) in ultrapure water after being thawed on ice, and directly analyzed in duplicate. Chromatographic separation (DionexTM UltiMate 3000 UHPLC, Thermo Fisher ScientificTM, Waltham, MA, USA) was achieved using a C18 column (2.1 × 100 mm, 1.8 µm, ACQUITY UPLC® HSS T3, Waters Corp., Milford, MA, USA) in reverse phase (RP) at 30 °C and 250 µl/min total flow rate. Phase A consisted of ultrapure water and phase B of acetonitrile, both with 0.1% formic acid (v/v). The gradient elution profile, previously validated (Gómez et al., 2016), was as follows: 0 min (0% B), 2 min (0% B), 5 min (70% B), 8 min (100% B), 13 min (100% B). The column was equilibrated for 6 min prior to each analysis.

The MS acquisition (Impact-II with conventional ESI ion source, Bruker Daltonics, Billerica, USA) was performed in the positive ionization mode in a scan range from m/z 100 to 1500 and 12 Hz spectra rate. Settings were as follows: nebulizer gas pressure, 2.1 Bar; gas temperature, 300 °C; capillary voltage, 4500 V; drying gas flow rate, 10 l/min. MS^2, obtained from CID fragmentation of the top three parent ions of each scan with collision energies ranging from 4 to 20 eV, was used for metabolite identification.

External calibration of the Q-TOF MS using a commercial mixture (ESI Low concentration Tune Mix, Agilent Technologies, Santa Clara, CAL, USA) and internal mass calibration with a solution of Na formate clusters (m/z range from 91 to 1383) were carried out as a quality control. Prior to each injection, the calibrant was infused at 180 µl/min with a syringe pump during column stabilization to re-calibrate each chromatogram and ensure constant mass accuracy during the whole analytical sequence.

Furthermore, for continuous quality monitoring, a 10ppb solution of triphenyl phosphate (Sigma Aldrich, St. Luis, MIS, USA) was injected each five samples to check detector sensibility, mass accuracy and chromatographic performance. A mix of all sequenced samples was also injected at the beginning and end of the sequence to obtain successful and stable chromatographic resolution of samples. All acquired data were exported by DataAnalysis v.4.2 (Bruker Daltonics, Billerica, USA).

2.4 Data processing and analysis

Raw data were preprocessed using the software MZmine v2.53 (Pluskal et al., 2010), applying peak detection, deconvolution, smoothing and alignment. All the parameters used for these processes are summarized in Suppl. Table 1. Peak areas were processed using the R package pRocessomics (https://github.com/Valledor/pRocessomics). Missing value imputation was performed with Random Forests (RF) algorithm and a threshold value of 0.25. Data were filtered using a consistency criterion (0.8 threshold) and abundance balancing using the average of the total intensities of all samples (normalization step). Thereafter, each sample concentration signal was subtracted with their corresponding blank. The resulting output data, with their corresponding retention time, m/z and peak area, were submitted to statistical analysis.

2.5 Experimental design

To measure metabolic variability that could obscure a correct embryonic sex definition, we designed a study with fixed, controllable factors: three Day-6 stages (i.e., M, EB and B); two Day-7 stages (i.e., ExB and FEB); two bull breeds, with four Asturiana de los Valles (AV; beef) and three Holstein (dairy) bulls; and two culture conditions prior to Day-6 (BSA and BSA + FCS). We focused on Day-7 ExB and FEB because of their highest interest due to superior pregnancy rates as fresh, V/W and F/T (Block et al., 2009; Murillo-Ríos et al., 2017).

Experimental procedures consisted of analytical sample formation as a first analytical step. We produced and used N = 167 CM samples ex professo in this study for Metabolomics (Table 1; and detailed description in Suppl. Table 2), of which N = 127 (N = 16 replicates) were from

amelogenin-sexed embryos and N = 40 corresponded to embryos transferred with their sex identified in calves at birth (ETs in N = 24 different rounds -days-). Importantly, all samples, both from sexed and transferred embryos, were analyzed in a same, single process.

However, to more accurately calculate sex ratio and embryo transition distribution, we used larger sets of metadata. Thus, Day-7 blastocyst sex ratios were calculated from samples described in Suppl. Table 3 (excluding calves) and further metadata of available sexed samples (Total: N = 249 in 21 replicates; Suppl. Table 3). Major effects affecting sex ratios were also analyzed (Suppl. Table 4). Subsequently, we calculated the transition distribution over all data available in our laboratory under the same culture conditions (i.e., N = 1,114 Day-6 to Day-7 embryos, mostly not sexed embryos; Suppl. Table 5). Such a distribution is necessary to measure the absolute impact of each sex-biomarker within each embryo culture condition and transition, and it does not refer to embryo development rates. Readers interested in development rates of the here-used culture systems, together with cell distribution, lipid contents, and apoptotic and pregnancy rates of embryos produced, are encouraged to consult (Gómez et al., 2017, 2020a; Murillo et al., 2017). Significantly, the same seven bulls were used in all sample sets. For each level of fixed effects with significant differences, least square means and their errors (± SEM) were estimated.

2.6 Statistical analysis

Major fixed metabolic effects were analyzed first in the entire feature dataset (N = 8,031 metabolite signals).

2.6.1 Multivariate Study

We used the software Metaboanalyst 4.0 (Chong et al., 2018). Partial Least Square-Discriminant Analysis (PLS-DA) and Orthogonal Partial Least Square-Discriminant Analysis (OPLS-DA), a supervised method that uses class information to maximize the separation between groups, were used. The accuracy of the discrimination was parametrized with R2 and Q2 indexes to test possible overfitting. Major effects examined were sex, culture, bull breed, Day-6 stage and Day-7 stage. Results of the multivariate study suggested dividing the entire data set into subsets.

2.6.2 Subset study

Subset Univariate Study.

We designed such a study to diagnose embryonic sex phenotypes by subsets defined by combinations of fixed factors that can be supervised in the laboratory, i.e., culture from Day-0 to Day-6 (BSA vs. BSA + FCS), bull breed (Holstein vs. AV), Day-6 stage, Day-7 stage and/or embryonic transition from Day-6 to Day-7. Embryonic sex was analyzed within subsets, which were randomized for replicate and bull. On the basis that embryo culture usually yields non-uniform, but measurable, developmental distribution, we hypothesized that a metabolomics study combined with the supervision of such factors would improve sex diagnosis. All subsets were analyzed with Metaboanalyst 4.0 (Chong et al., 2018) with the tool Volcano Plot defined by: (1) ANOVA and Kruskal-Wallis tests $P < 0.05$ (tendencies < 0.10); (2) Concentration fold-change > 2.

Biomarker Discovery within subsets.

Biomarkers of embryonic sex were sought in all subsets by univariate Receiver Operator Characteristic – Area under curve (ROC-AUC) (Metaboanalyst 4.0). Concentration fold-changes > 2, ROC-AUC > 0.70 and $P < 0.05$ ($P < 0.10$ tendencies) (T-test) allowed discovery of candidate biomarkers.

Metabolite Annotation.

Metabolite features fulfilling conditions that differed between male and female embryos as required in 2.6.1 and 2.6.2 were first tentatively assigned by comparing precursor masses to public databases (< 10 ppm of difference between measured and exact compound mass). Then, MS^2 spectra were matched against public databases (Human Metabolome Database (HMDB), MassBank, GNPS and NIST 14), using 5 ppm threshold, with MZmine and Sirius v4.4.29 (Dührkop et al., 2019). For increasing confidence in metabolite identity allocation, different isCID energies were applied to each parent mass and resulting MS^2 spectra were employed for database comparisons. Annotations were only considered when at least the precursor mass and three MS^2 ions were coincident. Suppl. Table 6 shows detailed annotated peak information. When available, commercial standards were analyzed in the same conditions to confirm the tentatively identified metabolites by comparison of retention time and fragmentation pattern (Suppl. Figure 1 and Suppl. Table 6).

2.6.3 General Univariate Study

We analyzed the integration of annotated metabolites and selected from subsets within a general model (Proc GLM module of SAS/STAT (version 9.2; SAS Institute Inc., Cary, NC)) for normally distributed metabolites, and Kruskal-Wallis test for non-normally distributed metabolites. Significant effects concerning each metabolite were determined by $P < 0.05$ and tendencies $P < 0.10$ were recorded. Bonferroni correction post-hoc test was included as a false discovery rate ($P < 0.10$). The major effects examined were sex, culture, bull breed, Day-6 stage and Day-7 stage.

Table 1
Samples (N = 167) of culture medium (CM) used in the metabolomics study of sex by UHPLC-MS/MS corresponding to each Day-6 (morula; EB: early blastocyst; B: blastocyst) and Day-7 (ExB: expanding blastocyst; FEB: fully expanded blastocyst) embryonic transitions after Day-0 to Day-6 embryo culture in synthetic oviduct fluid (SOF) containing BSA or BSA + FCS, and individual culture from Day-6 to Day-7 in SOF without protein supplements

		BSA			BSA + FCS		
Day-6	Day-7	Male	Female	Calves	Male	Female	Calves
B	ExB	0	1	1 F	7	7	1 F
B	FEB	8	11	3 M, 5 F	8	9	2 M, 3 F
EB	ExB	5	5	2 M	8	8	3 M
EB	FEB	7	9	2 M, 3 F	8	8	1 M, 1 F
Morula	ExB	8	8	1 M, 2 F	8	7	2 M, 1 F
Morula	FEB	6	8	4 M, 2 F	8	5	1 M
	Total	34	42	12 M, 13 F	47	44	9 M, 6 F

Data from N = 16 replicates / embryos transferred in N = 24 rounds (days)
F (female) and M (male) describe the numbers of samples of which sex was diagnosed in calves born from embryos produced in each condition analyzed

2.6.4 Impact of metabolite biomarkers

The impact of most relevant biomarkers was evaluated as follows:

1) By ROC-AUC within subsets: defining the efficiency of a metabolite to identify embryonic sex under specific breed, culture, Day-6 and Day-7 stages.

2) By Day-7 transition coverage: ROC-AUC weighed with the relative abundance of embryonic transitions (i.e., percentage of each embryonic stage in culture) and culture medium (BSA and BSA + FCS, as the distribution of transitions within culture is different) for each subset. The weighed ROC-AUCs reflected the proportion of embryos effectively sexed, as some high ROC-AUCs have low impact due to low-represented embryonic transitions.

3) By the number of subsets in which a metabolite is represented and the number of correctly sexed embryos within such subsets. Thus, the presence of a metabolite in multiple subsets was considered proof of cross-validation and increases the value of the metabolite.

The capacity of metabolites to diagnose sex was analyzed in two ways:

- Single metabolite biomarkers: the ability of a unique metabolite to identify embryonic sex within all subsets in which it is represented (with ROC-AUC > 0.700) for each condition defined by culture and breed.
- Combined biomarkers: the minimal numbers of metabolite combinations to reach the highest identification within subsets defined by culture conditions, breed and Day-6 and Day-7 stages.

3 Results

Metabolic profiles typically obtained by UHPLC- MS/MS are shown in Fig. 2.

A total of 118,564 aligned spectral features were obtained, and after peak area processing, 8,031 features were retained. The corresponding blanks were thereafter subtracted from sample signals. The resulting output data were submitted for statistical analysis.

3.1 Sex ratio analysis

The blastocyst sex ratio was only affected by culture conditions from Day-0 to Day-6, leading to a significant overall skew towards males within embryos cultured in BSA + FCS over those cultured in BSA alone (Suppl. Table 3) (BSA: 0.532 ± 0.079, FCS: 0.726 ± 0.076; P = 0.019). Although not significant, the numerically largest skew in sex ratio

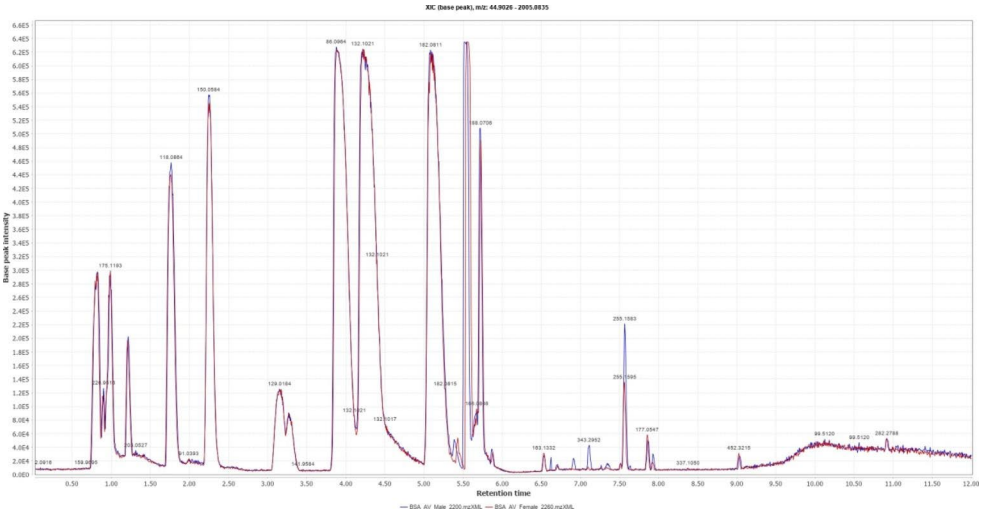

Fig. 2 Chromatogram obtained after UHPLC-MS/MS analysis of two culture medium (CM) samples spent by an embryo, in positive mode. The total ion count (TIC) chromatograms obtained from the analysis of CM spent by a female (red) and male (blue) embryo. LC-MS was performed on a quadruple time-of-flight MS/MS system combined with a UHPLC system

was recorded in the transition morula-FEB derived from BSA + FCS culture (0.782 ± 0.103) and the lowest in all groups of Day-7 ExB previously cultured with BSA (from Day-6, B: 0.500 ± 0.246; EB: 0.444 ± 0.164; Morula: 0.476 ± 0.107). The major effects Day-6 embryonic stage and Day-7 embryonic stage, the individual bull and bull breed did not affect the sex ratio (Suppl. Table 4). The frequencies of appearance of each embryo transition from Day-6 to Day-7 are shown as percentages in Suppl. Table 5. As explained, this table does not represent developmental rates Day-7/Day-6, but it does calculate the impact of each biomarker (i.e., percentage of ExB and FEB correctly sexed by each metabolite biomarker on Day-7). Approximately 57% of Day-7 blastocysts in BSA arose from Day-6 morulae, while 53% embryos in BSA + FCS derived from Day-6 EBs. The less represented transition was B to ExB, both in BSA (2.5%) and BSA + FCS (4.0%).

3.2 Multivariate Study

Sample classification was examined by PLS-DA and OPLS-DA for all metabolite signals. Independent studies were performed for each major fixed factor. Suppl. Table 7 shows the corresponding predictive classification measures, whereby the entire sample dataset models were not predictive for sex (Fig. 3A, 4A), Day-6 stage (Figs. 3B and 4B) and Day-7 stage (Fig. 3C, 4C) (i.e., very low Q2, distant from R2, and low accuracy). In contrast, the predictive ability was fair for culture (Figs. 3D and 4D) and high for bull breed (Figs. 3E and 4E), with high accuracy, and Q2 value close to R2. These classifications indicate that sex metabolic differences are of lower magnitude than other effects, and a study by subsets is a pertinent strategy to identify sex free of confounders.

3.3 Subset study

Subset Univariate Study.

The univariate analysis of the 8,031 features obtained after metabolic signal processing produced 1,196 subsets differentially affected by sex, corresponding to 760 unique features, on which the annotation was performed.

Biomarker Discovery and Tentative Identification within Subsets.

We obtained 31 significantly regulated metabolites as candidate biomarkers that varied with embryonic sex in 182 subsets. Only subsets ≥ 7 samples (≥ 3 each sex) were considered. Nine of the 31 metabolites - Cytosine, L-Proline, L-Valine, L-Threonine, L-Methionine L-Phenylalanine, L-Tyrosine, Citric acid and L-Tryptophan - were correctly validated with standards; mass fragment information is shown in Suppl. Tables 6, and MS/MS spectrum comparison between metabolites obtained in samples and the available standards is shown in Suppl. Figure 1. Validation of the 9 standards suggest that the analysis was appropriate for compound annotation. The remainder of the tentatively identified metabolites were supported by Suppl. Table 6. Suppl. Table 8 shows representative biomarker candidate metabolites sorted by bull breed (AV or Holstein) and culture until Day-6 (BSA or BSA + FCS), and ranked by their discriminatory power (Day-7 stage ID). Within subsets, 85 were identified in AV breed, 29 in Holsteins, and 68 without breed effect. Sex identifications based on metabolites were performed 1,921 times with female samples and 1,584 times with male samples. Notably, 76 subsets showed top ROC-AUC values (0.850-1.000), while 106 subsets were in the range of 0.700 to < 0.850.

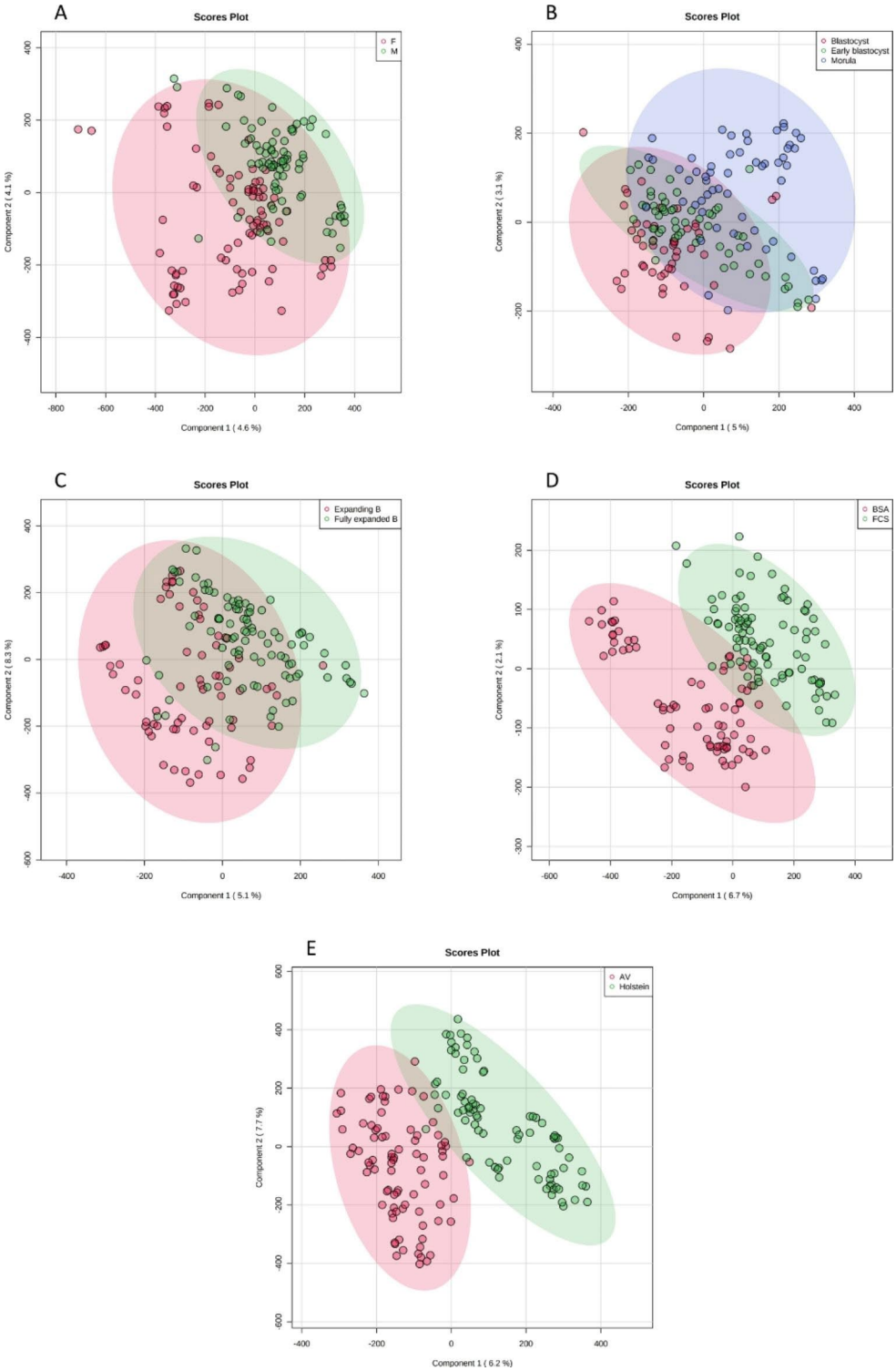

Fig. 3 Partial least square discriminant analysis (PLS-DA) performed with all retained metabolite signals for sex (3A), Day-6 stage (3B), Day-7 stage (3C), culture supplementation (3D) and bull breed (3E). Colored circles represent 95% confidence intervals. Separation by permutation was non-significant ($P > 0.05$) for any factor despite closer Q2 and R2Y values being observed for culture (3D; $Q2 = 0.596$; $R2Y = 0.934$) and bull breed (3E; $Q2 = 0.738$; $R2Y = 0.958$)

3.4 General Univariate Study

Metabolites tentatively identified in the subset study were analyzed in the entire dataset. Effects included in the study were culture (prior to Day-6), Day-6 stage, Day-7 stage, bull breed, and individual bull as a random factor (Table 2).

All annotated metabolites were differentially affected by variation sources (major effects), with cytosine, L-Histidine and L-Threonine (5 significant effects influenced each metabolite concentration) as the most variable biomarkers, exception made of Indole, which was not affected by the analyzed effects. However, these effects did not hide

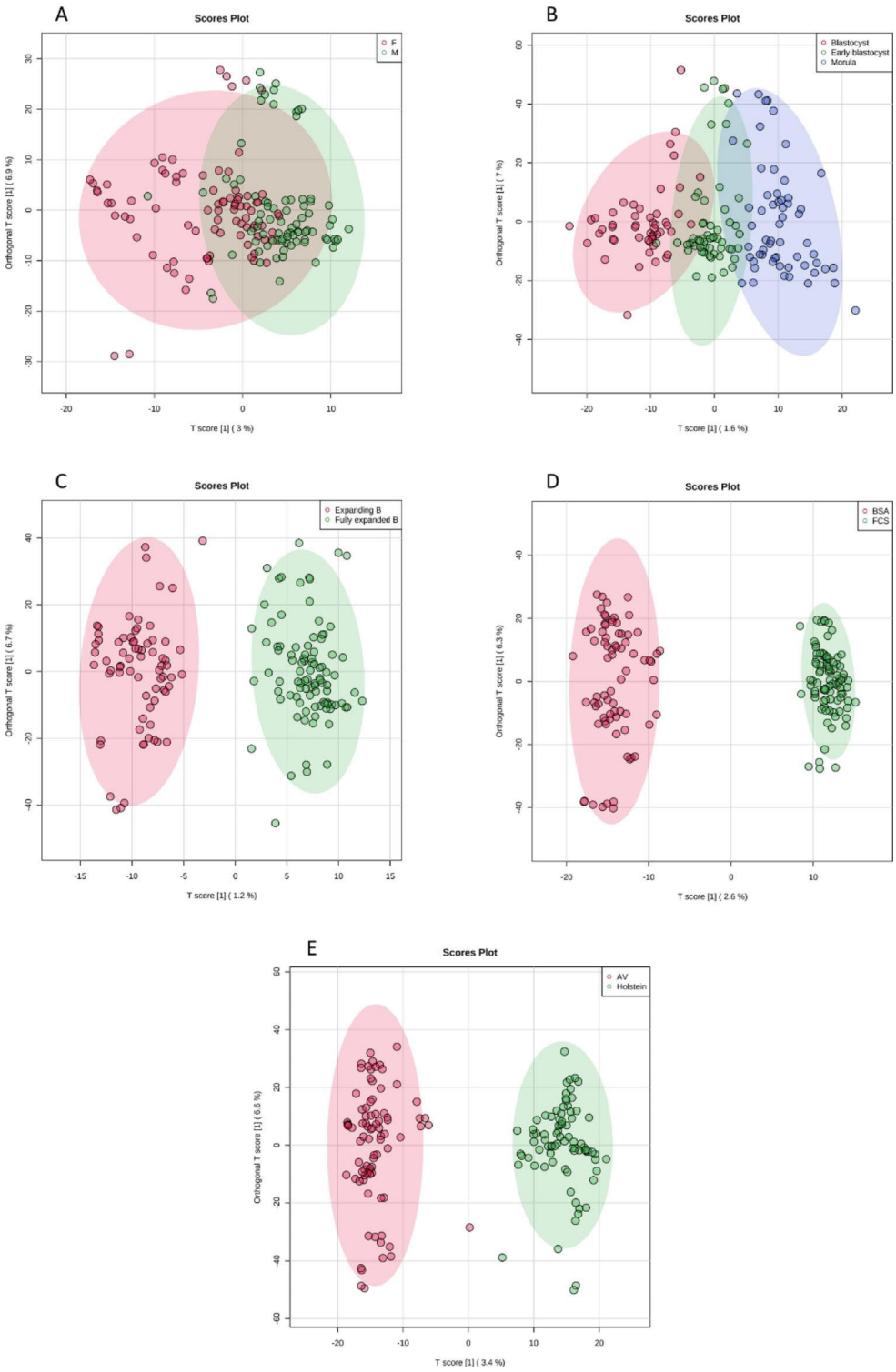

Fig. 4 Orthogonal partial least square discriminant analysis (OPLS-DA) performed with all retained metabolites does not separate samples by embryonic sex (4A: Q2 = 0.0675, P = 0.09; R2Y = 0.441, P = 0.77), Day-6 stage (4B: Q2 = 018.8, P = 0.01; R2Y = 0.841, P = 0.49) and Day-7 stage (4C: Q2 = 0.0998, P = 0.01; R2Y = 0.926, P = 0.19), while significant separation with closer Q2 and R2Y values is shown for embryo culture with BSA or BSA + FCS (4D: Q2 = 0.673, P = 0.01; R2Y = 0.981, P = 0.01) and bull breed (4E; Q2 = 0.796, P = 0.01; R2Y = 0.957, P = 0.01)

differences between male and female embryos for some metabolites (e.g., L-Threonine), which were highly significant for cytosine ($p < 0.001$). Sex was the effect with the lowest overall incidence (5 metabolites affected). Culture stage had an important effect on 12 metabolites (Day-6) and 11 metabolites (Day-7), with 18 metabolites affected by one or another stage and/or day. Culture and bull breed significantly influenced 19 and 15 metabolites, respectively, being both the highest values shown for the fixed effects analyzed. However, the most striking metabolic influence was exerted by each single bull, showing both the highest impact in metabolites affected (20) and the highest significance

Table 2 Analysis of major effects affecting individual metabolites, which include sex, Day-6 and Day-7 stage, culture prior to Day-6 (BSA or BSA + FCS), bull breed and individual bull

Metabolite	Class	Sex	Day-6 stage	Day-7 stage	Culture	Bull Breed	Bull	VS
L-Arginine	Amino acids				0.005			1
l-Glutamic acid	Amino acids						< 0.001	1
l-Histidine	Amino acids		0.011	< 0.001	**0.096**	0.034	< 0.001	5
l-Lysine*	Amino acids		< 0.001	**0.078**		0.009	< 0.001	4
l-Methionine*	Amino acids		0.043	< 0.001	0.001		< 0.001	4
l-Phenylalanine	Amino acids	**0.086**	**0.075**	0.023		0.048		4
Pipecolic acid	Amino acids				**0.099**	0.013		2
l-Proline	Amino acids		**0.068**			< 0.001	< 0.001	3
Pyroglutamic acid	Amino acids			**0.077**	0.011	0.026	0.002	4
l-Threonine	Amino acids	**0.083**	0.012	0.004		< 0.001	< 0.001	5
l-Tryptophan	Amino acids		0.018			0.015	0.005	3
l-Tyrosine	Amino acids				< 0.001	0.02	0.001	3
l-Valine	Amino acids				< 0.001		< 0.001	2
Benzoic acid	Benzenoids	**0.07**		**0.091**	**0.071**		< 0.001	4
Indole	Benzenoids							0
p-Cresol	Benzenoids		**0.089**		< 0.001		< 0.001	3
Phenylacetaldehyde	Benzenoids			0.012	< 0.001			2
Citric acid	Carboxylic acid		0.041				< 0.001	2
5-Hydroxy-L-tryptophan*	Indole			0.003	**0.083**	0.004	< 0.001	4
Dihydro-alpha-ionone	Lipid		0.003		0.037			2
Dimethyl adipate	Lipid	0.044				0.018	< 0.001	3
5Z-Dodecenoic acid	Lipid				**0.06**	0.023		2
12-Hydroxydodecanoic acid	Lipid				0.028			1
Citramalic acid	Lipid				0.003			1
Linoleamide	Lipid				0.01		< 0.001	2
MG (16:0/0:0/0:0)	Lipid				0.006	< 0.001	< 0.001	3
Oleamide	Lipid		**0.096**				< 0.001	2
Palmitic amide	Lipid		< 0.001					1
Palmitoylethanolamide	Lipid				**0.076**			1
Phosphatidylethanolamine	Lipid			0.003		0.012	< 0.001	3
Cytosine	Pyrimidine	< 0.001		**0.059**	0.016	0.018	< 0.001	5
P < 0.05		2	8	7	13	15	20	
0.05 < P < 0.10		3	4	4	6	0	0	
Total P < 0.10		5	12	11	19	15	20	

*: indicates normal distribution. N = 167 culture medium samples analyzed. VS: variation sources of each metabolite, i.e., number of major effects affecting the metabolite. Tendencies are in **bold**

in their p values (i.e., 17 metabolites with p < 0.001 and 3 metabolites with p from 0.001 to 0.005). This suggests a very strong random effect in culture that can only be met by introducing variability (i.e., 7 individual bulls included in this study).

3.5 Impact of metabolite biomarkers

3.5.1 Biomarkers class impact

We summarized information extracted from Suppl. Table 8 as a dataset. Thus, classification by chemical taxonomy (Table 3) showed lipid and lipid-like molecules (93 subsets; 11 metabolites; average -av- ROC-AUC: 0.8260) and Amino acids (55 subsets; 13 metabolites; av ROC-AUC: 0.8365) as the most relevant classes for metabolite sex classification.

Notably, all lipids tentatively identified were or contained fatty acid (FA), and/or had a role in the FA metabolism. Interestingly, most amino acids were components of the SOF formulation used (marked with asterisks in Table 3). The next class in importance was benzenoids (17 subsets; 4 metabolites; av ROC-AUC: 0.8912), followed by three single metabolite classes: citric acid (a component of SOF; Tricarboxylic acids and derivatives), 5-Hydroxy-L-tryptophan (Tryptamines and derivatives), and cytosine (Aminopyrimidines and derivatives). We based the relevance

Table 3 Predictive power of each candidate metabolite biomarker grouped by class, based on ROC-AUC average, total number of subsets and total number of correctly identified embryos

Metabolite	ROC-AUC		N Subsets	Sex ID		
	Average	Range		F	M	Total
Lipid and lipid-like molecules						
Linoleamide	0.9034	1.0000-0.7551	5	48	27	75
Dihydro-alpha-ionone	0.8609	0.8800-0.8512	3	27	19	46
12-Hydroxydodecanoic acid	0.8494	1.000 -0.7479	22	262	230	492
5Z-Dodecenoic acid	0.8333	0.8667-0.8000	2	12	15	27
Citramalic acid	0.8176	0.9143 – 0.7222	12	125	107	232
Palmitic amide	0.8309	1.0000-0.7100	6	68	42	110
Phosphatidylethanolamine	0.8209	0.8929 – 0.7650	10	143	81	224
Palmitoylethanolamide	0.7903	0.9167 – 0.7075	7	80	65	145
MG (16:0/0:0/0:0)	0.8007	0.9107 – 0.7021	16	187	157	344
Dimethyl adipate	0.7930	0.8800-0.7083	5	46	48	94
Oleamide	0.7850	0.8571 – 0.7266	5	42	30	72
	0.8260		93	1040	821	1861
Amino-acids, peptides and analogues						
L-Tryptophan*	0.8843	0.9500-0.7763	4	28	40	68
l-Lysine*	0.8571	ND	1	7	5	12
L-Histidine*	0.8477	0.8889-0.8000	3	23	17	40
l-Proline*	0.8476	0.9167 – 0.7521	10	86	80	166
Glutamic acid*	0.8910	1.0000-0.8035	4	41	24	65
Pyroglutamic acid	0.8129	0.8809 – 0.7009	7	63	63	126
l-Tyrosine*	0.8333	ND	1	6	10	16
L-Threonine*	0.8331	0.8750 – 0.8047	5	44	35	79
l-Arginine*	0.8875	0.9000-0.8750	2	10	16	26
l-Phenylalanine*	0.8089	0.9667 – 0.7132	6	66	75	141
l-Valine*	0.8085	0.9167 – 0.7633	7	78	58	136
l-Methionine*	0.7794	0.7969 – 0.7619	2	22	14	36
Pipecolic acid	0.7837	0.8095 – 0.7583	3	46	20	66
	0.8365		55	520	457	977
Benzenoids						
p-Cresol	0.9437	1.0000-0.8977	3	15	21	36
Phenylacetaldehyde	0.8750	ND	1	5	8	13
Benzoic acid	0.8861	1.000-0.7095	10	120	105	225
Indole	0.8601	0.9143 – 0.7909	3	30	19	49
	0.8912		17	170	153	323
Carboxylic acids and derivatives						
Citric acid*	0.8537	1.0000-0.7096	6	79	49	128
Indoles and derivatives						
5-Hydroxy-L-tryptophan	0.8308	0.880-0.7633	6	53	52	105
Pyrimidines and pyrimidine derivatives						
Cytosine	0.7798	0.8333 – 0.7328	5	59	52	111

*: components originally contained in SOF by formulation

Total number of subsets: statistically significant ($p < 0.05$), and tendencies ($0.05 < p < 0.1$). The impact of the different classes of metabolites was calculated as the sum of total subsets of all metabolites included in the class and the average ROC-AUC of all biomarkers. Sex ID shows the total number of female (F) and male (M) samples correctly identified per metabolite.

of biomarkers primarily on the numbers of subsets they participate, and on the total number of embryos whose sex was correctly identified. The following metabolites were represented in ≥ 10 subsets: 12-hydroxydodecanoic acid, citramalic acid, phosphatidylethanolamine (18:2/20:2) and MG(16:0/0:0/0:0) (Lipid class); L-Proline (amino-acids, peptides and analogues); and benzoic acid (Benzenoids). All metabolites identified were present at least in 2 subsets, except L-Tyrosine, L-Lysine and Phenylacetaldehyde. Regarding the number of embryos correctly sexed within

all subsets of each biomarker, 12-Hydroxydodecanoic acid identified the highest number of cases, 492 (262 female and 230 males), followed by MG(16:0/0:0/0:0), which identified 344 (187 female and 157 male), thus increasing the value of such metabolites as a biomarker.

3.5.2 Variation in log fold change (FCh)

The impact of factors analyzed in embryonic sex also affected the FCh values (Suppl. Table 8), which showed differences between female vs. male embryos that depended on the concurrence of other factors. For instance, 12-Hydroxydodecanoic acid (N = 22 subsets) showed positive FCh for Holstein-sired embryos vs. negative FCh for AV-sired embryos. A similar reversed FCh, mainly attributable to breed, was also observed with Dihydro-alpha-ionone, Indole, L-Tryptophan, Pyroglutamic acid and Phosphatidylethanolamine (18:2/20:2). Day-6 embryonic stage also influenced FCh values, as observed with L-Proline, which showed a positive FCh in 9/10 subsets with Day-6 stage of morula, except in one subset for EB stage. Such effects were not strictly observed in all subsets analyzed, thus we cannot ignore the fact that complex interactions between factors led also to reverse FCh. The following metabolites showed at least one subset (total N = 19 subsets) with FCh of -99 and <-100 (i.e., males leading to higher concentration in CM than females): Glutamic acid, L-Tryptophan, Pyroglutamic acid, Benzoic acid, 12-Hydroxydodecanoic acid, Citramalic acid and MG(16:0/0:0/0:0). On the contrary, Linoleamide showed one subset with FCh of > 100. All subsets with these FCh values were identified in culture with FCS, except one subset of Pyroglutamic acid and MG(16:0/0:0/0:0) withing BSA, and one subset of Benzoic acid disregarding culture supplementation.

Metabolites originally contained in CM.

Regarding the metabolites originally contained in SOF, we also reported variations in FCh values in 12 metabolites (51 subsets involved). All these metabolites were amino acids, except citric acid. Remarkably, 29 subsets were identified within AV, while only five subsets were in Holsteins (and 17 disregarding breed). Differences observed within SOF components in each CM and breed can be summarized as follows:

Culture with BSA.

With AV, Day-6 male embryos at the morula stage showed higher depletion of L-Arginine, L-Proline and L-Valine, while female morula led to decreased L-Phenylalanine and L-Tyrosine. At the EB stage, female embryos depleted more L-Proline than males, and at EB and B stages, males led to greater abundance of L-Threonine and L-Valine than females.

With Holstein, male morulae had a higher consumption of L-Proline, and female morulae of L-Phenylalanine. Male EBs showed a higher depletion of L-Tryptophan, while female EBs and Bs had a higher depletion of L-Threonine and L-Valine.

Culture with FCS.

With AV, Day-6 female morulae depleted more glutamic acid and L-Tryptophan than males. Male embryos at EB and B stage had a higher consumption of glutamic acid. In contrast, female EBs led to decreased L-Histidine and L-Methionine, and EB and female Bs showed higher depletion of L-Threonine and citric acid.

With Holstein, female morulae showed the same pattern as AV, i.e., higher depletion of Glutamic acid and L-Tryptophan. Female EBs showed greater decrease of L-Methionine and L-Threonine than males, and female EB and Bs led to a greater decreased in citric acid.

3.5.3 Metabolite sex discrimination power

Single metabolite biomarkers.

The discrimination ability of individual candidate biomarkers for each fixed factor, i.e., breed (Holstein and AV) and culture until Day-6 (BSA or BSA + FCS) is shown in Table 4. The discrimination power was calculated as the sum of percentages of embryos whose sex was correctly identified in all Day-6 to Day-7 transitions within which such a metabolite participated under specific culture conditions. Candidate metabolites were analyzed in CM, by separate culture with BSA or BSA + FCS, and by breed.

Biomarkers in Culture Medium.

Seventeen metabolites were discriminant in BSA, and 16 in FCS, while seven were not affected by culture. In culture with BSA, with no other factor included, L-Proline produced the highest sex identification coverage (50.17% of embryos), but lower than when the breed effect was included (AV: 78.74% sexed embryos). In BSA + FCS, MG(16:0/0:0/0:0) gave the highest sex identification percentage (63.95%), lower than considering only AV breed (81.02%). When breed and culture effect were not analyzed, 29.43% was the highest percentage of coverage obtained, given by benzoic acid.

Biomarkers in Holstein.

Holstein was the least represented breed, with eight discriminatory metabolites in BSA, with citramalic acid and 12-Hydroxydodecanoic acid being the metabolites with the highest identification power (close to 50% of embryos having their sex identified on average within all subsets significant for this metabolite). On the contrary, only one metabolite, benzoic acid, identified sex with FCS, with a maximum coverage of 19.30%. The analysis of subsets

Table 4

Predictive power of single candidate metabolite biomarkers grouped by class, based on percentage of embryos whose sex was correctly identified on Day-7 by breed (Asturiana de los Valles -AV- and Holstein) and culture condition (i.e., BSA, BSA+ FCS or not dependent on culture supplementation -ND-)

	ID in Holstein			ID in AV			ID w/o breed effect		
Metabolite	BSA	FCS	ND	BSA	FCS	ND	BSA	FCS	ND
Lipid and lipid-like molecules									
Linoleamide	-	-	-	23.77	56.77	-	-	3.03	-
Dihydro-alpha-ionone	-	-	24.58	-	-	12.68	5.21	-	-
12-Hydroxydodecanoic acid	48.25	-	32.20	49.41	50.33	**77.83**	45.30	43.27	20.32
5Z-Dodecenoic acid	-	-	-	45.61	-	-	7.31	-	-
Citramalic acid	49.88	-	35.70	46.56	-	12.06	49.12	12.43	17.08
Palmitic amide	-	-	-	34.63	44.06	12.06	24.54	-	-
Phosphatidylethanolamine	8.70	-	12.22	-	55.41	43.69	29.23	-	-
Palmitoylethanolamide	-	-	-	25.44	-	28.65	16.31	36.90	-
MG (16:0/0:0/0:0)	11.41	-	-	48.46	**81.02**	11.44	10.40	**63.95**	-
Dimethyl adipate	23.06	-	-	48.46	-	-	38.22	-	-
Oleamide	-	-	-	31.65	19.81	-	-	17.05	-
Amino-acids, peptides and analogues									
L-Tryptophan*	27.03	-	32.20	-	9.16	-	-	9.67	-
L-Lysine*	-	-	20.19	-	-	-	-	-	-
L-Histidine*	-	-	-	-	42.61	20.12	-	-	-
L-Proline*	-	-	34.51	**78.74**	-	15.10	50.17	-	-
Glutamic acid*	-	-	-	-	46.59	-	-	16.13	-
Pyroglutamic acid	25.12	-	-	42.76	13.74	-	39.96	16.89	10.00
l-Tyrosine*	-	-	-	47.51	-	-	-	-	-
L-Threonine*	-	-	-	-	40.63	-	7.03	15.68	10.15
l-Arginine*	-	-	-	51.31	-	-	-	-	-
L-Phenylalanine*	-	-	-	55.11	-	31.12	44.92	-	19.02
l-Valine*	-	-	-	**77.50**	-	32.20	26.38	-	-
L-Methionine*	-	-	-	-	-	11.35	-	11.35	-
Pipecolic acid	-	-	-	-	52.89	21.80	-	-	-
Benzenoids									
p-Cresol	-	-	11.32	-	-	-	7.87	-	-
Phenylacetaldehyde	-	-	-	-	-	-	-	8.91	-
Benzoic acid	31.15	19.30	-	-	**63.28**	-	-	39.95	29.43
Indole	-	-	34.51	-	34.51	-	-	-	-
Carboxylic acids and derivatives									
Citric acid*	-	-	-	-	**62.38**	-	-	32.95	-
Indoles and derivatives									

(continued) Table 4

Metabolite	ID in Holstein			ID in AV			ID w/o breed effect		
	BSA	FCS	ND	BSA	FCS	ND	BSA	FCS	ND
5-Hydroxy-L-tryptophan	-	-	17.57	-	-	-	7.31	36.20	9.63
Pyrimidines and pyrimidine derivatives									
Cytosine	-	-	-	57.55	-	46.94	27.70	9.71	-

*: components originally contained in SOF by formulation

ID: percentage of Day-7 embryos that each metabolite can identify by breed, calculated as the sum of percentages of all possible transitions for a specific culture condition (percent of embryos with identified sex within individual subsets are presented in Suppl. Table 8). ID values > 60% are in **bold**. Repeat transitions (i.e., that appeared in more than one subset) were manually calculated

disregarding culture supplementation in Holstein showed 10 significant metabolites with significant diagnostic power (ranging from 11.32 to 35.70% coverage), which improved with the metabolites Phosphatidylethanolamine and L-Tryptophan (from 8.70 to 12.22%, and 27.03–32.20%, respectively), while with citramalic acid and 12-Hydroxydodecanoic acid it decreased (from 49.88 to 35.70%, and 48.25–32.20%, respectively).

Biomarkers in AV.

In AV, sex discrimination by metabolites was higher than in Holsteins, with 16 metabolites for BSA, 15 for BSA + FCS, and 14 independent of culture supplementation. In culture with BSA, L-Proline and L-Valine yielded the highest embryo sexing coverage -close to 80%, and nine more metabolites close to 50%: 12-Hydroxydodecanoic acid (49.41%), 5Z-Dodecenoic acid (45.61%), Citramalic acid (46.56%), MG(16:0/0:0/0:0) (48.46%), Dimethyl adipate (48.46%), L-Tyrosine (47.51%), L-Arginine (51.31%), L-Phenylalanine (55.11%) and Cytosine (57.55%). In BSA + FCS, MG(16:0/0:0/0:0) gave the highest percentage of sexed embryos (81.02%), followed by Benzoic and citric acid (63.28 and 62.38%, respectively). Furthermore, six other metabolites identified sex in almost 50% of embryos represented in such cultures: Linoleamide (56.77%), 12-Hydroxydodecanoic acid (50.33%), Palmitic amide (44.06%), Phosphatidylethanolamine (55.41%), Glutamic acid (46.59%) and Pipecolic acid (52.89%). When culture supplementation was not considered, the highest sex coverage obtained was 46.94% for cytosine. As occurred with Holstein, the identification power of each metabolite participating in one or both culture conditions decreased when culture was not considered (e.g., MG(16:0/0:0/0:0) decreased from 48.46% in BSA and 81.02% in FCS to 11.44%), except for 12-Hydroxydodecanoic acid and Palmitoylethanolamide, in which the diagnostic ability increased (from approximately 50% with BSA and BSA + FCS to 77.83%, and 25.44% with BSA to 28.65%, respectively).

Combined metabolite biomarkers.

Combinations of the best represented metabolites within fixed factors (bull breed and culture condition), and embryonic stages, were used to perform the highest sex diagnosis coverage in each culture condition analyzed (Table 5).

A total of 19 combinations of metabolites with a coverage ranking between 79.74% and 96.67% were obtained. The highest sex diagnosis coverage was obtained with AV and BSA by combining four metabolites (combination Nº 7, 96.67% of embryos sexed): L-Phenylalanine, Phosphatidylethanolamine, Linoleamide and p-Cresol. Furthermore, the highest identification with the minimum numbers of metabolites were the combinations Nº 5 (for AV and BSA, formed by Palmitic amide and L-Phenylalanine) and Nº

Table 5
Best combinations of metabolites that diagnosed sex, expressed as percentage of correct samples with their sex identified by a specific metabolite

Metabolite	Class	ROC-AUC	T-test	Bull Breed	Culture	Day-6	Day-7	Day-7 ID
Oleamide [1,2]	Lipid	0.8500	**0.0667**	AV	FCS	EB + B	ExB	19.8141
Benzoic acid [2]	Benzene	1.0000	0.0002	AV	FCS	EB + B	FEB	46.4391
l-Tryptophan [2]	aa	0.9000	**0.1102**	AV	FCS	M	FEB	9.1614
MG(16:0/0:0/0:0) [1,2]	Lipid	0.7857	**0.0534**	AV	FCS	M	ExB	15.7700
12-Hydroxydodecanoic acid [1]	Lipid	0.8889	0.0002	AV	FCS		FEB	50.3275
Combination 1	85.9116							
Combination 2	91.1846							
12-Hydroxydodecanoic acid [3,4]	Lipid	0.8417	0.0409	AV		EB + B	ExB	13.3580
l-Valine [4]	aa	0.7950	0.0261	AV		EB + B	FEB	32.1978
l-Proline [3,4]	aa	0.7521	0.0464	AV		M	ExB	15.0979
12-Hydroxydodecanoic acid [4]	Lipid	0.8750	**0.0545**	AV		M	FEB	20.6093
12-Hydroxydodecanoic acid [3]	Lipid	0.8006	0.0021	AV			FEB	51.2820
Combination 3	79.7379							
Combination 4	81.2630							
Palmitic amide [5]	Lipid	0.8056	**0.0839**	AV	BSA	EB + B		34.6331
l-Phenylalanine [5,6,7]	aa	0.9667	0.0095	AV	BSA	M		55.1069
Phosphatidylethanolamine [6,7]	Lipid	0.8929	0.0212	AV		B		11.8824
l-Proline [6]	aa	0.8571	0.0409	AV	BSA	EB		25.4439
Linoleamide [7]	Lipid	1.0000	**0.0573**	AV	BSA	EB	FEB	23.7677
p-Cresol [7]	Benzene	1.0000	0.0002		BSA	EB	ExB	5.9169
Combination 5	89.7400							
Combination 6	92.4332							
Combination 7	96.6739							
Citramalic acid [8,10]	Lipid	0.8163	**0.0539**		FCS	B	ExB	3.2718
12-Hydroxydodecanoic acid [8]	Lipid	0.7642	0.0019		FCS		FEB	43.2680
Citramalic acid [9]	Lipid	0.7541	0.0111		FCS	B		12.4335
Cytosine [10]	Pyrimidine	0.7777	0.0174		FCS	B	FEB	9.7056
Benzoic acid [8,9,10]	Benzene	1.0000	**0.0596**		FCS	EB	ExB	19.3027
12-Hydroxydodecanoic acid [9,10]	Lipid	0.8906	0.0008		FCS	EB	FEB	30.2449
l-Tryptophan [9,10]	aa	0.9500	0.0211		FCS	M	FEB	9.6703
MG(16:0/0:0/0:0) [8,9,10]	Lipid	0.9107	0.0045		FCS	M	ExB	18.2786
Combination 8	84.1212							
Combination 9	89.9301							
Combination 10	90.4740							
p-Cresol [11]	Benzene	0.9333	0.0049		BSA	EB + B	ExB	7.8690
l-Valine [11,12]	aa	0.7633	0.0164		BSA	EB + B	FEB	26.3821
Citramalic acid [11,13]	Lipid	0.8616	0.0005		BSA	M		49.1178
12-Hydroxydodecanoic acid [12]	Lipid	0.9583	0.0024		BSA	M	FEB	35.3890
Dimethyl adipate [12]	Lipid	0.7500	0.0351		BSA		ExB	21.3830
MG(16:0/0:0/0:0) [13]	Lipid	0.7812	0.0381		BSA	B		10.3964
p-Cresol [13]	Benzene	1.0000	0.0002		BSA	EB	ExB	5.9169
l-Valine [13]	aa	0.7937	0.0460		BSA	EB	FEB	18.8632
Combination 11	83.3689							
Combination 12	83.1541							
Combination 13	84.2943							
Phosphatidylethanolamine [14, 15,16]	Lipid	0.8205	0.0200	Holstein		B		12.2240
Citramalic acid [14]	Lipid	0.8182	0.0185	Holstein		M		35.6962
12-Hydroxydodecanoic acid [14,15]	Lipid	0.7763	0.0032	Holstein		EB		32.1963
5-Hydroxy-L-tryptophan [15,16]	Indole	0.8750	**0.0783**	Holstein		M	ExB	17.5659
Citramalic acid [15,16]	Lipid	0.9143	0.0382	Holstein		M	FEB	21.5346
p-Cresol [16]	Benzene	0.8977	0.0073	Holstein		EB	ExB	11.3202

(continued) Table 5

Dihydro-alpha-ionone [16]	Lipid	0.8516	0.0110	Holstein		EB	FEB	24.5790
Combination 14	80.1166							
Combination 15	83.5208							
Combination 16	87.2237							
12-Hydroxydodecanoic acid [17]	Lipid	0.7908	0.0077	Holstein	BSA	EB + B		33.9994
Citramalic acid [17,18,19]	Lipid	0.8750	0.0277	Holstein	BSA	M		49.8813
12-Hydroxydodecanoic acid [18]	Lipid	0.9643	0.0025	Holstein	BSA	EB		28.6244
MG(16:0/0:0/0:0) [18,19]	Lipid	0.8571	**0.0622**	Holstein	BSA	B		11.4065
12-Hydroxydodecanoic acid [19]	Lipid	1.0000	0.0414	Holstein	BSA	EB	FEB	23.7677
p-Cresol [19]	Benzene	1.0000	0.0002		BSA	EB	ExB	5.9169
Combination 17	83.8807							
Combination 18	89.9122							
Combination 19	90.9724							

AV: Asturiana de los Valles; EB: Early blastocyst; M: Morula; B: Blastocyst; ExB: Expanded blastocyst; FEB: Fully expanded blastocyst; aa: amino acid; Day-7 stage ID: percent of total embryos within the transition and culture conditions identified on Day 7; ROC-AUC: Receiver Operator Characteristic – area under curve. p value analyzed by t-test. Significant differences: $p < 0.05$. Tendency (**bold**) $0.05 < p < 0.1$. Empty cells within breed, culture and embryonic stage mean that the subset in question was not dependent on such a factor. Superscripts indicate the participation of the cognate metabolite in a specific combination. For each condition, several combinations can be obtained, depending on how many metabolites were used to reach the highest coverage of sex identification

Combinations of subsets shown were extracted from Suppl. Table 8 with the following exceptions:

1) An extra subset of L-Tryptophan with t-test p value = 0.11, as all AV samples were significant (P = 0.0211) as part of a larger subset that included both breeds (AV and Holstein).

2) In the conditions AV and BSA, a subset of Phosphatidylethanolamine obtained with samples of both culture conditions (BSA and BSA + FCS) was also used to identify a transition, but the Day-7 ID of the subset was manually calculated with the percentage of appearance of that transition corresponding only to BSA.

17 (for Holstein and BSA, formed with the metabolites 12-Hydroxydodecanoic acid and Citramalic acid), which had a sex coverage of 89.74% and 83.88%, respectively. Only one subset (benzoic acid) was significant for Holstein embryos cultured with FCS. However, the sex of Holstein embryos cultured with FCS was successfully diagnosed within subsets disregarding culture supplementation or breed. As examples of the above, Citramalic acid for the transition from M and B to ExB and FEB (48.13% coverage), and Benzoic acid for EB to FEB and ExB (39.95% coverage). No effective sex-diagnosis combination was shown if excluding breed and culture supplementation (see Table 5), as the maximum percentage of sexed embryos covered was 49.74%, with a combination of benzoic acid and 12-hydroxydodecanoic acid. Within all combinations described, the most used metabolite was 12-Hydroxydodecanoic acid, which participates in 12 of the 19 combinations, followed by MG(16:0/0:0/0:0) and citramalic acid, both appearing in eight combinations each.

4 Discussion

In this study we analyzed the metabolome of male and female embryos to non-invasively identify biomarkers of embryonic sex. High variability among samples was accomplished by producing embryos from blind ovary collection, fertilized with two bull breeds (AV and Holstein), cultured in two conditions before Day-6 (BSA or BSA + FCS), and including different embryonic stages on Day-6 and Day-7. To the best of our knowledge, this is the first metabolomic study for sex identification with such high variability in cattle, showing that culture supplementation and bull breed had important effects on the metabolome. Furthermore, although Day-6 and Day-7 stage had a lower predictive effect than culture and bull breed, omitting these factors might preclude a correct identification of embryonic sex. Therefore, the separation of samples only in accordance with embryonic sex is insufficient to obtain reliable markers. The metabolome analysis by subsets, i.e., fixed factors that can be controlled in the laboratory, thus improved the identification of embryonic sex.

4.1 Sex diagnosis: single biomarkers

We divided the datasets into controllable subsets, as suggested by discriminant analysis and previous findings (Gómez et al., 2020b; Gómez et al., 2020c; Gomez et al., 2021). Different sets of biomarkers were obtained with fixed factors (i.e., bull breed, culture and embryonic stages). We obtained more biomarkers within AV than in Holstein. Differences between breeds were not due to the numbers of samples used in the study (83 Holstein and 84 AV). Rather, the ability of metabolites to diagnose embryonic sex was

higher for AV than for Holstein, perhaps attributable to certain subsets with unbalanced male and female samples in Holsteins, as outcome sex ratio was not predictable under any analyzed condition. Both culture systems used prior to Day-6 had similar representation between subsets. However, the sex ratio of specific transitions was divergent between culture systems, leading to a discriminatory power with BSA + FCS lower than with BSA. This may be due to the low number of subsets identified for Day-6 morula transitions with BSA + FCS (7 subsets), which represent 30% blastocysts developed in Day-7.

Among individual biomarkers, L-Proline and L-Valine produced the highest sex identification coverage with AV and BSA, MG(16:0/0:0/0:0) in BSA + FCS, and 12-Hydroxydodecanoic acid in both culture conditions. In Holstein, citramalic acid was the best biomarker for sex identification with BSA and also disregarding culture conditions, and only benzoic acid was identified as a biomarker with BSA + FCS. Typically, the coverage was lower when breed and culture were not considered. Many metabolites had abundant representation through subsets as a proof of validation, which increases the biomarker value and reliability.

4.2 Combining biomarkers

Among the metabolites obtained, none showed total confident diagnosis of embryonic sex in a complex, multifactorial environment (i.e., culture conditions, stages and bull breed) as single biomarkers, especially in Holsteins. Such difficulty to obtain a single metabolite biomarker for embryos has already been reported (Nõmm et al., 2019) and is consistent with our recent studies (Gimeno et al., 2021; Gomez et al., 2021). For this reason, we proposed combining metabolites to improve sex identification, thus facilitating increases in sex coverage from 79.74 to 96.79%. With FCS, sex identification was inconsistent in Holstein, but in those cases, it is possible to diagnose sex with biomarkers which were independent of breed or culture supplementation. Furthermore, as some metabolites fulfilled different factorial conditions (mainly 12-Hydroxydodecanoic acid, MG(16:0/0:0/0:0) and citramalic acid), with a few more metabolites (e.g., oleamide, L-Proline, L-Phenylalanine, Phosphatidylethanolamine, benzoic acid, p-Cresol and L-Valine), it was possible to diagnose sex within all different factors.

4.3 Predictable and controllable factors: breed and culture

Breed and culture exerted striking influences in metabolite concentrations, but both factors can be controlled in the laboratory and be used as a supervised strategy to improve sex identification. Our experiments came from undefined oocyte sources, and the breed effect was based directly on the bull breed. However, we believe that breed-defined oocyte sources would reduce experimental variability and noise, leading to more homogeneous blocks, by which predictions within specific metabolites would be even more accurate and never worse.

In the present study, FCh of some metabolites changed with bull breed; e.g., female AV embryos depleted more pyroglutamic acid than males, while Holstein embryos showed the opposite pattern. It is recognized that crossbreed and purebred embryos differ in their amino acid metabolism, gene expression and development kinetics (Lazzari et al., 2011). Early embryos from different breeds also differ in expression of genes related with carbohydrate, lipid and small molecule metabolism (Baldoceda et al., 2015). Although we only considered the bull breed, we agree that breed is an important factor that can induce differences in metabolism of early embryos (Lazzari et al., 2011), and suggest that embryos of different breed might display diverse metabolic requirements. Hence, the embryonic breed must be included to minimize variations in metabolome and avoid misleading results.

Individual bulls had the highest significant effects on metabolite concentrations. Single bulls differ in embryo development and survival (Abraham et al., 2012; Alomar et al., 2008; Chenoweth, 2007), since the fertilizing spermatozoon contributes to the nascent embryo with transcription factors, proteins, specific epigenetic signatures, and metabolites (Kropp et al., 2017; Morgan et al., 2020; Velho et al., 2018). Biomarker research uses procedures that hierarchically rank metabolites by importance and/or discrimination power, such as ROC-AUC or variable importance plots (VIP). Therefore, at least in metabolomics, the use of a single bull is revealed as a strong confounding factor whose influence only can be offset introducing high variability (i.e., several bulls).

Regarding culture, serum is known to improve embryo development and to increase blastocyst rates (Murillo et al., 2017), although high serum concentrations alter embryo quality (Rizos et al., 2003; Sudano et al., 2011) and increase cytoplasmic lipid accumulation in blastocysts (Abe et al., 2002; Murillo et al., 2017). Serum contains unidentified compounds, such as fatty acids (FA), amino acids and growth factors, whose composition varies between batches (van der Valk et al., 2018). Serum alters FA composition of blastocysts (Sata et al., 1999) and changes the embryonic transcriptome towards inflammatory pathways and lipid metabolism (in particular, genes involved in cholesterol metabolism) (Cagnone & Sirard, 2014; Heras et al., 2016). Furthermore, gene overexpression induced by FCS is notably higher in male than in female embryos (1283 vs. 456

differentially up-regulated genes) (Heras et al., 2016). Culture systems also skew sex ratio in favor of male embryos, as reported in murine (Jiménez et al., 2003), rabbit (Garcia-Dominguez et al., 2020), cattle (Bermejo-Alvarez et al., 2011; Camargo et al., 2010; Gutiérrez-Adán et al., 2001) and human (Maalouf et al., 2014). This concurs with our study, since we observed skewed sex ratio towards males within embryos cultured in BSA + FCS. Therefore, culture conditions modify embryonic metabolism, precluding a correct attribution of embryonic sex by metabolomics.

4.4 Non-predictable, but controllable, factors: embryonic stages Day-6 and Day-7

In the present study, Day-6 (M, EB and B) and Day-7 (ExB and FEB) stages affected embryo metabolism. The metabolism and metabolic requirements of preimplantation embryos change through development (Gardner & Harvey, 2015; Obeidat et al., 2019; Steeves & Gardner, 1999). Cleavage stage embryos use lactate, pyruvate, amino acids, and lipids to produce energy through the tricarboxylic acid (TCA) cycle. Once the embryonic genome is activated, ATP demands increase to maintain blastocoel expansion and cell proliferation (Gardner & Harvey, 2015), triggering increases in metabolism and nutrient consumption between M and blastocyst stages (Obeidat et al., 2019; Thompson et al., 1996). Therefore, it is conceivable that M and blastocysts differ in energy requirements, probably depending on whether development ends at the ExB or proceeds to the FEB stage, and thus in metabolite concentrations in their cognate CM after 24 h of individual culture. In the mouse, M and blastocyst differ in gene expression related with energy and metabolism (Tanaka & Ko, 2004), and the bovine M shows higher lipid droplet accumulation than EB (Gómez et al., 2017; Murillo et al., 2017). In this study, the inclusion of Day-6, and to a lesser extent of Day-7, embryonic stages as a discrimination factor refined the capacity of candidate biomarker metabolites to diagnose embryonic sex, in line with our recent work (Gimeno et al., 2021; Gomez et al., 2021).

4.5 Metabolic differences between male and female embryos

During preimplantation development, male and female embryos differ in gene expression and epigenetic regulation, leading to differences in proteome and metabolome (Bermejo-Alvarez et al., 2010; Gardner et al., 2010) and sensitiveness to stress (Dallemagne et al., 2018; Leme et al., 2020; Marei et al., 2018). The existence of both X chromosomes transcriptionally active in female embryos between the times of embryonic genome activation and X chromosome inactivation at the blastocyst stage, is consistent with metabolite differences found between male and female embryos in the present study, concerning amino acids, lipids and benzenoids. Male and female bovine embryos differ in consumption and/or release of amino acids (Rubessa et al., 2018; Sturmey et al., 2010) as observed in our study, where SOF components changed with embryonic sex, specifically 11 amino acids and citrate. Distinct preferences towards certain amino acids between male and female embryos depended on Day-6 stage. Thus, Day-6 female EB and B depleted L-Histidine, L-Methionine, L-Proline, L-Threonine, L-Valine and Pipecolic acid independent of breed and culture conditions, while males depleted Glutamic acid and L-Tryptophan. In contrast, female Day-6 morula depleted glutamic acid, L-Phenylalanine, L-Tryptophan and L-Tyrosine, while CM from males contained lower L-arginine, L-Proline and L-Valine. However, our results differed with a profile in which female embryos depleted arginine, glutamate, and methionine, and male embryos depleted phenylalanine, tyrosine, and valine (Sturmey et al., 2010). Changing requirements of amino acid during embryo development have been described (Guerif et al., 2013), which may account for the differences we observed in amino acid concentrations between embryonic stages, and explain that different amino acids may act as sex biomarkers only for specific periods (Rubessa et al., 2018), which is consistent with our study. Furthermore, metabolites that differed in CM between male and female embryos might reflect contrasting, sex-based nutritional demands, as shown in vivo with changes in the composition of uterine fluid when the bovine endometrium reacts to embryonic sex (Gómez et al., 2013).

Citrate concentration differed between male and female embryos under particular culture conditions (i.e., BSA + FCS, AV and the transitions from EB and B to FEB), with female embryos showing higher depletion than males. Citrate is an embryotrophic factor (Gray et al., 1992) necessary to maintain junctional integrity for compaction and blastocoel formation. Citrate can be produced by the catabolism of molecules, such as pyruvate, FAs and some amino acids present in CM, and it participates in FA synthesis through the TCA cycle (Dumollard et al., 2009). However, citrate addition to CM is controversial, being beneficial for blastocyst development (together with myo-inositol) (Holm et al., 1999) or showing an effect depending on the protein supplement used, either BSA or HSA (Lane et al., 2003). Such differences can be explained because citrate is a BSA contaminant (Gray et al., 1992), and the contents of commercial BSA can differ between brands (Sung et al., 2004). Our study in a protein-free culture step excludes such undesirable effects.

The analysis of the embryonic metabolism allows for understanding of the proper environment for the correct

development of embryos. Such knowledge could be applied to adjust culture conditions for each sex, particularly within amino acids, extending such studies to earlier culture periods, and making more tailored embryonic-sex culture conditions, provided that sex-sorted spermatozoa are increasingly used in vitro. In our experiments, mineral oil covered several culture droplets, which potentially may allow passage from less-polar metabolites from one drop to another, but none of the layers will be saturated by such solutes because their low concentrations in absolute terms. Thus, we can assume a steady partition coefficient of metabolites soluble both in oil and media. Such assumption means that absolute amounts of some compounds will be no measurable, but their ratio paraffin oil/culture media would be in a direct proportion to the non-partitioned concentration, which allows confident relative comparisons between culture media from male and female embryos.

Finally, we also reported differences in lipids released by male and female embryos (Gómez et al., 2018a, b). G6PD, a X chromosome linked enzyme involved in the PPP, is expressed at a four times greater level in female than male embryos (Tiffin et al., 1991). The PPP produces $NADP^+$ for the synthesis of lipids and other molecules. However, in our study, only one lipid, Dimethyl adipate, was affected by sex when we did not include culture, breed and stages in our models, suggesting that metabolic differences are hidden by such factors. In fact, when subdividing by subsets, we found 11 lipids that changed with sex, all of them were or contained FA, and/or had a role in the FA metabolism. In line with our findings in Holstein and culture with FCS, no differences in the constitutive lipid profile in the embryonic cells have been reported between Holstein male and female embryos cultured with estrus cow serum (Janati Idrissi et al., 2021). However, active lipid release can reflect metabolic differences induced by different in vitro production systems and breeds.

5 Conclusions

In this study, we have shown that the non-invasive metabolomic study of sex biomarkers requires paying particular attention to embryonic stages, culture factors and embryonic breed. On this basis, the use of subsets improves sex identification. Diagnosis of embryonic sex cannot be performed with a single biomarker, and combinations of biomarkers are needed for a more correct sex identification. The depletion and release of specific metabolites can help understand metabolic needs to make more suitable culture conditions in favor of embryos bearing one or other sex. Ultimately, a better knowledge of the embryonic metabolism and biomarkers can sustain a finely tuned evaluation of embryonic quality, currently based on "static" markers and morphological evaluations.

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Compliance with Ethical Standards

Conflict of interest I. Gimeno declares she has not conflict of interest; P. García- Manrique declares he has not conflict of interest; S. Carrocera declares she has not conflict of interest; C. López-Hidalgo declares she has not conflict of interest; M. Muñoz declares she has not conflict of interest; L. Valledor declares he has not conflict of interest; D. Martín-González declares he has not conflict of interest; E. Gomez declares he has not conflict of interest.

Ethical approval All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

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4| CAPÍTULO II

THE METABOLIC SIGNATURE OF IN VITRO PRODUCED BOVINE EMBRYOS HELPS PREDICT PREGNANCY AND BIRTH AFTER EMBRYO TRANSFER

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The Metabolic Signature of In Vitro Produced Bovine Embryos Helps Predict Pregnancy and Birth after Embryo Transfer

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Abstract: In vitro produced (IVP) embryos show large metabolic variability induced by breed, culture conditions, embryonic stage and sex and gamete donors. We hypothesized that the birth potential could be accurately predicted by UHPLC-MS/MS in culture medium (CM) with the discrimination of factors inducing metabolic variation. Day-6 embryos were developed in single CM (modified synthetic oviduct fluid) for 24 h and transferred to recipients as fresh (28 ETs) or frozen/thawed (58 ETs) Day-7 blastocysts. Variability was induced with seven bulls, slaughterhouse oocyte donors, culture conditions (serum + Bovine Serum Albumin [BSA] or BSA alone) prior to single culture embryonic stage records (Day-6: morula, early blastocyst, blastocyst; Day-7: expanding blastocyst; fully expanded blastocysts) and cryopreservation. Retained metabolite signals (6111) were analyzed as a function of pregnancy at Day-40, Day-62 and birth in a combinatorial block study with all fixed factors. We identified 34 accumulated metabolites through 511 blocks, 198 for birth, 166 for Day-62 and 147 for Day-40. The relative abundance of metabolites was higher within blocks from non-pregnant (460) than from pregnant (51) embryos. Taxonomy classified lipids (12 fatty acids and derivatives; 224 blocks), amino acids (12) and derivatives (3) (186 blocks), benzenoids (4; 58 blocks), tri-carboxylic acids (2; 41 blocks) and 5-Hydroxy-L-tryptophan (2 blocks). Some metabolites were effective as single biomarkers in 95 blocks (Receiver Operating Characteristic – Area Under the Curve [ROC-AUC]: 0.700–1.000). In contrast, more accurate predictions within the largest data sets were obtained with combinations of 2, 3 and 4 single metabolites in 206 blocks (ROC-AUC = 0.800–1.000). Pregnancy-prone embryos consumed more amino acids and citric acid, and depleted less lipids and cis-aconitic acid. Big metabolic differences between embryos support efficient pregnancy and birth prediction when analyzed in discriminant conditions.

Keywords: bovine; embryo; metabolomics; mass-spectrometry; liquid-chromatography; dimethyl adipate; 12-hydroxydodecanoic acid

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1. Introduction

Accurate selection of competent in vitro produced (IVP) embryos for transfer to recipients is essential to maximize pregnancy and birth rates. Currently, the selection of viable IVP embryos is based on morphology and development stage, subjective criteria that rarely allow birth rates over 45% with either fresh or cryopreserved embryos [1–5]. The interest in identifying reliable markers of embryonic viability is higher in IVP, and mainly with respect to cryopreserved embryos, because of their intrinsic reduced viability to term. The lack of embryonic competence entails the use of more than usual numbers

of embryos and recipients in order to achieve the planned born calf objectives, as well as more labor and farm inputs, thus leading to increased costs. Pregnancy biomarkers can lead to greater confidence in the preliminary embryo production and cryopreservation step prior to making the embryo transfer (ET) to recipients. Therefore, finding efficient markers of embryonic quality is a major objective for reducing both experimental and productive costs on farms.

In search of biomarkers, the individual quality of produced embryos has been assayed (i.e., predicted) by invasive and non-invasive approaches.

Biopsy is a typically invasive approach performed to collect embryonic cells or blastocoele fluid. Cell biopsy allows the study of chromosomal stability and expression of certain regulated genes linked to highly competent embryos [6–9]. With cell biopsy there is a risk of embryo damage that may lower pregnancy rates, leading to the misidentification of embryos with pregnancy potential, which may be analyzed as false negatives [7,9]. Biopsy for the extraction of blastocoele fluid seems to inflict less damage than cell removal [10], and metabolic profiles, proteins and genomic DNA are detectable in such fluid [11–13]. Biopsy requires great expertise, and, currently, no molecules have been identified in cattle blastocoele fluid that are associated with pregnancy. As minimal or no damage to each embryo is required in biomarker identification, non-invasive systems are potentially more advantageous than invasive systems.

Non-invasive systems include time-lapse monitoring of embryo morphology and development kinetics, which has been reported to be effective in predicting pregnancy with sophisticated equipment [14,15]. Time-lapse can also be coupled with measurement of oxygen consumption, a non-invasive tool that can be used as a predictor itself [16,17]. A third type of non-invasive procedure aims to identify and quantify molecules released into and consumed from the CM by the embryo that can be associated with pregnancy success. Collection of CM from a single embryo culture step, immediately before any operation that the embryo would undergo within normal embryo culture (i.e., transfer or cryopreservation), does not impact embryonic viability. Thus, proteins, metabolites and the contents of extracellular vesicles (EV), such as small RNAs that the embryo releases, can be identified in CM [18–20]. Up to now, however, studies in CM of cattle embryos had described a metabolic fingerprint profile (Fourier Transform Infrared Spectroscopy) associated with pregnancy competence [21,22] or embryonic sex [23], but without identification of the molecules involved. The real association of molecules with the pregnancy potential of cattle embryo has only recently been shown in metabolites, by analysis of CM from vitrified/warmed (V/W) embryos by gas chromatography coupled with quadrupole time of flight mass spectrometry (GC-qTOF-MS) [24] and by electrospray ionization [25]. In our study, short-chain fatty acid (FA) metabolites were identified as candidate biomarkers predictive of birth [24].

However, other metabolomics techniques, such as Ultra High-Performance Liquid Chromatography (UHPLC), yielded a wider range and greater sensitivity than GC-qTOF-MS as untargeted platforms for metabolite identification and quantification [23,26–28]. To facilitate metabolomic studies of embryo CM, we developed a 24 h, single culture step (SCS) in synthetic oviduct fluid (SOF) w/o protein, which allows direct injection (i.e., without protein extraction) into the chromatograph, allowing for reliable metabolite identification for pregnancy prediction [24] and diagnosis of embryonic sex [26–28]. The SCS takes place as a last culture step, and it covers all embryonic stages comprised between compact morula and fully expanded blastocyst (FEB). Crucially, the SCS produces embryos with high viability after transfer as fresh, frozen/thawed (F/T) and V/W, and it can potentially be preceded by other embryo culture systems (e.g., serum and/or BSA containing) with or without group culture [1].

Interestingly, UHPLC-MS has not yet been used as a platform to investigate the pregnancy and birth potential of cattle embryos cultured in SCS. Nevertheless, the great complexity of factors that influence the bovine embryo metabolism in culture needs to be deciphered and overcome to reach positive results in metabolite biomarkers research.

The morula to blastocyst is a very dynamic period in metabolic changes within early in vitro development [29,30]. In parallel to progress in blastulation, the embryo increases glycolytic flux and mitochondrial respiration [31,32] because of strict epigenetic control [33]. Such major changes are the tip of the iceberg in terms of the metabolic changes that arise when combined with breed, embryonic stage and culture conditions, whose certainly considerable impact has been evaluated in a companion study with UHPLC-MS during the development of morula and blastocysts in SCS [26]. Furthermore, the discrimination of embryonic stages has been shown to perfect pregnancy predictions in metabolomics [24].

We hypothesized that UHPLC-MS may be an efficient platform for discovery of metabolite pregnancy biomarkers with frozen and fresh IVP embryos. Together with a high variability in random factors, we included several fixed, controllable factors, supportive of a supervised strategy that improved predictions within embryonic stages, culture conditions, breed and cryopreservation status of the embryos.

2. Results

A total of n = 84 ETs were performed in 21 ET rounds. Supplementary Table S1 shows a summary of samples used in this study, while pregnancy and birth rates are shown in Table 1.

Table 1. Descriptive pregnancy rates (%) at gestational endpoints Day-40, Day-62 and birth of frozen and fresh in vitro produced embryos arranged by culture conditions from Day-0 to Day-6.

Cryopreservation	Culture	Day	*n*	Day-40	Day-62	Birth
Fresh	BSA	7	17	12 (70.6)	11 (64.7)	9 (52.9)
Fresh	BSA + FCS	7	11	8 (72.7)	8 (72.7)	7/10 (70.0) [(1)]
Frozen	BSA	7	30	18 (60)	18 (60)	14 (46.7)
		8	8	2 (25)	2 (25)	1 (12.5)
Frozen	BSA + FCS	7	18	11 (61.1)	10 (55.5)	8 (44.4)

[(1)] One recipient deceased after pregnancy Day-62 and did not reach birth. Day: age of the cultured embryo counted from the onset of in vitro fertilization.

We obtained a total of 118,564 aligned spectral features which, after peak area processing, led to 6111 features retained. Sample signals were thereafter subtracted with their corresponding blank. The resulting output data were submitted to statistical analysis. Metabolic profiles typically obtained by UHPLC-TOF MS-MS are shown in Figure 1.

2.1. Multivariate Statistics for Sample Separation at Gestational Endpoints and Non-Random Factors

Multivariate analysis within the entire dataset with 6111 retained features did not yield significant sample separation by sPLS-DA and oPLS-DA at any pregnancy stage. Sample separation by including each of non-random factors (i.e., either breed, embryonic stage(s), culture or cryopreservation) did not lead to significant sample separation either. Using combinations of two factors, however, we identified differences when sample separation was performed by culture condition and cryopreservation. Thus, frozen embryos cultured in BSA showed clear separation by sPLS-DA (Figure 2A,C,E) and significant separation by OPLS-DA (Figure 2B,D,F) at gestational endpoints Day-40, Day-62 and birth, respectively.

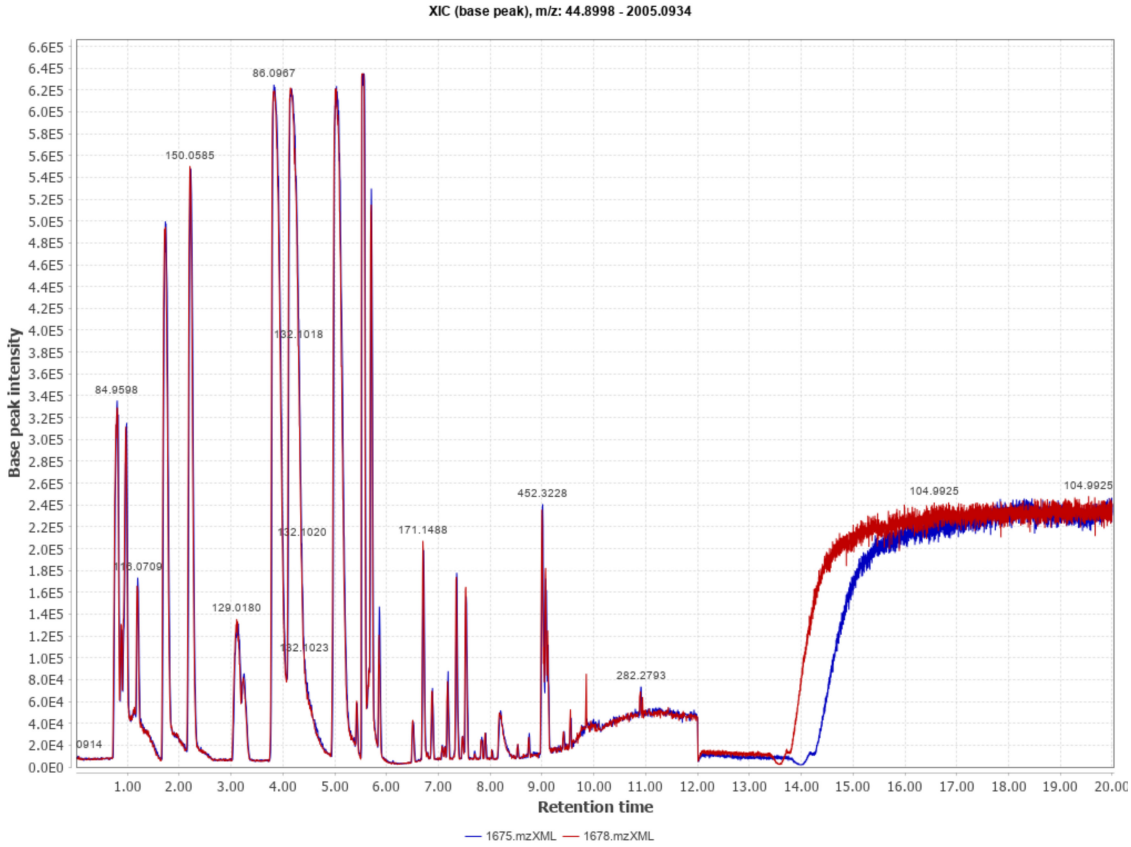

Figure 1. Metabolic spectral profile obtained by UHPLC-TOF MS-MS from two samples of embryo culture medium (CM) in positive-ion mode (range 100–1500 *m/z*). The total ion count (TIC) chromatograms correspond to a CM sample from one embryo diagnosed as pregnant (blue) and one embryo diagnosed as non-pregnant (red) on Day-40.

Interestingly, this separation included both Day-7 and Day-8 embryos and represented the largest di-factorial dataset (n = 38 samples) in the multivariate study. On the contrary, embryos with FCS (n = 18 samples frozen, and n = 11 samples fresh), did not show clear discrimination at any endpoint. However, fresh embryos cultured with BSA (n = 17) were predictive of pregnancy at Day-62 by sPLS-DA and OPLS-DA (Figure 3C,D), although not at Day-40 (Figure 3A,B) and birth (Figure 3E,F). No other combination with two or more factors was significant for discrimination by multivariate analysis.

2.2. The Bull: Influence of a Random Factor

We analyzed the individual bull (n = 7) as a random factor by multivariate analysis as a function of pregnancies on Day-62. Bull data were not analyzed at birth because one bull had only two birth samples, one bull had only two no-birth samples, and a third bull lost one recipient by sudden death after Day-62. Between-bull sample separation was not evident by sPLS-DA, with just Bull B-open and Bull G-pregnant outside of a narrow cluster formed by the remainder groups (Figure 4A). Separation by single bull and pregnant status was nevertheless more evident and significant within OPLS-DA (Figure 4B); interestingly, overlap between bulls and type of samples was not observable, suggesting that precluding individual variability (i.e., use of a single bull in biomarker studies) can hide the identification of metabolites that would act as biomarkers.

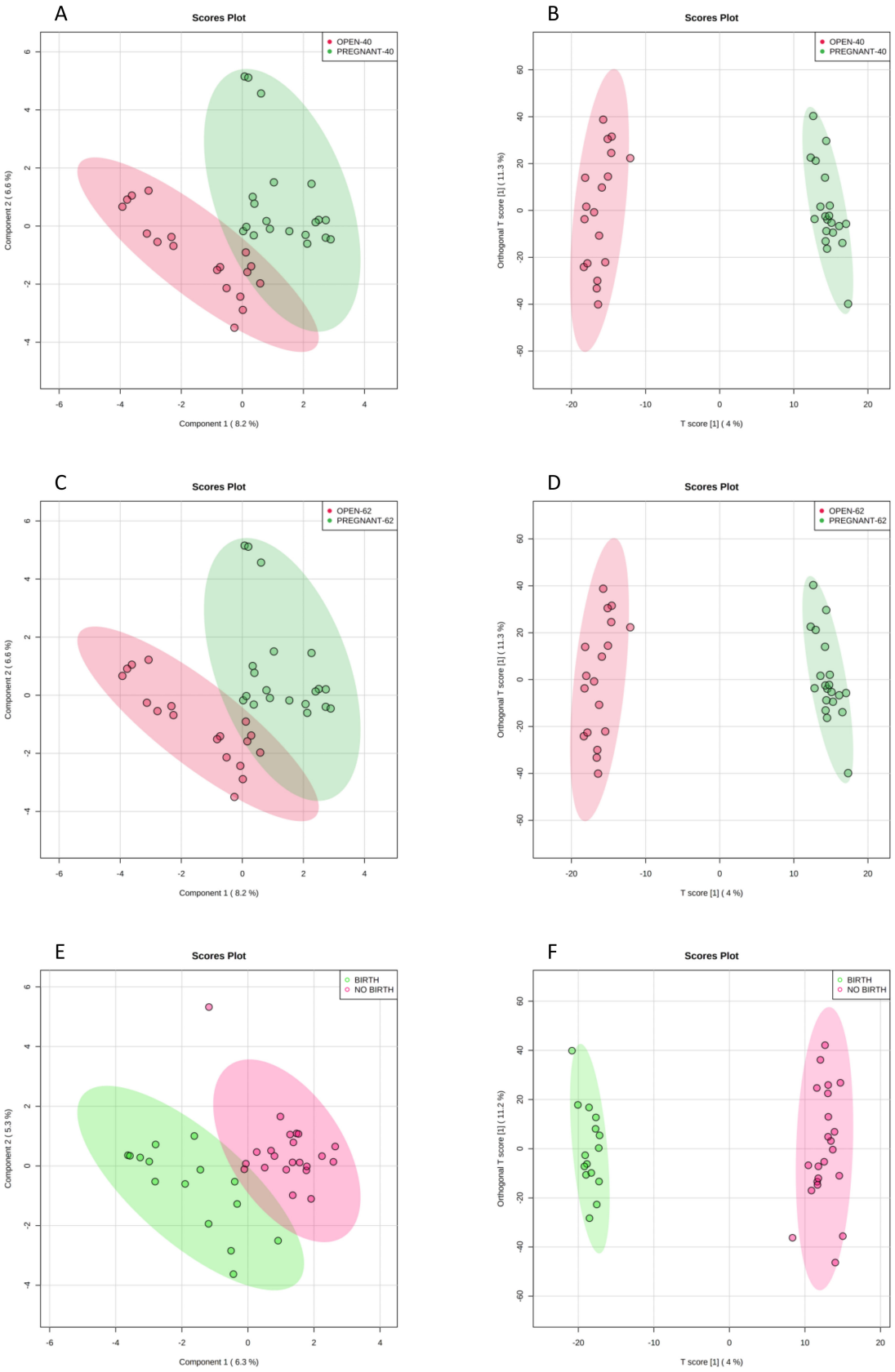

Figure 2. Separation of frozen embryos cultured in BSA by sparse partial least square-discriminant analysis (sPLS-DA) (**A**,**C**,**E**) and by orthogonal partial least square-discriminant analysis (OPLS-DA) (**B**,**D**,**F**) measured by gestational endpoints Day-40, Day-62 and birth, respectively. Figure 3B, Q2: $p < 0.06$, R2Y: $p < 0.03$. Figure 3D: Q2: $p < 0.07$, R2Y: $p < 0.04$. Figure 3F: Q2: $p < 0.03$, R2Y: $p < 0.01$. Empirical p-values were obtained after 100 permutations.

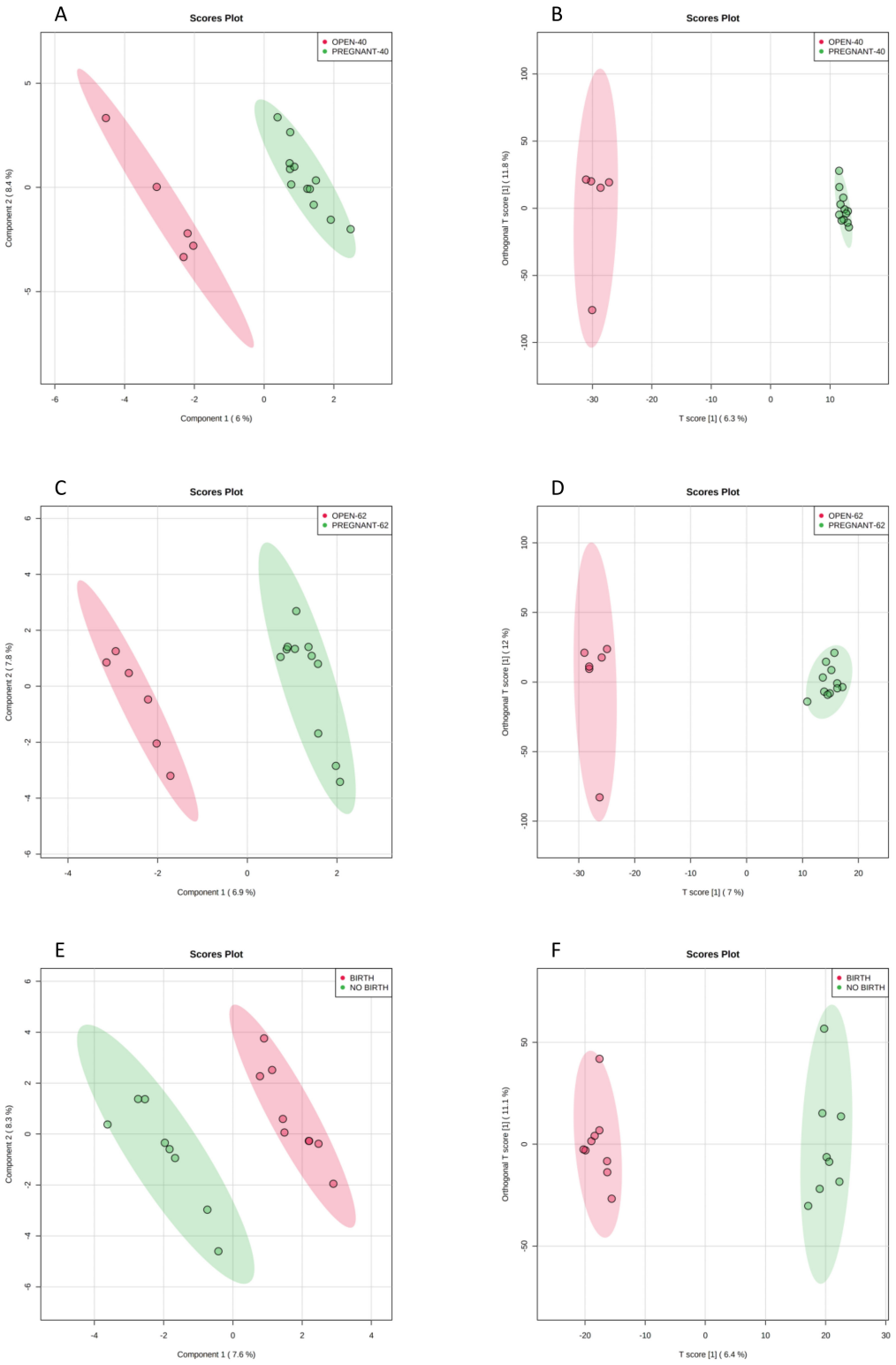

Figure 3. Separation of fresh embryos cultured with BSA was predictive of pregnancy at Day-62 (**C**,**D**) (Q2: $p < 0.07$, R2Y: $p < 0.04$), although not for pregnancy at Day-40 (**A**,**B**) and birth (**E**,**F**) (Q2 and R2Y > 0.10). Empirical p-values were obtained after 100 permutations.

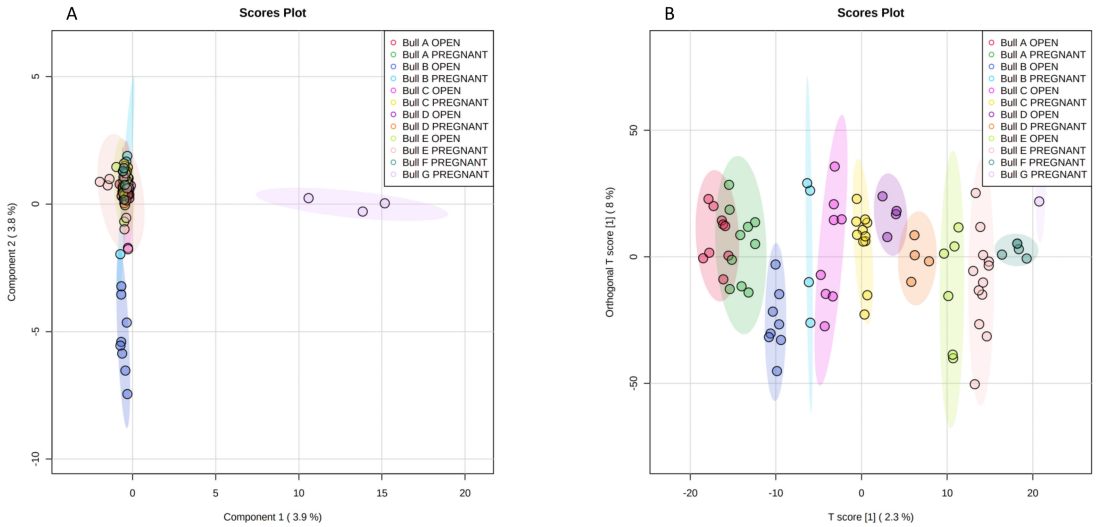

Figure 4. Separation of pregnant and non-pregnant samples from 7 different bulls as a function of pregnancy on Day-62, by sPLS-DA (**A**) and by OPLS-DA (**B**) (Q2: $p < 0.01$, R2Y: $p < 0.01$; 2 samples of Bull G-pregnant hidden down the legend). Empirical *p*-values were obtained after 100 permutations.

2.3. Block Analysis with Fixed Factors to Identify Metabolite Biomarkers

The multi-factorial combination of the 6111 retained metabolite signals led to obtaining 17,331 blocks starting from metabolite signals defined by combining the fixed factors controllable in the laboratory, i.e., bull breed, culture medium, cryopreservation, embryonic stage at the onset of single culture (Day-6 or Day-7) and embryonic stage at the end of single culture (Day-7 or Day-8). Of those, 3946 blocks contained metabolite signals predictive of pregnancy at Day-40, Day-62 and birth. Metabolite identity was explored at this stage, and only those metabolites with a compound mass below 10 ppms to their exact mass, and also with at least three MS2 ions, were considered to have been confidently identified (see Materials and Methods and Supplementary Table S2).

After metabolite identification, the study yielded 511 blocks with 34 significantly accumulated metabolites (frozen and fresh), 198 for birth, 166 for Day-62 and 147 for Day-40 (Supplementary Table S3). Log FChs in metabolite relative concentrations expressed in all tables and figures are shown as pregnant/non-pregnant ratio. In their majority, Log FChs can be considered as qualitative (e.g., when >|99.000|), with higher abundance of metabolites in CM from non-pregnant recipients (positive FCh: 51; negative FCh: 460). The value of a metabolite as a biomarker was therefore given both by its ROC-AUC value > 0.700 and by the proportion of pregnancies correctly predicted under the embryo culture conditions of each block. Of 34 significantly accumulated hits, 33 metabolites were represented in blocks of frozen embryos (with 16 metabolites of frozen embryos not represented within fresh embryos); 17 metabolites were in blocks independent of embryo cryopreservation; and only one block was exclusive of fresh embryos (5-Hydroxy-L-tryptophan) (Supplementary Table S3). Three metabolites had more blocks at birth within fresh than within frozen embryos (L-glutamic acid, L-lysine and phenylacetaldehyde). As reviewed in our published database for sex analysis within developmental transitions [26], eight metabolites present in 15 blocks were significantly affected by embryonic sex. 12-Hydroxydodecanoic acid was the metabolite most affected by sex, with 5/43 blocks involved. As Supplementary Table S3 is large and contains blocks that partially overlap, the most relevant information was extracted to be shown in smaller tables through the main text.

The overall performance within the selected blocks under study is shown in Figure 5.

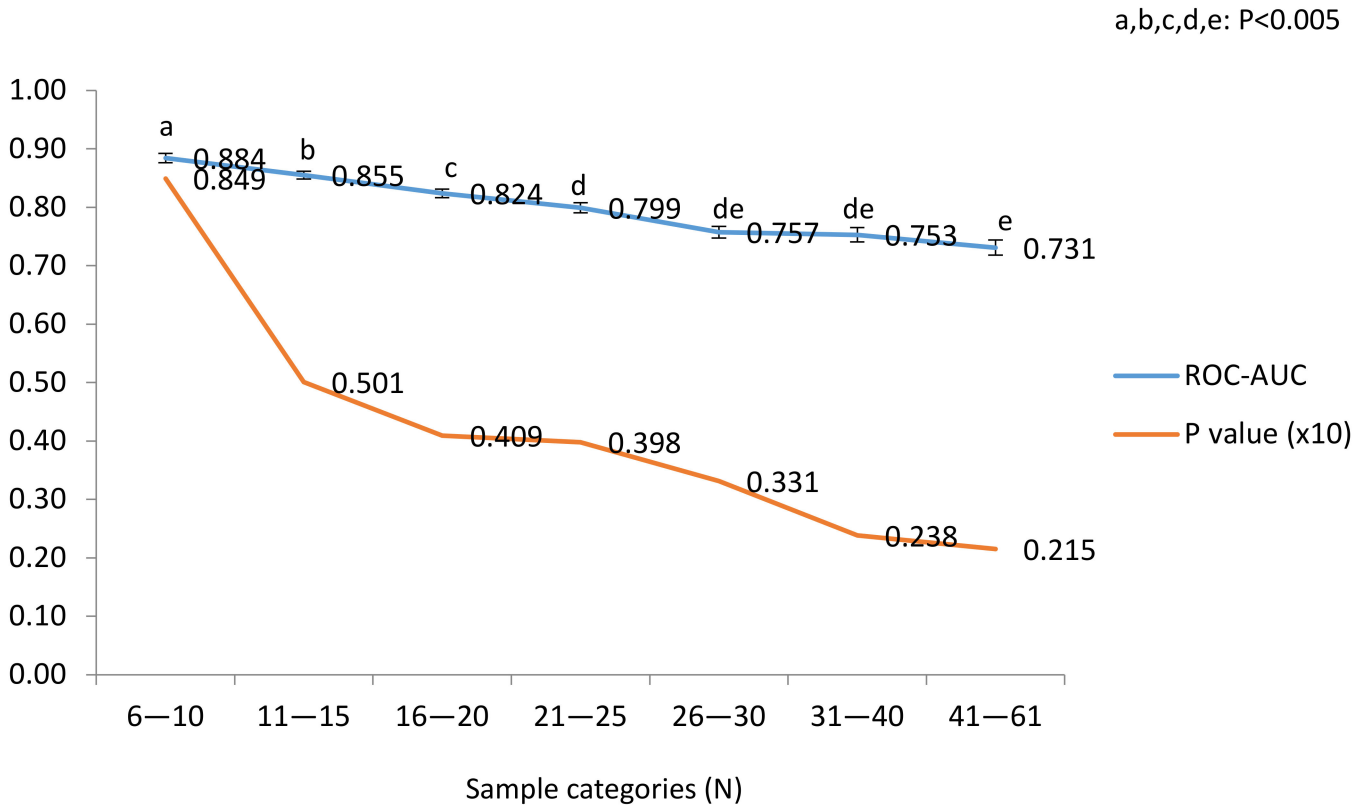

Figure 5. ROC-AUC and their *p*-value averages plotted by categories of sample numbers (Pearson's correlation coefficients are shown). *p*-values given in a ×10 basis for scale consistency with ROC-AUC. Data from $n = 511$ blocks distributed into 78, 131, 106, 85, 52, 30 and 29 blocks from lower to higher sample categories.

Average ROC-AUC values (LSM ± SEM) decreased with sample numbers, but their significance (*p*-values, shown in a ×10 basis for consistency of scale) increased. Negative correlations were recorded between ROC-AUC and sample numbers (R: −0.6086; $p < 0.0001$) and between *p*-values and sample numbers (R: −0.34114; $p < 0.0001$). Interestingly, the ROC-AUC slope was attenuated in categories above >21 samples, close to the plateau ROC-AUC ≈ 0.700 and within the number of samples covered in this study.

2.4. Univariate Statistics with Candidate Biomarker Metabolites

The metabolites previously identified in blocks were analyzed for pregnancy endpoints in the entire dataset using a GLM model that included all effects identified (cryopreservation, culture, bull breed, embryonic stages—0 h and 24 h—and embryonic age), and a Bonferroni correction ($p < 0.10$) as a false discovery rate test (Table 2).

L-Lysine was the only metabolite that significantly differed between pregnant and open recipients at the three pregnancy endpoints (Day-40, Day-62 and Birth), while L-Leucine, Palmitoylethanolamide and Lauroyl diethanolamide differed for predicting birth but not for earlier endpoints. On the contrary, concentrations of L-Valine, Dimethyl adipate and Phosphatidylethanolamine (18:2/20:2) changed as a function of early pregnancy endpoints, but not birth. Miscarriages that occurred after Day-40 were $n = 11$ cases; interestingly, embryos that experienced such late miscarriage differed in their levels of Dimethyl adipate (discriminated both pregnancy to term embryos vs. late miscarriage—Figure 6—and vs. open embryos; $p = 0.0011$).

Table 2. Probability of changes in relative concentrations of metabolites that differed in culture medium between transferred embryos later diagnosed as pregnant or open at Day-40, Day-62 and birth, and between embryos that miscarried after Day-40 vs. embryos that never reached pregnancy and vs. embryos that reached pregnancy to term.

	Birth		Day-62		Day-40		Miscarriage	
Metabolite	***p*-Value**	**Bon**	***p*-Value**	**Bon**	***p*-Value**	**Bon**	***p*-Value**	**Bon**
L-Leucine	0.0454	0.05						
L-Lysine	0.0494	0.05	0.009	0.05	0.0143	0.05		
Palmitoylethanolamide	0.0782	0.10						
L-Valine			0.0439	0.05	0.0729	0.05		
L-Glutamic acid			0.0740	0.05	0.0844	0.05		
Dimethyl adipate			0.0774	0.05	0.0266	0.05	0.0018	0.05
Lauroyl diethanolamide	0.0112	0.05						
Phosphatidylethanolamine(18:2/20:2)			0.0518	0.05	0.0168	0.05		

GLM analysis parametrized with the following factors: embryo cryopreservation, culture, bull breed, embryonic stages -0 h and 24 h-, and embryonic age. Significant *p*-values are stated as $p < 0.05$, and tendencies $0.05 > p < 0.10$. Bonferroni (Bon) as a false discovery rate value at levels $p < 0.05$ and $p < 0.10$.

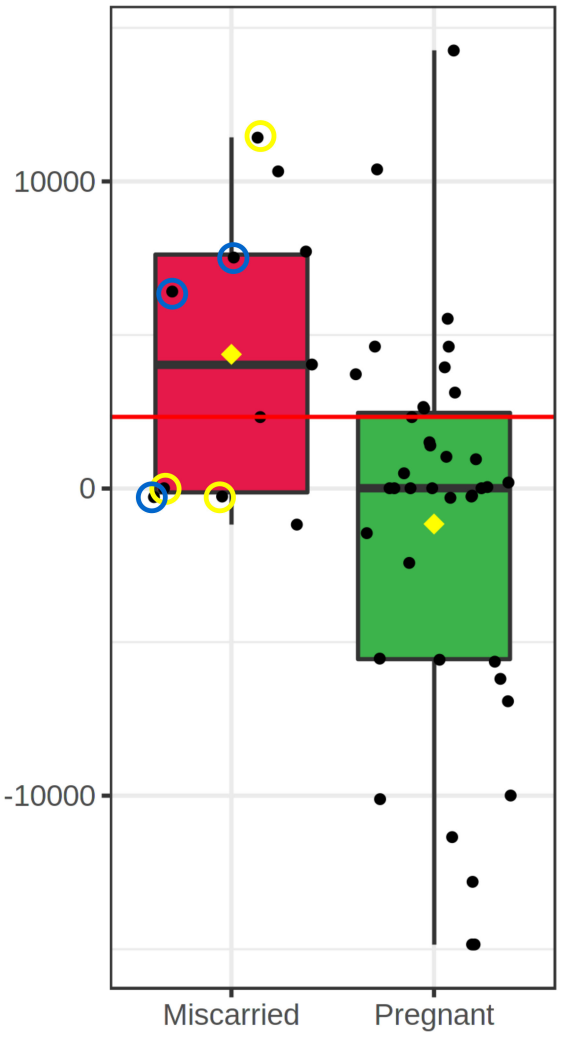

Figure 6. Boxplot for Methyl adipate concentrations that differ between embryos that miscarried after Day-40 and embryos that led to pregnancy on Day-40. Circled samples indicate fresh embryos cultured in BSA (yellow line), and frozen embryos cultured with FCS (blue line); non-circled samples are frozen embryos cultured with BSA (no miscarried samples from fresh embryos cultured with FCS).

Class metabolite analysis in all identified blocks. Metabolites were grouped into five taxonomical classes identified in total blocks at the three developmental endpoints (Table 3): Class (1) Lipid and lipid-like molecules (224 blocks—the most predictive class; 12 metabolites); Class (2) amino acids (186 blocks; 15 metabolites); Class (3) benzenoids (58 blocks; 4 metabolites); Class (4) Tricarboxylic acids (41 blocks; 2 metabolites); and Class (5) Tryptamines and derivatives (2 blocks; 1 metabolite) (metabolite subclasses are defined in Supplementary Table S2).

Table 3. Taxonomical classification (Classes were lipids—1, amino acids and derivatives—2, benzenoids—3, tri-carboxylic acids—4 and tryptamines and derivatives—5) of metabolites identified as significantly predictive within blocks and pregnancy endpoints D40, D62 and Birth (numbers of blocks with Fz: Frozen embryos; Fh: Fresh embryos; FF: Frozen and Fresh embryos).

		Blocks (N) at Gestational Endpoints											
		Day-40			**Day-62**			**Birth**			**Total**		
Metabolite	**Class**	**Fz**	**Fh**	**FF**	**Fz**	**Fh**	**FF**	**Fz**	**Fh**	**FF**	**Fz**	**Fh**	**FF**
Citramalic acid	1	1	0	0	0	0	0	1	1	0	2	1	0
5Z-Dodecenoic acid	1	3	0	0	2	0	0	1	0	1	6	0	1
Dimethyl adipate	1	14	0	4	12	1	6	1	1	2	27	2	12
Lauroyl diethanolamide	1	4	0	0	3	0	3	7	1	5	14	1	8
Linoleamide	1	6	0	4	8	0	2	5	0	1	19	0	7
Oleamide	1	2	0	0	3	1	2	0	2	3	5	3	5
Palmitic amide	1	0	0	0	0	0	0	2	1	2	2	1	2
Palmitoylethanolamide	1	1	0	0	0	0	1	7	0	0	8	0	1
Phosphatidylethanolamine(18:2/20:2)	1	4	2	2	3	2	5	4	1	3	11	5	10
MG(16:0/0:0/0:0)	1	1	0	0	1	3	0	12	1	3	14	4	3
12-Hydroxydodecanoic acid	1	9	0	2	11	0	3	14	0	4	34	0	9
Dihydro-alpha-ionone	1	1	0	0	1	0	0	2	0	3	4	0	3
			60			**73**			**91**			**224**	
L-Threonine	2	4	0	1	1	0	0	4	0	0	9	0	1
L-Arginine	2	4	0	0	1	0	2	5	0	6	10	0	8
L-Glutamic acid	2	4	1	1	4	1	1	2	4	1	10	6	3
L-Glutamine	2	4	0	1	1	0	3	5	0	3	10	0	7
L-Leucine	2	1	0	0	1	2	0	2	1	1	4	3	1
L-Lysine	2	0	3	1	1	2	3	0	2	1	1	7	5
L-Methionine	2	2	0	1	3	0	0	7	0	3	12	0	4
L-Proline	2	3	0	1	2	0	1	3	0	2	8	0	4
L-Tryptophan	2	0	0	1	1	0	3	0	1	3	1	1	7
L-Tyrosine	2	0	0	1	2	0	2	0	0	2	2	0	5
L-Valine	2	5	1	2	3	0	1	0	0	0	8	1	3
Pipecolic acid	2	0	0	0	2	0	0	1	1	0	3	1	0
L-Histidine	2	2	0	2	2	0	5	3	0	2	7	0	9
L-Phenylalanine	2	1	0	0	1	0	1	1	0	1	3	0	2
Pyroglutamic acid	2	6	0	2	4	0	3	3	0	2	13	0	7
			55			**59**			**72**			**186**	
Benzoic acid	3	4	0	1	1	0	1	2	0	4	7	0	6
Indole	3	2	0	2	1	0	3	5	1	2	8	1	7
Phenylacetaldehyde	3	1	0	0	1	1	0	1	3	0	3	4	0
p-Cresol	3	7	0	1	8	0	3	2	0	1	17	0	5
			18			**19**			**21**			**58**	
cis-Aconitic acid	4	8	1	1	9	0	1	5	2	2	22	3	4
Citric acid	4	2	2	0	1	2	1	2	1	1	5	5	2
			14			**14**			**13**			**41**	
5-Hydroxy-L-tryptophan	5	0	0	0	0	1	0	0	0	1	0	1	1
Cumulative			**147**			**166**			**198**			**511**	

All lipids identified contained or were fatty acids (FA), and/or had a role in the FA metabolism. Interestingly, all amino acids (except pipecolic acid) and citrate were components of the SOF formula used. The abundance of predictive blocks increased with gestational endpoint, being maximal for birth (198 blocks), intermediate for Day-62 (166 blocks), and lowest for Day-40 (147 blocks). This increasing abundance was mainly observed through Classes 1, 2 and 3. As counted by embryo cryopreservation, frozen embryos recorded 309 blocks, and fresh embryos 51 blocks, while 151 blocks were independent of cryopreservation. The following metabolites had outstanding representation in blocks: 12-Hydroxydodecanoic acid (43 blocks) and methyl adipate (41 blocks), followed by cis-Aconitic acid, Linoleamide, phosphatidylethanolamine(18:2/20:2), Lauroyl diethanolamide, p-Cresol, MG(16:0/0:0/0:0), and pyroglutamic acid, with 29 to 20 blocks each.

2.5. Pregnancy Endpoint Analysis

2.5.1. Overview

Relevant predictions from early pregnancy stages (i.e., Day-40 and Day-62) were drawn from factors analyzed without using embryo cryopreservation and breed as factors. However, contrary to earlier diagnosis endpoints, accurate birth predictions required discrimination by cryopreservation stage (i.e., frozen vs. fresh), which was improved when combined with breed, culture conditions or both. In contrast, single embryonic stages showed lower ROC-AUCs than cryopreservation, breed or culture, although stages greatly improved birth predictions for all these latter factors (as observed in Supplementary Table S3). The discordance between early and late pregnancy predictive block patterns reflects a different late pregnancy course between fresh and frozen embryos, with marked metabolite differences, as seen also between embryos that led to miscarriage, failure of embryos to set pregnancies on Day-40 and pregnancies to term.

2.5.2. Impact of Biomarkers through Pregnancy Endpoints

The tracking of the impact of each biomarker through the developmental endpoints is depicted as a heatmap in Figure 7.

Metabolites were ranked by the number of "Total" blocks that were predictive at Day-40. The color scale accounts for the numbers of blocks for each metabolite through pregnancy endpoints with frozen and fresh embryos, blocks independent of cryopreservation, and total number of blocks. Generally, the arrangement by total blocks at Day-40 showed abundant metabolite blocks at later pregnancy endpoints (mainly in the top of Figure 7), in particular among the most abundant frozen over fresh embryos. Low abundance metabolites in Figure 7 at Day-40 are at the bottom, in a region that shows increasing abundance of metabolites through pregnancy endpoints, with contrasting metabolites that predicted at birth but not earlier (i.e., MG(16:0/0:0/0:0), indole and palmitoylethanolamide, as relevant metabolites). On the contrary, other metabolites displayed no or lower predictive ability at birth, but higher block numbers for Day-40 and/or Day-62 (i.e dimethyl adipate, pyroglutamic acid, linoleamide, p-cresol and L -valine). Interestingly, dimethyl adipate was strongly involved in miscarriage (see above "Univariate statistics within candidate biomarker metabolites"), and accounted for the most striking differences between abundant blocks on Day-40 (18 blocks) and on Day-62 (19 blocks), as compared with blocks at birth (4 blocks); such differences were more pronounced with frozen embryos. In contrast, Lauroyl diethanolamide, the other metabolite involved in miscarriage, showed increasing numbers of total blocks through Day-40, Day-62 and birth (i.e., 4, 6 and 13 blocks).

		Pregnancy D40				Pregnancy D62				Birth				
Metabolite	Class	Frozen	Fresh	Both	Total	Frozen	Fresh	Both	Total	Frozen	Fresh	Both	Total	
Dimethyl adipate	1	14	0	4	18	12	1	6	19	1	1	2	4	
12-Hydroxydodecanoic acid	1	9	0	2	11	11	0	3	14	14	0	4	18	
cis-Aconitic acid	4	9	1	1	11	8	1	0	9	5	2	2	9	
Linoleamide	1	6	0	4	10	8	0	2	10	5	0	1	6	
Phosphatidylethanolamine(18:2/20:2)	1	4	2	2	8	3	2	5	10	4	1	3	8	
Pyroglutamic acid	2	6	0	2	8	4	0	3	7	3	0	2	5	
p-Cresol	3	7	0	1	8	8	0	3	11	2	0	1	3	
L-Valine	2	5	1	2	8	3	0	1	4	0	0	0	0	19
L-Glutamic acid	2	4	1	1	6	4	1	1	6	2	4	1	7	18
L-Glutamine	2	4	0	1	5	1	0	3	4	5	0	3	8	16
Benzoic acid	3	4	0	1	5	1	0	1	2	2	0	4	6	14
L-Threonine	2	4	0	1	5	1	0	0	1	4	0	0	4	13
Lauroyl diethanolamide	1	4	0	0	4	3	0	3	6	7	1	5	13	12
L-Arginine	2	4	0	0	4	1	0	2	3	5	0	6	11	11
Indole	3	2	0	2	4	1	0	3	4	5	1	2	8	10
L-Histidine	2	2	0	2	4	2	0	5	7	3	0	2	5	9
L-Proline	2	3	0	1	4	2	0	1	3	3	0	2	5	8
Citric acid	4	2	2	0	4	1	2	1	4	2	1	1	4	7
L-Lysine	2	0	3	1	4	1	2	3	6	0	2	1	3	6
L-Methionine	2	2	0	1	3	3	0	0	3	7	0	3	10	5
5Z-Dodecenoic acid	1	3	0	0	3	2	0	0	2	1	0	1	2	4
Oleamide	1	2	0	0	2	3	1	2	6	0	2	3	5	3
MG(16:0/0:0/0:0)	1	1	0	0	1	1	3	0	4	12	1	3	16	2
Palmitoylethanolamide	1	1	0	0	1	0	0	1	1	7	0	0	7	1
Dihydro-alpha-ionone	1	1	0	0	1	1	0	0	1	2	0	3	5	0
L-Tryptophan	2	0	0	1	1	1	0	3	4	0	1	3	4	
L-Leucine	2	1	0	0	1	1	2	0	3	2	1	1	4	
Phenylacetaldehyde	3	1	0	0	1	1	1	0	2	1	3	0	4	
L-Tyrosine	2	0	0	1	1	2	0	2	4	0	0	2	2	
L-Phenylalanine	2	1	0	0	1	1	0	1	2	1	0	1	2	
Citramalic acid	1	1	0	0	1	0	0	0	0	1	1	0	2	
Palmitic amide	1	0	0	0	0	0	0	0	0	2	1	2	5	
Pipecolic acid	2	0	0	0	0	2	0	0	2	1	1	0	2	
5-Hydroxy-L-tryptophan	5	0	0	0	0	0	1	0	1	0	0	1	1	

Figure 7. Heatmap representative of abundance of blocks within metabolites that predicted pregnancy at the developmental endpoints D-40, D-62 (predictive blocks shown correspond to fresh and frozen, as well as non-cryopreservation dependent, embryos). The number of blocks per metabolite is ranked by the column "Total" within blocks at Pregnancy Day-40. Taxonomical classes (Class): (1) Lipids; (2) Amino acids; (3) Benzenoids; (4) Carboxylic acids; (5) Tryptamines and derivatives.

2.6. Single Biomarker Metabolites Predict Pregnancy with >70% Effectiveness

Hits bearing ROC-AUC > 0.700, with which it is possible to accurately predict pregnancy in specific blocks with ≥70% effectiveness as single metabolites, are shown in Table 4.

This selection consisted of 95 blocks, all independent from the embryonic stage at the onset of individual culture (0 h), and only 6 blocks in which fresh embryos were dependent on embryonic stage at 24 h (end of individual culture). This dependence at 24 h meant the exclusion of two embryos (one early blastocyst and one blastocyst) transferred in the fresh dataset, and one block with FEB fresh embryos. Therefore, insofar as ExB and FEB are involved, the embryonic stage generally did not influence the predictions with single metabolites shown in Table 3. Birth was once again the most predictive endpoint (37 blocks), followed by 29 blocks for Day-62 and 28 blocks for Day-40. Twenty blocks predicted with fresh embryos, while 74 blocks predicted with frozen embryos, and 1 block was independent of cryopreservation (indole). By breed, 73 blocks predicted within AV and eight in Holstein; while 13 blocks were independent of breed. Class summary reflects 23 blocks with nine metabolites (lipids); 46 blocks with 11 metabolites (amino acids and derivatives); 15 blocks with four metabolites (benzenoids); and 12 blocks with two metabolites for carboxylic acids. The following top 11 hits predicted with 90% to 100% accuracy: Lauroyl diethanolamide, L-Glutamic acid, L-Proline, L-Methionine, Pyroglutamic acid, L-Glutamic acid, L-Arginine, L-Lysine, L-Threonine, L-Glutamine, L-Methionine, all belonging to the amino acid and derivatives class, except for one lipid.

Table 4. Single metabolites that predicted pregnancy (Day-40 and Day-62) and birth (Endpoint) with >0.700 coverage within culture medium of IVP embryos transferred to recipients. Each block had specific embryo cryopreservation, bull breed and culture conditions, and is supported by embryonic stages. Embryos were sired by Holstein—H—or Asturiana de los Valles—AV—bulls, and cultured with albumin (BSA) or with albumin + fetal calf serum (FCS) followed by an individual 24 h culture step (IC stage) developmentally defined by embryonic stages at 0 h (M: morula; EB: early blastocyst; B: Blastocyst) and at 24 h (ExB: expanding blastocyst; FEB: Fully expanded blastocyst) that led to embryos aged 7, 8 and both days that were transferred fresh or frozen (Cryo) to Day-7 estrus synchronized recipients. N: samples used to calculate predictions within each metabolite block (P: pregnant; O: Open). Impact: proportions of embryos represented within IC stages developed under each culture conditions. Unfilled cells indicate the independence of this factor. P1: *p*-value by *t*-test; P2: *p*-value by GLM or Kruskal–Wallis test. LogFCh: logarithm of fold change pregnant/non-pregnant metabolite concentration values. AUC: area under curve.

	ROC-Analysis								IC Stage			N		Coverage	
Metabolite	**AUC**	**P1**	**LogFCh**	**P2**	**Endpoint**	**Cryo**	**Breed**	**Culture**	**0 h**	**24 h**	**Age**	**P**	**O**	**Impact**	**Predicted**
Lauroyl diethanolamide	1.000	0.000	−99.000	0.003	Birth	Frozen	AV	BSA				8	5	100.000	100.000
L-Glutamic acid	1.000	0.110	−99.000	0.004	Birth	Fresh	H	BSA				8	4	100.000	100.000
L-Proline	1.000	0.000	−99.000	0.003	P40	Frozen	AV	BSA				9	4	100.000	100.000
L-Proline	1.000	0.000	−99.000	0.003	P62	Frozen	AV	BSA				9	4	100.000	100.000
L-Proline	0.952	0.010	−99.000	0.005	Birth	Frozen	AV	FCS				6	7	100.000	95.238
L-Proline	0.952	0.010	−99.000	0.005	P40	Frozen	AV	FCS				6	7	100.000	95.238
L-Proline	0.952	0.010	−99.000	0.005	P62	Frozen	AV	FCS				6	7	100.000	95.238
L-Methionine	0.944	0.024	−99.000	0.011	P40	Frozen	AV	BSA				9	4	100.000	94.444
L-Methionine	0.944	0.024	−99.000	0.011	P62	Frozen	AV	BSA				9	4	100.000	94.444
Pyroglutamic acid	0.929	0.084	−99.000	0.008	Birth	Frozen	AV	FCS				6	7	100.000	92.857
Pyroglutamic acid	0.929	0.084	−99.000	0.008	P40	Frozen	AV	FCS				6	7	100.000	92.857
Pyroglutamic acid	0.929	0.084	−99.000	0.008	P62	Frozen	AV	FCS				6	7	100.000	92.857
L-Glutamic acid	0.920	0.110	−99.000	0.019	Birth	Fresh	H					10	5	100.000	92.000
L-Arginine	0.905	0.016	−99.000	0.014	Birth	Frozen	AV	FCS				6	7	100.000	90.476
L-Arginine	0.905	0.016	−99.000	0.014	P40	Frozen	AV	FCS				6	7	100.000	90.476
L-Arginine	0.905	0.016	−99.000	0.014	P62	Frozen	AV	FCS				6	7	100.000	90.476
L-Lysine	0.905	0.008	−99.000	0.008	P62	Fresh	AV					7	6	100.000	90.476
L-Threonine	0.905	0.014	−99.000	0.014	Birth	Frozen	AV	FCS				6	7	100.000	90.476
L-Threonine	0.905	0.014	−99.000	0.014	P40	Frozen	AV	FCS				6	7	100.000	90.476
L-Threonine	0.905	0.014	−99.000	0.014	P62	Frozen	AV	FCS				6	7	100.000	90.476
L-Glutamine	0.900	0.021	−99.000	0.003	Birth	Frozen		FCS				8	10	100.000	90.000
L-Methionine	0.900	0.025	−99.000	0.019	Birth	Frozen	AV	BSA				8	5	100.000	90.000
L-Methionine	0.900	0.025	−99.000	0.019	Birth	Frozen	AV	BSA				8	5	100.000	90.000
12-Hydroxydodecanoic acid	0.889	0.029	−99.000	0.035	P40	Frozen	AV	BSA				9	4	100.000	88.889
12-Hydroxydodecanoic acid	0.889	0.029	−99.000	0.035	P62	Frozen	AV	BSA				9	4	100.000	88.889
Benzoic acid	0.889	0.021	−99.000	0.034	P40	Frozen	AV	BSA				9	4	100.000	88.889
Benzoic acid	0.889	0.021	−99.000	0.034	P62	Frozen	AV	BSA				9	4	100.000	88.889
Dihydro-alpha-ionone	0.889	0.019	−99.000	0.036	P40	Frozen	AV	BSA				9	4	100.000	88.889

Table 4. *Cont.*

	ROC-Analysis								IC Stage			N		Coverage	
Metabolite	**AUC**	**P1**	**LogFCh**	**P2**	**Endpoint**	**Cryo**	**Breed**	**Culture**	**0 h**	**24 h**	**Age**	**P**	**O**	**Impact**	**Predicted**
Dihydro-alpha-ionone	0.889	0.019	−99.000	0.036	P62	Frozen	AV	BSA				9	4	100.000	88.889
L-Lysine	0.889	0.011	−3.512	0.026	Birth	Fresh	AV					6	6	100.000	88.889
Citric acid	0.881	0.069	−99.000	0.022	P62	Fresh	AV					7	6	100.000	88.095
citramalic acid	0.861	0.027	−99.000	0.045	Birth	Fresh	AV					6	6	100.000	86.111
Phenylacetaldehyde	0.860	0.021	−99.000	0.045	Birth	Fresh	H					10	5	100.000	86.000
MG(16:0/0:0/0:0)	0.857	0.062	−99.000	0.035	P62	Fresh	AV					7	6	100.000	85.714
cis-Aconitic acid	0.850	0.023	−99.000	0.047	Birth	Frozen	AV	BSA				8	5	100.000	85.000
Phenylacetaldehyde	0.846	0.070	−99.000	0.082	P62	Fresh	H					13	3	100.000	84.615
L-Histidine	0.833	0.020	−99.000	0.051	Birth	Frozen	AV	FCS				6	7	100.000	83.333
L-Histidine	0.833	0.020	−99.000	0.051	P40	Frozen	AV	FCS				6	7	100.000	83.333
L-Histidine	0.833	0.020	−99.000	0.051	P62	Frozen	AV	FCS				6	7	100.000	83.333
MG(16:0/0:0/0:0)	0.833	0.098	−99.000	0.065	Birth	Fresh	AV					6	6	100.000	83.333
Dimethyl adipate	0.818	0.003	−20.168	0.003	P40	Frozen	AV					15	11	100.000	81.818
Dimethyl adipate	0.818	0.003	−20.168	0.003	P62	Frozen	AV					15	11	100.000	81.818
p-Cresol	0.813	0.050	11.024	0.050	P62	Frozen		FCS			7	10	8	100.000	81.250
Citric acid	0.810	0.085	2.460	0.073	Birth	Frozen	AV	FCS				6	7	100.000	80.952
Citric acid	0.810	0.085	−2.460	0.073	P40	Frozen	AV	FCS				6	7	100.000	80.952
Citric acid	0.810	0.085	−2.460	0.073	P62	Frozen	AV	FCS				6	7	100.000	80.952
Citric acid	0.800	0.043	−99.000	0.093	P40	Fresh	AV					8	4	100.000	80.000
Indole	0.800	0.054	−1.835	0.093	Birth	Frozen	AV	BSA				8	5	100.000	80.000
Indole	0.800	0.054	−1.835	0.093	Birth	Frozen	AV	BSA				8	5	100.000	80.000
L-Lysine	0.800	0.091	3.031	0.093	P40	Fresh	AV					8	5	100.000	80.000
L-Proline	0.800	0.050	−99.000	0.093	Birth	Frozen	AV	BSA				8	5	100.000	80.000
L-Proline	0.800	0.050	−99.000	0.093	Birth	Frozen	AV	BSA				8	5	100.000	80.000
12-Hydroxydodecanoic acid	0.799	0.007	−99.000	0.011	Birth	Frozen	H					9	21	100.000	79.894
5-Hydroxy- L -tryptophan	0.798	0.063	−99.000	0.086	P62	Fresh	AV					7	6	100.000	79.762
L-Valine	0.791	0.014	−22.286	0.014	P40	Frozen	AV					15	11	100.000	79.091
L-Valine	0.791	0.014	−22.286	0.014	P62	Frozen	AV					15	11	100.000	79.091
12-Hydroxydodecanoic acid	0.788	0.003	−99.000	0.003	Birth	Frozen		BSA				15	33	100.000	78.841
Indole	0.786	0.052	−99.000	0.100	Birth	Frozen	AV	FCS				6	7	100.000	78.571
Indole	0.786	0.052	−99.000	0.100	P40	Frozen	AV	FCS				6	7	100.000	78.571
Indole	0.786	0.052	−99.000	0.100	P62	Frozen	AV	FCS				6	7	100.000	78.571
MG(16:0/0:0/0:0)	0.784	0.020	5.508	0.018	P62	Fresh						19	9	100.000	78.363
L-Glutamic acid	0.780	0.002	−99.000	0.004	P40	Fresh	AV			ExB + FEB		8	3	100.000	77.950

Table 4. *Cont.*

Metabolite	ROC-Analysis								IC Stage			N		Coverage	
	AUC	P1	LogFCh	P2	Endpoint	Cryo	Breed	Culture	0 h	24 h	Age	P	O	Impact	Predicted
p-Cresol	0.771	0.045	11.457	0.045	P40	Frozen	AV				7	15	8	100.000	77.083
p-Cresol	0.771	0.045	11.457	0.045	P62	Frozen	AV				7	15	8	100.000	77.083
L -Leucine	0.770	0.031	−99.000	0.017	P62	Fresh						19	9	100.000	77.005
L-Glutamic acid	0.762	0.018	−14.573	0.018	P62	Fresh	AV				7	7	6	100.000	76.190
L-Glutamine	0.762	0.033	15.617	0.033	Birth	Frozen	AV				7	14	9	100.000	76.190
12-Hydroxydodecanoic acid	0.762	0.017	−99.000	0.049	Birth	Frozen	H	BSA				7	18	100.000	76.190
MG(16:0/0:0/0:0)	0.759	0.026	5.619	0.026	P62	Fresh				ExB + FEB		19	7	100.000	75.940
Citric acid	0.759	0.033	−99.000	0.024	P62	Fresh						19	9	100.000	75.936
Dimethyl adipate	0.756	0.017	17.092	0.017	Birth	Frozen	AV					14	12	100.000	75.595
Dimethyl adipate	0.750	0.054	−18.047	0.054	P40	Frozen	AV				7	15	8	100.000	75.000
Lauroyl diethanolamide	0.750	0.006	−9.767	0.006	Birth	Frozen	AV					14	12	100.000	75.000
L-Leucine	0.750	0.065	−99.000	0.065	P62	Fresh	AV					7	6	100.000	75.000
Indole	0.744	0.017	−99.000	0.037	Birth	Frozen	AV					14	12	100.000	74.405
cis-Aconitic acid	0.739	0.026	−21.627	0.026	P40	Frozen	AV					15	11	100.000	73.939
Pyroglutamic acid	0.739	0.060	−99.000	0.041	P40	Frozen	AV					15	11	100.000	73.939
Pyroglutamic acid	0.739	0.060	−99.000	0.041	P62	Frozen	AV					15	11	100.000	73.939
L-Methionine	0.739	0.034	−99.000	0.013	Birth	Frozen		BSA				15	33	100.000	73.913
cis-Aconitic acid	0.738	0.014	−16.641	0.014	P40	Frozen		BSA			7	18	12	100.000	73.843
L-Glutamic acid	0.738	0.043	8.994	0.043	Birth	Frozen	AV					14	12	100.000	73.810
Phosphatidylethanolamine(18:2/20:2)	0.738	0.094	−99.000	0.090	Birth	Frozen		FCS				8	10	100.000	73.750
L-Valine	0.736	0.006	4.109	0.018	P40	Fresh	AV			ExB + FEB		8	3	100.000	73.602
cis-Aconitic acid	0.733	0.015	−99.000	0.020	P40	Fresh	AV			ExB + FEB		8	3	100.000	73.292
Palmitoylethanolamide	0.730	0.086	−99.000	0.085	Birth	Frozen	H	BSA				7	18	100.000	73.016
L-Glutamine	0.725	0.064	−13.100	0.064	P40	Frozen	AV				7	15	8	100.000	72.500
L -Methionine	0.720	0.064	−99.000	0.063	Birth	Frozen	H					9	21	100.000	71.958
Oleamide	1.000	0.011	3.786	0.009	P62	Fresh		BSA		FEB		6	3	71.490	71.490
Lauroyl diethanolamide	0.713	0.022	−99.000	0.022	Birth	Frozen		BSA				15	33	100.000	71.304
Citric acid	0.713	0.052	2.972	0.088	P40	Fresh						20	8	100.000	71.250
Indole	0.706	0.011	−4.439	0.032	Birth		AV					20	18	100.000	70.556
L-Lysine	0.705	0.011	3.605	0.040	P40	Fresh	AV			ExB + FEB		8	3	100.000	70.497
L-Threonine	0.702	0.037	−99.000	0.085	Birth	Frozen	AV					14	12	100.000	70.238
Phosphatidylethanolamine(18:2/20:2)	0.700	0.055	−5.441	0.055	P40	Frozen	AV				7	15	8	100.000	70.000

2.7. Combinations of Biomarker Metabolites Increase Pregnancy Prediction Rates

We combined the predictive power of single metabolites to obtain overall predictions >0.800 within blocks and series. A series is defined by the same conditions for cryopreservation, breed and culture, supported by one or more developmental IC-stages. Metabolite combinations generally (but not in all cases) gave higher predictions for larger data sets than did single metabolites. A general list of predictive-compliant series (17) with their blocks and metabolites that can be used for combinations is shown in Supplementary Table S4. For clarity, the best combination from each predictive series is shown in Table 5.

Overall combinations (Supplementary Table S4) were more informative for birth (9 series; 89 blocks), and decreased towards Day-62 (5 series; 56 blocks) and Day-40 (3 series; 45 blocks). By classes, lipids were the most represented in blocks (90), followed by amino acids (68), benzenoids (17), carboxylic acids, (13) and tryptamines and derivatives (1). Dimethyl adipate (18), 12-Hydroxydodecanoic acid (17), linoleamide (13), pyroglutamic acid (9) and cis-aconitic acid (9) were the most represented metabolites. This representation is consistent with the general relevance of each single metabolite. Interestingly, nine series (88 blocks) were independent on cryopreservation, the remainder being 83 blocks for frozen embryos (five series) and 18 blocks for fresh embryos (three series). Culture showed nine independent series (103 blocks), with BSA and FCS being necessary in six series (75 blocks) and two series (11 blocks), respectively. Combined predictions showed great independence of bull breed (14 series), and only the Holstein breed was required as a factor (three series; nine blocks), with AV breed not being necessary. Collectively, efficient predictions can be made with little or no dependence on fixed factors. Thus, as shown in Table 5, predictive information was obtained from very basic conditions (i.e., independent of cryopreservation, culture and bull) at the three endpoints with three (Day-40), four (Day-62) and five (birth) combined metabolites (as seen in series 3, 8 and 17, respectively). On the contrary, 11 series with only two combined metabolites each required only one or more conditions to be predictive, as shown with series 4 and 5, showing combinations of three and four metabolites on Day-62, and series 15 with all metabolites at birth. Interestingly, whatever the path, all original metabolites shown in Supplementary Table S4 can form combinations resulting in predictive values >0.800 and, in some cases, also reaching the highest predictive values summarized in Table 5.

2.8. Validation

Biomarker validation: The strong random and pre-planned variability with which our study was designed through different block studies ensures a principle of proof validation (different populations, samples and conditions). Thus, we based the relevance of biomarkers primarily on the numbers of blocks in which they participate with ROC-AUC > 0.700, either singly or combined, and, for practical purposes, on the total number of embryos whose pregnancy probability was correctly identified (as a function of ROC-AUC and frequency of appearance of the embryos in question within each block).

Table 5. Best metabolite block combinations (combined coverage) that predicted pregnancy (Day-40 and Day-62 and birth) (Endpoint) with >0.800 coverage within IVP embryos transferred to recipients. Each series consists of blocks with the same embryo cryopreservation, bull breed and culture conditions, and supported by embryonic stages. IVP embryos (sired by Holstein—H—or Asturiana de los Valles—AV—bulls) were cultured with albumin (BSA) or with albumin + fetal calf serum (FCS) followed by a single 24 h culture step (IC stage) developmentally defined by embryonic stage at 0 h (M: morula; EB: early blastocyst; B: Blastocyst) and at 24 h (ExB: expanding blastocyst; FEB: fully expanded blastocyst) that led to embryos aged 7 or 8 days that were transferred fresh or frozen (Cryo) to Day-7 estrus synchronized recipients. N: samples used to calculate predictions within each metabolite block (P: pregnant; O: Open). Impact: proportion of embryos represented within IC stages developed under each culture conditions. No filled data indicates the independence of this factor. Asterisks indicate blocks aggregated within a series to form the combined average value that is shown below the respective asterisks.

							IC Stage			N		Single Coverage		Combined
Series	**Metabolite**	**Class**	**Endpoint**	**Cryo**	**Breed**	**Culture**	**0 h**	**24 h**	**Age**	**P**	**O**	**Impact**	**Predicted**	**Coverage**
1	p-Cresol	3	P40	Frozen	H		EB + B			11	10	56.371	44.071	*
1	citramalic acid	1	P40	Frozen	H		M			5	4	43.630	39.267	83.3385
2	Dimethyl adipate	1	P40	Frozen			EB + B			21	20	56.371	47.781	*
2	Linoleamide	1	P40	Frozen			M			10	5	43.630	38.394	86.1751
3	L -Proline	2	P40				EB + B	ExB		8	2	15.871	12.998	*
3	Linoleamide	1	P40				EB + B	FEB		28	23	40.500	34.977	*
3	Linoleamide	1	P40				M	ExB		8	3	20.080	18.825	*
3	L -Tyrosine	2	P40				M	FEB		7	3	23.550	23.550	90.3504
4	12-Hydroxydodecanoic acid	1	P62	Frozen		BSA	EB	FEB		3	8	23.770	22.780	*
4	Pyroglutamic acid	2	P62	Frozen		BSA	B	FEB		10	6	10.790	9.531	*
4	L -Tyrosine	2	P62	Frozen		BSA	M	FEB		6	3	36.930	36.930	*
4	L -Histidine	2	P62	Frozen		BSA		ExB		7	3	28.510	25.795	95.0353
5	Dimethyl adipate	1	P62	Frozen			EB			6	13	41.470	35.090	*
5	Phosphatidylethanolamine(18:2/20:2)	1	P62	Frozen			B		7	13	6	14.900	11.462	*
5	Linoleamide	1	P62	Frozen			M			4	3	43.630	38.176	84.7278
6	Palmitoylethanolamide	1	P62			BSA		ExB		7	3	28.510	28.510	*
6	Dimethyl adipate	1	P62			BSA		FEB		24	19	71.490	53.539	82.0489
7	Pyroglutamic acid	2	P62			FCS		ExB		8	3	43.380	37.958	*
7	L -Glutamine/D-Glutamine	2	P62			FCS		FEB		10	8	56.620	49.543	87.5000
8	Dimethyl adipate	1	P62				EB			15	16	41.470	29.374	*
8	12-Hydroxydodecanoic acid	1	P62				B			20	10	14.900	10.430	*
8	L -Lysine	2	P62				M	ExB		7	4	20.080	17.211	*
8	L -Tyrosine	2	P62				M	FEB		7	3	23.550	23.550	80.5658

Table 5. *Cont.*

							IC Stage			N		Single Coverage		Combined
Series	**Metabolite**	**Class**	**Endpoint**	**Cryo**	**Breed**	**Culture**	**0 h**	**24 h**	**Age**	**P**	**O**	**Impact**	**Predicted**	**Coverage**
9	citramalic acid	1	Birth	Frozen	H		M			5	4	43.630	39.267	87.3472
9	MG(16:0/0:0/0:0)	1	Birth	Frozen	H		EB + B			4	17	56.370	48.080	*
10	12-Hydroxydodecanoic acid	1	Birth	Frozen		BSA	EB + B			9	19	42.992	36.956	*
10	Pipecolic acid	2	Birth	Frozen		BSA	M			6	4	57.010	46.321	83.2765
11	Pyroglutamic acid	2	Birth	Frozen			M			10	5	43.628	34.902	*
11	12-Hydroxydodecanoic acid	1	Birth	Frozen			EB + B			13	28	56.370	46.924	81.8259
12	L -Glutamic acid	2	Birth	Fresh	H		EB + B			8	5	56.370	50.733	*
12	L-Lysine	2	Birth	Fresh			M			4	4	43.630	40.903	91.6361
13	L -Glutamic acid	2	Birth	Fresh		BSA	EB + B			6	4	42.990	42.990	*
13	Indole	3	Birth	Fresh		BSA	M			3	4	57.010	57.010	100.0000
14	cis-Aconitic acid	4	Birth	Fresh		BSA		FEB		5	4	71.490	64.341	*
14	Phenylacetaldehyde	3	Birth	Fresh		BSA		ExB + B6		4	3	28.510	28.510	92.8510
15	Oleamide	1	Birth			BSA	EB			8	11	29.684	25.299	*
15	cis-Aconitic acid	4	Birth			BSA	B			7	12	13.308	12.199	*
15	Indole	3	Birth			BSA	M	ExB		3	5	20.079	20.079	*
15	L -Tyrosine	2	Birth			BSA	M	FEB		6	3	36.927	36.927	94.5039
16	Citric acid	4	Birth			FCS		ExB		7	4	43.381	37.184	*
16	L -Glutamine	2	Birth			FCS		FEB		8	9	56.618	49.541	86.7245
17	L -Proline	2	Birth				EB	ExB		4	4	12.610	11.034	*
17	Dimethyl adipate	1	Birth				EB	FEB		8	15	28.863	23.090	*
17	L -Methionine	2	Birth				B			13	16	14.898	11.531	*
17	L -Glutamine	2	Birth				M	ExB		7	4	20.075	18.068	*
17	L -Tyrosine	2	Birth				M	FEB		6	4	23.553	23.553	87.2759

Taxonomical classes (Class): (1) Lipids; (2) Amino acids; (3) Benzenoids; (4) Carboxylic acids; (5) Tryptamines and derivatives.

3. Discussion

In this study, we used fresh and frozen IVP embryos transferred to recipients to non-invasively identify high numbers of metabolite biomarkers that predicted pregnancy in spent CM.

Thus, multivariate analysis pointed out that supervision with at least two factors favors discrimination between pregnant and open recipients, focused on the fresh and frozen BSA datasets. Interestingly, the frozen dataset included Day-7 and Day-8 embryos, suggesting that quality profiles of competent embryos do not differ with age or retarded development. Rather, the lower survival of Day-8 embryos [5,24,34] could be due to lower rates of high-quality embryos that essentially do not differ in metabolism from Day-7 counterparts. The bull was the more powerful effect identified (in line with 26), with almost complete separation of single bulls and pregnancy status (given as a function between metabolite signals and pregnancy on Day-62). This random bull effect must be counteracted with individual variability (seven bulls in our study), since the way to discriminate biomarkers (e.g., higher ROC-AUC, high FCh, and statistical tests with the lowest *p*-values) is hierarchical, and therefore highly dependent on specific bull interactions (i.e., a potential risk of misidentification with a single bull). In our study, we identified abundant numbers of metabolites that predicted pregnancy at the three stages diagnosed. Herein, smaller sample datasets with fixed factors (i.e., blocks) helped to discover metabolite biomarkers, as hypothesized. In contrast, in our previous study with lower sample numbers to accomplish a factorial study (n = 36 V/W embryos from Holstein and AV) [24], embryos were clustered together by discriminant analysis. The present analysis (n = 84 embryos), nevertheless, did benefit from discriminant factorial strategies, as many more biomarkers were obtained.

The number of predictive metabolites and blocks was higher in frozen than in fresh embryos, which is not surprising, because the number of samples in the frozen dataset was approximately double that in the fresh dataset. The interest, within the field of biomarker studies, is potentially higher in frozen embryos, as their birth rates are lower than fresh embryos (i.e., more added value is expected), and international exchanges of high-cost, genetic-merit embryos are made with cryopreservation. However, more than a few metabolites qualified both for fresh and F/T embryos, suggesting that common predictions are possible. Thus, metabolites with different representation between frozen and fresh embryos could shed light on the metabolic facts than make them different. Among these, the biggest differences were recorded for birth within 12-Hydroxydodecanoic acid, lauroyl diethanolamide, L -Methionine, MG (16:0/0:0/0:0) and palmitoylethanolamide, and, at Day-40 and Day-62, dimethyl adipate, 12-Hydroxydodecanoic acid, cis-aconitic acid, linoleamide and p-Cresol. Again, this greater representation of lipids is in agreement with our recent study with V/W embryos [24]. The only metabolite not represented exclusively in frozen embryos (5-Hydroxy- L -tryptophan) had low incidence, being present in two blocks. Interestingly, the low impact of embryonic sex on specific developmental transitions, metabolites and blocks, confirmed in a sex-specific larger dataset [26], permitted us to discard sex as a possible confounder for pregnancy prediction (consistent with [25]). The scarcity of metabolites with sex dependence within blocks facilitates pregnancy prognosis. Independence of sex is interesting, even with sex-sorted spermatozoa, since the efficiency of sex sorting is high but not 100% [35]. The vast amount of information obtained in our study was unexpected, given the limited information in previous studies analyzing metabolomic biomarkers in CM for pregnancy or even for short-term in vitro developmental endpoints [24,25,36].

Testing single metabolites with ROC-AUC > 0.700 and/or multiple combinations of metabolites with ROC-AUC > 0.800 took advantage of a variety of factors to achieve higher pregnancy predictive coverage of embryos that are metabolically diverse. Difficulties in obtaining a single biomarker predictive of embryo development have been cited [36], and are consistent with recent studies [24,25]. Fortunately, the factors selected (breed, cryopreservation, stages, and culture supplements) are under normal control in an IVP laboratory, and new agreement and validation studies are needed to refine which of the

identified candidate biomarkers are finally effective. Dairy, beef and crossbred cattle breeds markedly differ in metabolism [37–39]. Although breed-linked metabolism does not have an extensive influence on early embryos [24], metabolomic differences measured in embryo CM yield breed-specific (dairy and beef) changes in certain metabolites [26]. Culture systems are also potential sources of metabolic variation, as oxygen tension and culture composition (glucose, culture with FCS and BSA) can induce changes in the carbohydrate, lipid and amino acid metabolism in embryos [31,40]. However, although testing the large assortment of laboratory culture conditions is impractical, the use of a common analytical SCS (24 h), which is highly tolerant with embryonic competence, can led to efficient inter-laboratorial biomarker identification. Thus, failure to identify the abundance and/or consistency of cattle biomarkers in previous studies could be attributable to lower sample data sets, no incorporation of specific factors or lack of normalizing conditions.

The breed bulls in our study are used worldwide (Holstein), while the AV is a double-muscled cattle breed comparable to Belgian Blue, Piamontese and other commercial beef breeds; therefore, our general findings and those in AV have wide impact. Metabolic differences imposed by individual bull (random) and breed bull (fixed) effects (with 20 and 15 metabolites affected, respectively; [26]) suggest counteracting random variability in biomarker studies using sufficient bulls (seven in our study). Our study also lacks some stage transitions with low incidence in culture and/or shows unbalanced pregnant and open samples (e.g., in culture with FCS, Day-6 morulae develop to Day-7 at very low rates and with low viability). We should also confront our results within recipients evaluated for their pregnancy competence [24]; the information obtained from embryos could be obtained with an even greater precision and confidence, and with wider prediction ability. The adequacy of our procedures, with blocks showing increased statistical significance value above the 21-sample threshold, suggests appropriate predictive consistency with stability over the value ROC-AUC = 0.700.

Amounts of predictive blocks were higher for birth and decreased through earlier pregnancy stages, as shown with previous studies with fresh [21] and V/W embryos [21,24]. In contrast, superior-quality in vivo embryos collected from FSH-superstimulated cows that were cultured in vitro for 24 h showed higher predictive ability by FTIR on Day-60 than at birth [22]. Such differences could entail a differential signature for embryo losses (between Day-60 and birth) present within IVP embryos, not within in vivo embryos. In this regard, biomarkers that differed in their abundance between pregnancy endpoints could be predictive of specific embryo developmental competence. Miscarriage is a difficult topic to investigate because of obscure etiology, low incidence, and the influence of conditions such as cryopreservation and the presence of serum [3]. The cases of dimethyl adipate (and, to a lesser extent, p-cresol), highly pregnancy predictive for Day-40 and Day-62, but with low predictive ability at birth, are consistent with their pregnancy and miscarriage identification in the general dataset. The effects of dimethyl adipate on reproductive organs are unknown. However, in rats, the compound may induce heat shock protein [41], although it is not known as teratogenic [42]. A contrasting pattern was shown by other metabolites, with MG (16:0/0:0/0:0) and palmitoylethanolamide being relevant, and predictive for birth in many blocks in frozen embryos, but not for earlier pregnancy endpoints. Such metabolites were not involved in general miscarriage, but they could inform of such differences in frozen embryos. Interestingly, the abundant presence of lipids as biomarkers is consistent with our former study wherein V/W embryos that did not reach pregnancy to term released higher amounts of non-esterified saturated FA (NEFAs) (stearic, capric and palmitic acids) and glyceryl-monostearate into the CM [24]. In the present study, all lipids identified were FAs or FA derivatives, with palmitic acid being present through many compounds (i.e., Phosphatidylethanolamine (18:2/20:2), palmitic amide, palmitoylethanolamide), an indication that the lipid stock and its breakdown determine embryonic quality. Interestingly, palmitic acid concentrations are higher within the less viable IVP than in vivo developed embryo [43], and FCS increases lipid contents and palmitic, palmitoleic, oleic and stearic acids [44] and reduces tolerance of embryos to cryopreservation [45–47]. In domestic

species, lipids are stored in blastomeres as trygliceride [48,49] with higher stocks in IVP embryos than in vivo collected embryos [43]. Lipid granules decrease through blastulation concurrent with an increase in lipolytic gene expression [40,50,51]. The lipid breakdown in embryos is consistent with the embryonic ability to develop in a medium deprived of exogenous substrate [52], and lipid granules decrease in our protein-free SCS [40].

IVP embryos show more oxidized but scarce glycerophospholipids, a group of membrane constituents. Slow freezing alters glycerophospholipids in in vivo and IVP embryos [43] (lysophosphatidylcholines). Phosphatidylcholines are methylated phosphatidylethanolamines whose increase in membranes enhances embryo survival to cryopreservation [53]. In our work, phosphatidylethanolamine(18:2/20:2) had, however, a comparable predictive impact through frozen and fresh blocks. Palmitoylethanolamide accumulates during cellular stress, and it can counteract cellular stress and inflammation [54], and is found in amniotic and other reproductive fluids [55].

An excess of palmitic and other FAs impacts DNA methylation, therefore leading to epigenetic alteration, as observed within different cell types [56], and in fasting to postprandial transitions in obese humans [57,58]. These events are conceptually similar to the dynamic reprogramming during embryonic development, by which environmental stimuli (including assisted reproductive technologies) lead to acquisition of a stable, modified genotype later in life [59] and, in this case, loss of pregnancy competence. NEFAs are mainly responsible for low embryonic fitness [24,60–63]. Our results confirm findings with V/W embryos [24] in which fully viable embryos were those with less active lipid catabolism, as shown by reduced FA contents in their CM.

Although we sought a very active lipid metabolism generally associated with non-pregnancy-prone embryos, embryos that led to pregnancy and birth incorporated more amino acids. Therefore, a more active amino acid metabolism could be supportive of pregnancy, which is in agreement with a recent study [25]. Endogenous FA and amino acids can be used as nutrients by early embryos, but it is unclear whether our results match with an efficient use of metabolic resources by the most viable embryos, as postulated [64]. The use of amino acids as markers of embryo development was proposed in human embryos by Houghton [65]. Actually, embryos from different species, development stages, quality, morphology and sex differ in their profile of amino acid consumption [27,28,65–74]. Such metabolic complexity can distort the identification of viable embryos, hence the limited pregnancy predictive metabolomic studies in bovine embryos and the lack of molecular specification [21,22,24,25]. Most amino acid blocks showed negative FCh (163 blocks) vs. 23 blocks with positive FCh, reflecting higher amino acid intake in embryos that reached pregnancy and birth. Additionally, as with lipids, most blocks showed large FCh (qualitative) differences, typical within the exogenous protein-free CM we used over BSA containing CM [28].

Amino acids had a relevant presence as single biomarkers (23 out of 24 top ROC-AUC > 0.90 were amino acids and derivatives), as essential (L -Methionine, L -Arginine, L -Lysine, L -Threonine) and non-essential (L -Glutamic acid, L -Proline and L -Glutamine), together with the amino acid derivative Pyroglutamic acid. Within combinations of biomarkers, amino acids (20 hits) had a presence comparable to lipids (19 hits). A recent ESI-MS study identified higher depletion of glutamic acid by embryos that led to pregnancy on Day-60 [25]. Glutamic acid was present in 19 blocks, seven of them birth-predictive. Such authors also identified high pyruvate and low lactate as being predictive of pregnancy at Day-60 in a cohort of FEB embryos. We identified a regulated metabolite with mass corresponding to lactate, whose identification did not reach our standards (not shown); it had negative FCh in four blocks, consistent with [25].

Benzenoids were present in 58 blocks; 53 with negative and five positive FCh. Benzoic acids derivatives (e.g., hippuric acid) were the second most abundant subclass of metabolites present in the cow uterine fluid (UF) in the cycle days 0 and 5 [75]. Thus, P-cresol, an end-product of protein degradation which could act as a signaling metabolite, peaks in UF on Day-5 and decreases onwards [75]. Phenylacetaldehyde is an oxidation-related

aldehyde that may form from styrene [76]; styrene is used in laboratory plasticware. Indole is a potent antioxidant. Tryptophan is an indole derivative and the precursor of the neurotransmitters serotonin and melatonin (N-acetyl-5 methoxytriptamine). However, indole itself is a microbial metabolite, while 5-Hydroxy- L -tryptophan had a low representation in our study (two blocks), suggesting an unclear role. The Krebs cycle metabolites citric acid and cis-aconitic acid also decreased in CM from pregnant recipients. Citrate is a constituent of our SOF medium.

Our findings with amino acids, tri-carboxylic acids and FA metabolism, together with the influence of certain lipids on membrane function, can be integrated with mitochondrial function and morphology, as representative of the success of the embryonic viability [77]. Embryos with true pregnancy potential show active amino acid and citric acid intake, but lower lipid metabolism and lower depletion of cis-aconitic acid. Lower lipid breakdown corresponds to less release of lipids into CM; such embryos incorporate citrate for oxidative metabolism, which follows the Krebs cycle through cis-aconitic acid, which would be used and therefore non-excreted, as opposed to non-pregnancy-prone embryos. We suggest that less competent embryos accumulated more lipids in their cells.

The block design and the metabolic complexity observed between embryonic stages, under the influence of breed, culture conditions and ultimately cryopreservation, made it not possible to provide either a pathway study or a general in-depth description of the metabolic changes between embryonic transitions. Embryologists may check whether specific biomarkers identified in our work fit well with their particular embryo culture, breed and cryopreservation conditions. We consider it a practical advantage that several amino acids, citric acid and cis-aconitic acid from the embryo CM have analytical kits available in the market. The same occurs with some lipids identified (e.g., linoleamide and dodecenoic acid). Progress in refining pregnancy predictions is expected when more studies and trials become available.

An unexpected number of metabolites were predictive of pregnancy and birth under contrasting conditions. There was, however, a window for single biomarkers, and a larger window for combinations of biomarkers. Cryopreserved and fresh embryos share pregnancy and birth biomarkers, although many were specific to fresh or frozen embryos. Discovering embryonic biomarkers for pregnancy and birth is possible under the control of developmental stage, culture conditions and breed, as traits that make embryos differ in their metabolism. The information on the pregnancy predictive capacity of the metabolome in CM from cattle IVP embryos is novel, and selecting appropriate metabolites for targeted research work in particular laboratorial conditions is possible.

4. Materials and Methods

All reagents were purchased from SIGMA (Madrid, Spain) unless otherwise stated. Figure 8 describes the experimental procedures workflow.

4.1. Rationale

In this work, we described the conditions whereby metabolites can perform as biomarkers within in vitro embryo culture. The predictive value of metabolites is not only defined by specific ROC-AUCs, but also, from a practical point of view, the incidence of particular embryo stages for which a metabolite is predictive must be defined within a specific culture system.

This study analyzed the metabolomic profiles in CM from F/T and fresh (control) embryos. Embryos were transferred to recipients which were diagnosed as pregnant or open (i.e., non-pregnant) at specific time endpoints (Day-40, Day-62 and birth). Metabolites in CM were analyzed as a function by which to predict pregnancy at each endpoint. We previously showed that metabolites identified and quantified in the CM surrounding embryos, cultured singly, depend on controllable factors, such as culture conditions and embryonic stages at the onset and end of the embryo culture step, as well as, perhaps, the cryopreservation status, since recipients made pregnant with V/W and fresh embryos

differ in terms of their metabolic profiles [37,78], probably reflecting the fact that embryos also differ.

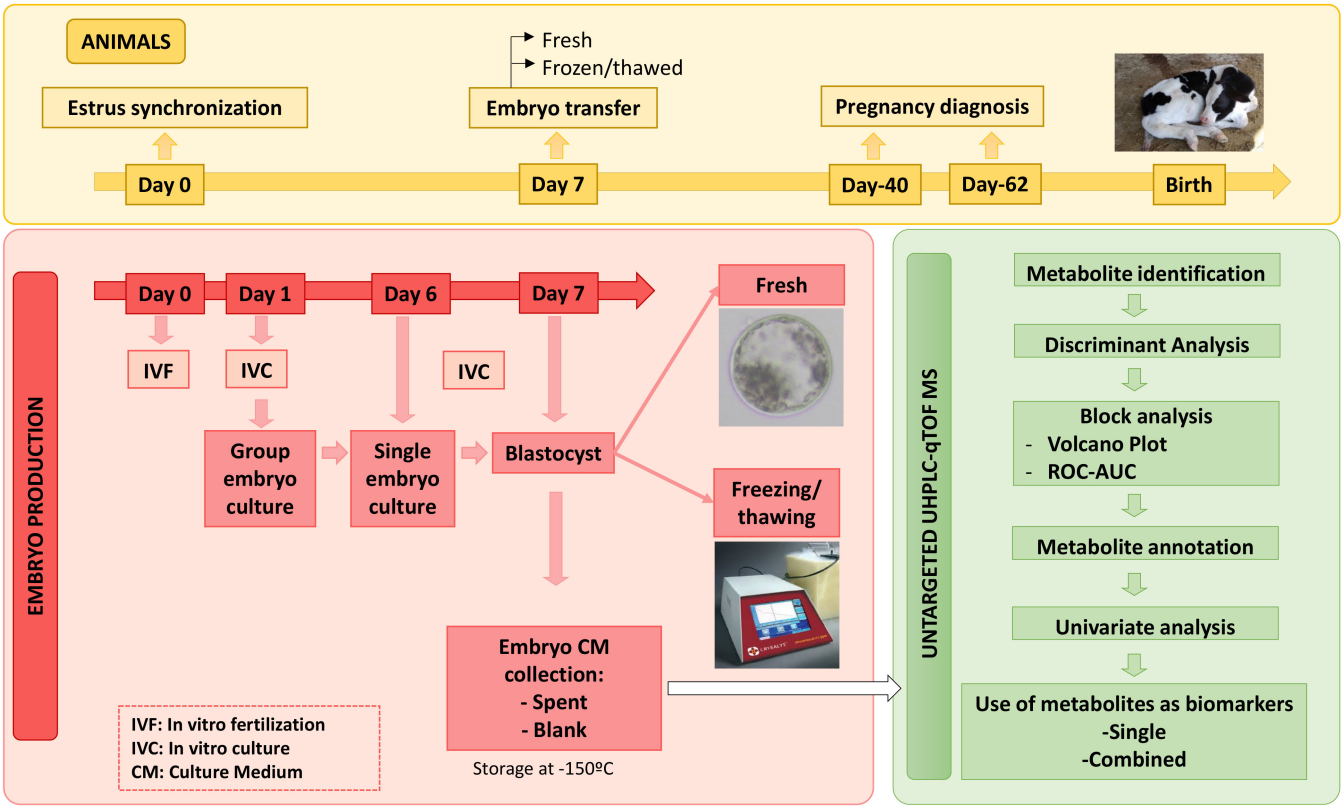

Figure 8. General experimental workflow.

Biomarker studies are governed by principles of population science. Thus, the samples analyzed must be sufficient in number and randomness to be representative of the population under study [78–80]. Within embryos, random sources refer to different bulls, oocytes from different mothers and, to a certain extent, different embryo production systems, since it is not feasible to adapt each biomarker to the wide assortment of laboratorial conditions in cattle. Thus, although the most valuable biomarkers are those that behave as predictive in populations with high individual variability, discrimination by supervised, controllable, non-random factors can in turn improve the accurateness of predictions. In the case of embryos, discrimination by cryopreservation, breed, culture system and developmental stage can improve the predictions [24,26]. Biomarkers identified in the so-called discovery population increase their value when identified in independent populations and/or sample groups [81,82].

4.2. Oocyte Collection and In Vitro Maturation (IVM)

The procedures for in vitro embryo production (IVP) were recently described [1]. In brief, ovaries were collected from slaughtered cows (Matadero de Guarnizo, Spain; Matadero Municipal de Leon, Spain). Antral follicles (3–8 mm diameter) were aspirated and transferred to holding medium (HM) TCM199 (Invitrogen, Barcelona, Spain), 25 mM HEPES and 0.4 mg/mL BSA. Good-quality oocytes (more than three cumulus cell layers and homogenous cytoplasm) were selected for IVM. Cumulus-oocyte complexes (COCs) were rinsed three times in HM and washed three times in maturation medium (MM) consisting of TCM199 $NaHCO_3$ (2.2 mg/mL) supplemented with 10% FCS (*v*/*v*) (F4135), 1.5 µg/mL of porcine FSH-LH (Stimufol; ULg FMV, Liège, Belgium) and 1 µg/mL 17 β-estradiol. COCs (n = 30–50) were matured in a four-well dish with 500 µL of MM at 38.7 °C, 5% CO_2 and high humidity for 22 to 24 h.

4.3. In Vitro Fertilization (IVF)

Oocytes were in vitro fertilized (Day-0) with commercial frozen/thawed semen from Asturiana de los Valles (AV) bulls (n= 4) and Holstein (n= 3) bulls with proven fertility. Motile sperm were obtained following a swim-up protocol [83], incubating for 1 h with pre-equilibrated Sperm-TALP (Tyrode's albumin lactate pyruvate). Then, the supernatant upper layer, which contained motile sperm, was recovered and centrifuged for 7 min at 200× g, and the resultant supernatant was removed. COCs were washed twice in HM and transferred to 4-well dishes containing 500 µL of pre-equilibrated fertilization medium (Fert-TALP) with heparin (10 µg/mL; Calbiochem, La Jolla, CA, USA). COCs and sperm cells (2×10^6 cells/mL) were co-incubated for 18 to 20 h at 38.7 °C in a 5% CO_2 atmosphere with saturated humidity.

4.4. In Vitro Culture (IVC)

Cumulus cells were detached using a vortex, and fertilized oocytes were cultured in modified synthetic oviduct fluid (mSOF) containing 45 µL/mL BME amino acids solution (B6766), 5 µL/mL MEM non-essential amino acids solution (M7145), citrate (0.1 µg/mL), myo-inositol (0.5 µg/mL), and BSA (A3311) (6 mg/mL) with or without 0.1% (v/v) FCS (SIGMA F4135), under mineral oil. IVC was carried out in groups (n = 35–50) at 38.7 °C, 5% CO_2, 5% O_2, 90% N_2 and saturated humidity until Day-6. On Day-6 (143 h PI) good quality morulae, early blastocysts and blastocysts were selected and cultured individually in 12 µL mSOF with 0.5 mg/mL polyvinyl-alcohol PVA (P8136), without BSA or FCS, under mineral oil for 24 h. On Day-7, embryos at the expanding blastocyst stage (ExB) and fully expanded blastocysts (FEB) were collected and transferred fresh or were frozen. Moreover, Day-7 early blastocysts and blastocysts that had developed from Day-6 morulae were individually re-cultured again for 24 h with new CM and those that reached the FEB stage on Day-8 were frozen, while the remaining embryos were discarded. The SCS in protein-free medium step allows for direct chromatographic analysis of CM for non-invasive studies without previous sample processing, and leads to high pregnancy and birth rates with cryopreserved (vitrified and frozen) and fresh embryos [1,84]. All embryos were transferred to recipients synchronized on cycle Day-7 (168 h PI). Spent CM of each embryo and blank samples (i.e., CM incubated without embryo), collected from the last 24 h of culture, were snap-frozen in LN_2 and stored at −150 °C until metabolomics analysis.

4.5. Embryo Freezing and Thawing

These procedures were recently described in detail [1]. Briefly, ExB and FEB were washed individually three times in PBS + 4 g/L BSA and loaded in freezing medium containing PBS (P4417), 1.5 M EG and 20% CRYO3 (5617, Stem Alpha, St Genis Largentiere, France) for 10 min. Embryos were aspirated in a French straw, loaded between 2 columns with PBS + 0.75 M EG + 20% CRYO3, and 2 further columns PBS + 0.75 M EG + 20% CRYO3 separated by air. The straw was closed with a plug and loaded into a programmable freezer (Crysalis, Cryocontroller PTC-9500; Biogenics INC, Harriman, TN, USA) at −6 °C for 2 min and seeded once with supercooled forceps. Straws remained for eight further min at −6 °C and were subsequently dehydrated at −0.5 °C/min up to −35 °C. Finally, the straws were stored in LN_2 until use. For thawing, the straws were held for 10 s in air and 30 s in a bath at 35 °C and carefully dried with 70% ethanol. Each thawed straw with a single embryo was mounted in an ET catheter and directly transferred to recipients.

4.6. Recipient Management, Embryo Transfer and Pregnancy Diagnosis

Embryos were transferred to recipient heifers from Asturiana de los Valles (1.74 years), Holstein (1.84 years), and their crosses (1.76 years), in the experimental herd. Recipient traits, feeding, nutrition, management and ET procedures were described in detail [78,85]. Briefly, recipients were synchronized in estrus with an intravaginal progestogen device (PRID Alpha; CEVA, Barcelona, Spain) for 8–11 days, followed by a prostaglandin $F_2\alpha$ analogue (Dynolitic, Pfizer, Madrid, Spain) injected 48 h before progestogen removal. Blood

plasma was collected on Day-0 and Day-7 (before ET) in ethylenediamine tetraacetic acid (EDTA) vacuum tubes via coccygeal vein puncture for progesterone (P4) measurement. An enzyme-linked immunosorbent assay (ELISA) test operating on a 0–40 ng/mL^{-1} scale (EIA-1561, DRG DiagnosticsSpringfield, NJ, USA) was used. The test was sensitive starting from 0.5 ng/mL^{-1}, and cross-reactivity from steroids other than P4 was less than 1%. Intra- and inter-assay coefficients of variation were 6% and 7%, respectively.

The criteria for selection of recipients for transfer included observation of standing estrus by experienced carekeepers 2–3 times per day, and heat monitoring with an automated sensor system (Heatphone, Medria, Humeco, Huesca, Spain). In the absence of clear estrous signs, progesterone levels were used to select recipients, with P4 fold change Day-7/Day-0 >8 and Day-7 P4 values >3.5 ng/mL. Before ET, all recipients were clinically examined for detection of a healthy corpus luteum in one ovary by ultrasonography. Pregnancy was diagnosed by ultrasonography on Day-40 and Day-62, and birth rates were monitored.

ETs were performed with fresh and frozen/thawed embryos, non-surgically and under epidural anesthesia. All frozen and transferred embryos were ExB and FEB (n = 56), while embryos transferred fresh (n = 28) exceptionally included one early blastocyst and one blastocyst. Fresh embryos were washed twice in Embryo Holding Media (019449, IMV Technologies, L'Aigle, France) and mounted in straw in the same medium, while frozen/thawed embryos were directly transferred.

4.7. Untargeted Metabolomic Analysis and UHPLC-TOF MS Conditions

All samples were diluted 1:3 (*v/v*) in ultrapure water after being thawed on ice, and directly analyzed in duplicate.

Chromatographic separation (Dionex^{TM} UltiMate 3000 UHPLC, Thermo Fisher Scientific^{TM}, Waltham, MA, USA) was achieved using a C18 column (2.1 × 100 mm, 1.8 µm, ACQUITY UPLC® HSS T3, Waters Corp., Milford, MA, USA) in reverse phase (RP) at 30 °C and 250 µL/min total flow rate. Phase A consisted of ultrapure water and phase B acetonitrile, both with 0.1% formic acid (*v/v*). The gradient elution profile, previously validated [28], was as follows: 0 min (0% B), 2 min (0% B), 5 (70% B), 8 min (100% B), 13 min (100% B). The column was equilibrated for 6 min prior to each analysis.

The MS acquisition (Impact-II with conventional ESI ion source, Bruker Daltonics, Billerica, MA, USA) was performed in the positive ionization mode in a scan range from m/z 100 to 1500 and 12 Hz spectra rate. Settings were as follows: nebulizer gas pressure, 2.1 Bar; gas temperature, 300 °C; capillary voltage, 4500 V; drying gas flow rate, 10 L/min MS^2, obtained from CID fragmentation of the top 3 parent ions of each scan with collision energies ranged from 4 to 20 eV, was used for metabolite identification.

External calibration of the Q-TOF MS using a commercial mixture (ESI Low concentration Tune Mix, Agilent Technologies, Santa Clara, CA, USA) and internal mass calibration with a solution of Na formate clusters (m/z range from 91 to 1383) were carried out as a quality control. Prior to each injection, calibrant was infused at 180 µL/min with a syringe pump during column stabilization to re-calibrate each chromatogram and ensure constant mass accuracy during the whole analysis sequence.

Furthermore, for continuous quality monitoring, a 10 ppb solution of triphenyl phosphate (Sigma Aldrich, St. Luis, MI, USA) was injected every 5 samples to check detector sensitivity, mass accuracy and chromatographic performance. A mix of all sequenced samples was also injected at the beginning and end of the sequence to obtain successful and stable chromatographic resolution of samples. All acquired data were exported by DataAnalysis v.4.2 (Bruker Daltonics, Billerica, MA, USA).

4.8. Data Processing

Raw data were extracted and converted to the appropriate format for processing. Data treatment, peak selection, deconvolution, alignment and identification were performed with the software MZmine v2.53 [86] and the R package pRocessomics (https://github.com/Valledor/pRocessomics, accessed on 4 April 2020). Supplementary Table S5 describes

the parameters under which data were processed. Data processing included missing value imputation with the Random Forest (RF) algorithm (threshold value = 0.25). Data were filtered using a consistency criterion (0.8 threshold) and abundance balancing using the average of the total intensities of all samples (normalization step). Thereafter, each sample concentration signal was subtracted with their corresponding blank. The resulting output data, with their corresponding retention time, m/z and peak area, were submitted to statistical analysis.

Potential metabolite markers were tentatively identified by matching the obtained MS/MS data to those published in free-access databases: Human Metabolome Database (HMDB), MassBank, GNPS and NIST 14, within a mass accuracy window of 5 ppm.

A total of 118,564 initial variables were obtained after MZmine analysis, and peak areas were then processed using R. Missing value imputation was performed with the Random Forest (RF) algorithm and a threshold value of 0.25. Data were filtered using a consistency criterion (0.8 threshold), and abundance balancing using the average of the total intensities of all samples (normalization step). After processing, a total of 6111 features remained, which were thereafter subtracted with their corresponding blank. The resulting output data were submitted to statistical analysis.

4.9. Statistical Analysis

Metabolites in embryo CM were analyzed as a function to predict pregnancy at each pregnancy endpoint (Day-40, Day-62 and birth). The analysis charted the following steps:

4.9.1. Multivariate Statistics

This analysis was performed with Metaboanalyst [87].

We used the discriminant analysis (DA) algorithms sparse Partial Least Square (sPLS-DA) and Orthogonal Partial Least Square (OPLS-DA) for supervised sample separation. Multivariate analysis started by analyzing the entire feature dataset (6111 retained features) as a function of pregnancy at the gestational endpoints D40, D62 and Birth. Subsequently, sample separation was explored by combinations of breed and/or embryonic stages, or their combinations with culture condition and cryopreservation. The effect of each single bull as a random factor was also analyzed.

4.9.2. Block Analyses

A design with fixed, controllable (non-random) factors was used to identify blocks with significant predictive ability, excluding random factors (Metaboanalyst). Thus, blocks consisted of combinations of the fixed factors embryo, culture medium from Day-0 to Day-6 (BSA vs. BSA + FCS) and bull breed (Holstein vs. AV), since each of these factors can alter embryonal metabolism [26]. Furthermore, metabolomics supervised with stage factors improves pregnancy prediction [24]. The block study also included embryo cryopreservation (fresh/F/T) and age of embryo (7/8 days), and analyses were performed at each pregnancy end-point (Day-40, Day-62 and birth). The random factors date (i.e., round of ET performed), bull (n = 7) and recipient were not considered within blocks. Two significance levels were used. The first level consisted of Volcano Plots formed by Fold Changes (FCh) >|2| and $p < 0.05$ or tendencies $0.05 > p < 0.10$ (parametric and non-parametric statistics by ANOVA and Kruskal–Wallis test, respectively). Metabolites fulfilling such requirements were analyzed at a second level by Receiver Operator Characteristic—area under curve (ROC-AUC) >0.700 and FCh >|2|). The statistical significance (t-test) with p-value required was <0.10, with minor exceptions to these criteria within blocks having lower sample numbers and the highest ROC-AUCs (0.850–1.000) for specific embryonic transitions for convenience; such low sample sets were only used as complementary information for larger blocks. The study analyzed all factorial combinations from a set with n = 84 embryos transferred (n = 28 Day-7 fresh; and n = 48 Day-7 frozen, and n = 8 Day-8 frozen). All embryos (except two fresh) were ExB and FEB transferred to recipients synchronized on

cycle Day-7. Only blocks yielding metabolite signals fulfilling the above criteria were described.

4.9.3. Metabolite Identification

Metabolites were first tentatively assigned by comparing very accurate precursor masses to public databases (<10 ppm of difference between measured and exact compound mass), taking advantage of the high resolution of the qTOF instrument. Only those m/zs fulfilling significant conditions in the block study were further validated by using MS/MS data. MS2 spectra were then matched against public databases (Human Metabolome Database (HMDB), MassBank, GNPS and NIST 14, using a 5 ppm threshold) to validate metabolite identification. Different collision energies were applied to each parent mass and resulting spectra were employed for database comparisons. Identifications were only validated when at least the precursor mass and three MS2 ions were coincident.

4.9.4. Univariate Studies in the Whole Dataset

This study investigated how the candidate biomarker metabolites identified in the block study behaved in the entire data set (SAS/STAT; Version 9.2; SAS Institute Inc. Cary, NC, USA). Generalized Linear Models were applied, and Bonferroni correction ($p < 0.10$) was used as a false discovery rate post hoc test. The variable endpoints analyzed were P40, P62, birth, miscarriage after Day-40, and metabolic differences between Day-8 and Day-7 embryos.

4.9.5. Taxonomical Analysis (Class Metabolite Analysis and Block Validation)

Metabolites were grouped in taxonomical classes identified in total blocks at the three developmental endpoints. The overall value of each metabolite as a pregnancy predictor was defined by the numbers of blocks in which the metabolite was significantly predictive (here termed as intrinsic value), and by the number of samples correctly sexed within such blocks (absolute value). Thus, the validation proof of a metabolite as a predictor increases when represented within higher numbers of blocks, different culture and cryopreservation conditions, and increased number of samples correctly identified.

4.9.6. Endpoint Analysis

The endpoint analysis gave an overview on the general impact of metabolites through the pregnancy endpoints, with significant differences in the predictive mode (i.e., early or late pregnancy endpoints) of each metabolite.

4.9.7. Use of Metabolites as Biomarkers

The effectiveness of a metabolite as a pregnancy predictor under specific culture, stage and/or cryopreservation conditions is dependent not only on its ROC-AUC value, but also on the relative abundance of embryonic transitions (i.e., the proportion of each embryonic stage in culture) and culture medium (BSA and BSA + FCS) within blocks represented by each metabolite. Thus, we weighed ROC-AUC with the relative abundance of the group of embryos represented in culture. For this purpose, we used a metadatabase with more embryos (n = 1114) than in this study to accurately define the proportions of metabolites expected [26] (see Supplementary Table S6, with the stage distribution abundance). Metabolite biomarkers were used in two ways:

Single Biomarker Metabolites

Hits bearing ROC-AUC > 0.700, with which it is possible to accurately predict pregnancy in specific blocks with $\geq$0.70% effectiveness as single metabolites.

Combined Biomarker Metabolites

We combined the predictive power of single metabolites to obtain overall predictions >0.800 within blocks and series. A series is defined by the same conditions for cryopreservation, breed and culture, supported by one or more developmental IC-stages.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/metabo11080484/s1, Table S1: Summary of embryos (sample n°) under study, including culture from Day-0 to Day-6 (BSA or BSA + FCS), cryopreservation status (Cryo), embryonic stages before (Day-6) and after (Day-7) individual culture period, age of the embryo (days), bull breed (AV: Asturiana de los Valles) and progress through the diagnosed gestational endpoints, Table S2: Peak identification, m/z, retention time and database of the annotated metabolites in the study after comparison between MS/MS fragments to available metabolite databases using SIRIUS v4.4.29. Details of fragmentation trees, molecular formula identification, median mass error (ppm) of explained peaks and CSI: FingerID are shown. Metabolites with different RT but the same annotation, and the two adducts identified for L-Arginine, were considered together in the biomarker analysis. Three of the fragments detected for each metabolite are also shown. Validation: metabolites with annotation confirmed in the MS3 analysis. Explained peaks: number of peaks in the spectrum which can be explained by the fragmentation tree. Similarity: numbers in percentage between the predicted fingerprint and the fingerprint of each candidate, Table S3: General results of metabolomic block analysis consisting of Receiver Operator Characteristic (ROC)-analysis (defined by Area Under Curve –AUC– > 0.700, *p*-value by *t*-test < 0.10 and fold change |2| (written as LogFCh) concentrations between embryos that led to non-pregnant/pregnant recipients) combined with GLM or Kruskal–Wallis (KW) test *p*-value < 0.10. Minor exceptions to these criteria were used within blocks with lower sample numbers and only the highest ROC-AUCs (0.850–1.000) for specific embryonic transitions; such blocks were only used as complementary information (*p*-values shown in bold). Pregnancies were diagnosed at specific gestational times (Endpoint) within embryos obtained from oocytes fertilized in vitro with Asturiana de los Valles (AV) or Holstein (H) bulls, and cultured from 6 days or 7 days in synthetic oviduct fluid medium supplemented with albumin (BSA) or with albumin + fetal calf serum (FCS) followed by a single 24 h culture step developmentally defined by embryonic stage at 0 h (M: morula; EB: early blastocyst; B: Blastocyst) and at 24 h (ExB: expanding blastocyst; FEB: Fully expanded blastocyst) that led to embryos aged (Age) 7 or 8 days that were transferred fresh or frozen (Cryo) to Day-7 estrus synchronized recipients. Each line represents a block in which a metabolite concentration significantly discriminates between pregnant and non-pregnant recipients under each condition, numerically represented by the impact of embryos under the defined culture, stage and age conditions as a proportion of the total embryos, leading to a proportion of embryos in culture predicted for pregnancy by such a metabolite. The effect of embryonic sex within such culture condition or block (not explored in this work) was defined from reviewing Gimeno et al., 2021 (submitted) in a specific, larger study for identification purposes. Taxonomical classes (Class): (1) Lipids; (2) Amino acids; (3) Benzenoids; (4) Carboxylic acids; (5) Tryptamines and derivatives, Table S4: Metabolites quantified in embryo culture medium that, in combinations, predicted pregnancy (Day-40 and Day-62) and birth (Endpoint) with ROC-AUC > 0.800 within embryos obtained from oocytes fertilized in vitro with Asturiana de los Valles (AV) or Holstein (H) bulls, and cultured 6 days or 7 days in synthetic oviduct fluid medium supplemented with albumin (BSA) or with albumin + fetal calf serum (FCS) followed by a single 24 h culture step (IC stage) developmentally defined by embryonic stage at 0 h (M: morula; EB: early blastocyst; B: Blastocyst) and at 24 h (ExB: expanding blastocyst; FEB: Fully expanded blastocyst) that led to embryos aged (Age) 7 or 8 days that were transferred fresh or frozen (Cryo) to Day-7 estrus-synchronized recipients. Block combinations are shown arranged in series with the same embryo cryopreservation, bull breed and culture conditions, and supported by embryonic stages. Each block fulfilled the criteria of FCh >|2| and significant $p < 0.05$ or tendencies $0.05 > p < 0.10$ (parametric and non-parametric statistics by ANOVA and Kruskal–Wallis test) and ROC-AUC > 0.800 with significant *t*-test $p < 0.05$ or tendencies $0.05 > p < 0.10$. For convenience, minor exceptions to these criteria within blocks with lower sample numbers and only the highest ROC-AUCs (0.850–1.000) for specific embryonic transitions (*p*-values highlighted in yellow). Each metabolite within a block shows "single coverage", defined by the percentage frequency of such embryonic transition (i.e., "Impact", obtained within a larger dataset from Gimeno et al., (2021) and the "Predicted value" (i.e., Impact*ROC-AUC). The combined

coverage adds up single impacts of metabolites (upper squares marked with asterisks) to give a total coverage, (values ranked from the highest predictive combination –Predicted 1- and following ones –Predicted 2, Predicted 3–, on the right). Colored lines reflect one of the highest predictive metabolite combinations within a series, although any other metabolite in the table can also be combined to give predictive values >0.800. Taxonomical classes (Class): (1) Lipids; (2) Amino acids; (3) Benzenoids; (4) Carboxylic acids; (5) Tryptamines and derivatives, Table S5: Chromatography and Mzmine data processing workflow with details of steps, parameters and values used, Table S6: Descriptive percent distribution of transitions between Day-6 embryonic stages (M: morula; EB: early blastocyst; B: blastocyst) to Day-7 expanding blastocyst (ExB) and fully expanded blastocysts (FEB) after Day-0 to Day-6 culture with BSA or BSA + FCS).

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Institutional Review Board Statement: The study was conducted according to the guidelines of the Declaration of Helsinki and approved by the Animal Research Ethics Committees of SERIDA and University of Oviedo (PROAE 33/2020; Resolución de 13 de Noviembre de la Consejería de Medio Rural y Recursos Naturales), in accordance with European Community Directive 86/609/EC.

Informed Consent Statement: Not applicable.

Data Availability Statement: The data presented in this study are available on reasonable request from the corresponding author. The data are not publicly available due to privacy and/or ethical concerns.

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5| CAPÍTULO III

BIOMARKER METABOLITE MATING OF VIABLE FROZEN/THAWED IVP BOVINE EMBRYOS WITH PREGNANCY-COMPETENT RECIPIENTS LEADS TO IMPROVED BIRTH RATES

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ABSTRACT

Selection of competent recipients prior to embryo transfer (ET) is indispensable for improving pregnancy and birth rates in cattle. However, pregnancy prediction can fail when the competence of the embryo is ignored. We hypothesized that the pregnancy potential of biomarkers could improve with information on embryonic competence. In vitro produced (IVP) embryos cultured singly for 24 h (from Day-6 to Day-7) were transferred to Day-7 synchronized recipients as fresh or after freezing/thawing. Recipient blood was collected on Day-0 (estrus; N=108) and Day-7 (4-6 h before ET; N=107) and plasma was analyzed by Nuclear Magnetic Resonance ($1H^{+}$NMR). Spent embryo culture medium (CM) was collected and analyzed by UHPLC-MS/MS in a subset of N=70 samples. Concentrations of metabolites quantified in plasma (N=35) were statistically analyzed as a function of pregnancy diagnosed on Day-40, Day-62 and birth. Univariate analysis with plasma metabolites consisted of a block-study with controllable fixed factors (i.e., embryo cryopreservation, recipient breed, and day of blood collection) (Wilcoxon test and T-test). Metabolite concentrations in recipients and embryos were independently analyzed by iterations that reclassified embryos or recipients using the support vector machine (SVM). Iterations identified some competent embryos, but mostly competent recipients that had a pregnancy incompetent partner embryo. Misclassified recipients that could be classified as competent were reanalyzed in a new iteration to improve the predictive model. After subsequent iterations, the predictive potential of recipient biomarkers was recalculated. On Day-0, creatine, acetone and L-phenylalanine were the most relevant biomarkers at Day-40, Day-62, and birth, and on Day-7, L-glutamine, L-lysine, and ornithine. Creatine was the most representative biomarker within blocks (N=20), with a uniform distribution over pregnancy endpoints and type of embryos. Biomarkers showed higher abundance on Day-7 than Day-0, were more predictive for Day-40 and Day-62 than at birth, and the pregnancy predictive ability was lower with frozen/thawed (F/T) embryos. Six metabolic pathways differed between Day-40 pregnant recipients for fresh and F/T embryos. Within F/T embryos, more recipients were misclassified, probably due to pregnancy losses, but were accurately identified when combined with embryonic metabolite signals. After re-calculation, 12 biomarkers increased ROC-AUC (>0.65) at birth, highlighting creatine (ROC-AUC=0.851), and 5

new biomarkers were identified. Combining metabolic information of recipient and embryos improves the confidence and accuracy of single biomarkers.

INTRODUCTION

Optimizing the selection of recipients and in vitro produced (IVP) embryos for transfer is essential to improve pregnancy and birth rates in cattle. Pregnancy rates, in particular with cryopreserved IVP embryos, remain far from meeting the needs of the market, and efforts have been made to identify markers and develop strategies allowing selection of the most appropriate embryos and recipients to obtain successful pregnancies (Diskin et al., 2016). Within recipients, since the common selection procedures for reproductive assessment often excludes fertile animals from transfer (Dickinson et al., 2019), there is a demand for biomarkers based on simple and logistically feasible methods (Kanazawa et al., 2016; Daly et al., 2020; Demetrio et al., 2020). Although the genomic selection of fertile recipients is efficient (Geary et al., 2016), genomic tools do not allow the selection of specific reproductive cycles in which pregnancy will proceed, as management, nutrition and environmental factors also impact on pregnancy (Robles and Chavatte-Palmer, 2017; Caton et al., 2020).

Omics are gaining relevance as sophisticated methods to explain the biological processes inherent to bovine reproduction (Canovas et al., 2014; Rabaglino et al., 2021). Among the different omic levels, the metabolome has been specially addressed as the closest indicator of changes in the biological function, integrating the external (e.g., diet) and internal (e.g., genotype) factors that influence metabolism (Goldansaz et al., 2017; Moore et al., 2017). Thus, using one-proton Nuclear Magnetic Resonance ($1H^{+}$NMR), we and others identified and quantified low molecular weight metabolites in Day-0 and Day-7 recipient plasma that correlate and predict the reproductive performance within the cognate estrus cycle (Phillips et al., 2018; Gómez et al., 2020a; Gomez et al., 2020b; Funeshima et al., 2021). In addition, the competence of fresh and cryopreserved [i.e., frozen/thawed (F/T) and vitrified/warmed (V/W)] IVP embryos to establish pregnancy and reach birth after transfer was non-invasively determined in embryo culture medium (CM) by UHPLC-MS/MS (Gimeno et al., 2021) and GC-MS/MS (Gómez et al., 2021). In this way, the combination of one biomarker identified in recipients with one biomarker from the embryonic side permits considerable improvement in pregnancy prediction to term (>90%) (Gómez et al., 2021).

Given the strong metabolomic differences between recipient breeds (Gómez et al., 2020c), the control of recipients and certain laboratory factors is a must to identify reliable, pregnancy predictive metabolite biomarkers. We therefore should consider the range of metabolite concentrations in the scope of the recipient breed, to a certain extent the embryo CM, and the status of the embryo (fresh or cryopreserved), although specific metabolites are independent of such effects (e.g., ornithine) (Gómez et al., 2020a; Gomez et al., 2020b). The analysis by $1H^{+}$NMR of the plasma metabolome allows for accurate quantification of absolute metabolite concentrations, as wells as reliable results, allowing comparisons through different experiments (Goldansaz et al., 2017; Emwas et al., 2019).

Together with V/W embryos, IVP embryos cryopreserved by conventional slow freezing have recently become of interest because of the possibility of direct transfer and simplicity of use, without a need for laboratory equipment (Sanches et al., 2016; Jia et al., 2018; Zolini et al., 2019; Gómez et al., 2020d; Álvarez-Gallardo et al., 2021). We have recently identified metabolite biomarkers in the CM of such embryos prior to embryo transfer (ET) (Gimeno et al., 2021), which are useful for identifying "true" competent recipients as previously shown with CM of V/W IVP embryos analyzed by GC-MS/MS (Gómez et al., 2021). However, the identification of pregnancy and birth metabolite biomarkers among recipients transferred with F/T embryos has not yet been investigated.

In this work we investigated the metabolite biomarkers in blood plasma on Day-0 (estrus time) and Day-7 (hours prior to ET time) which can predict pre-defined stages of pregnancy and birth in heifers transferred with F/T embryos. The biomarkers and metabolite signals we previously identified in a CM subset from the cognate embryos (Gimeno et al., 2021) were used to obtain an accurate definition of the pregnancy potential of the recipient – and the tandem embryo/recipient- by detecting recipients acting as false negatives using a novel iterative in silico strategy. We hypothesized that, as shown with V/W and fresh embryos and their recipients, the predictive capacity of the recipient would increase once supported by accurate definition of the embryonic competence.

MATERIALS AND METHODS

Experimental procedures were conducted following the guidelines of the Declaration of Helsinki and approved by the Animal Research Ethics Committees of SERIDA (PROAE 26-2016;

Resolución de 25 de Julio de 2016 de la Consejería de Medio Rural y Recursos Naturales), in accordance with the European Community Directive 86/609/EC.

Reagents were purchased from SIGMA (Madrid, Spain) unless otherwise stated.

Embryo production

Embryos were produced in vitro from slaughterhouse ovaries following detailed procedures previously described for oocyte collection, in vitro maturation, and in vitro fertilization, which was performed with N=7 single different bulls, (N=4 Asturiana de los Valles -AV-, and N=3 Holstein bulls; Gómez et al., 2020a). For in vitro culture (IVC), presumptive zygotes were cultured first in groups in modified synthetic oviduct fluid (mSOF) with amino acids [MEM non-essential amino acids solution (#M7145), 3.3 µL/mL; and BME amino acids solution (#B6766), 45 µL/mL], citrate (0.1 µg/mL), myo-inositol (0.5 µg/mL), and 6 mg/mL BSA (#A3311) with or without 0.1% (v/v) FCS (#F4135). In vitro culture (IVC) was carried out in droplets under mineral oil at 38.7°C, 5% CO_2, 5% O_2, 90% N_2, and saturated humidity. On Day-6 (143h PI), excellent and good quality (grade 1 and grade 2) morulae and early blastocysts were selected and cultured individually for 24 h in 12 µL mSOF with 0.5 mg/mL polyvinyl-alcohol PVA (P8136) under mineral oil. Day-7 embryos showing very-good to excellent morphological quality were either transferred as F/T or fresh. Embryonic stages transferred were expanded blastocysts (cryopreserved and fresh) and blastocysts (fresh). The culture medium (CM) of embryos (10 µL) was collected and stored at -150°C and analyzed by UHPLC-MS/MS to identify pregnancy and birth biomarkers, as described in a previous work (Gimeno et al., 2021).

Embryo freezing and thawing

Slow freezing procedures were described in detail (Gomez et al., 2020d). Briefly, expanded and fully expanded blastocysts were washed in PBS + 4 g/L BSA and loaded in a freezing medium containing PBS (P4417), 1.5 M ethylene-glycol and 20% CRYO3 (5617, Stem Alpha, France) for 10 min. Embryos were aspirated in a French straw, loaded between 2 columns with PBS + 0.75 M EG + 20% CRYO3, and 2 further columns PBS + 0.75 M EG + 20% CRYO3 separated by air. The straw was closed with a plug and loaded into a programmable freezer (Crysalis, Cryocontroller PTC-9500) at −6°C for 2 min and seeded with super-cooled forceps. Straws remained for eight further min at −6°C and were subsequently cooled at −0.5°C/min up to −35°C. Finally, the straws were stored in LN_2 until use. For thawing, the straws were held for 10 s in air and 30 s in a water bath at 35°C and carefully dried with 70% ethanol. Each thawed straw with a single embryo was mounted in a 35°C ET catheter and directly transferred to Day-7 synchronized recipients.

Procedures involving animals

Animals were housed in the experimental herd of the Centro de Biotecnología Animal, SERIDA, Deva (Spain). Recipients used in our study were healthy, tested free from the seven most prevalent infectious diseases virtually affecting reproduction in the region, and underwent a gynecological examination.

1. Animal feeding and management

Animals were held in a body score condition 2.5 – 3.5 (scale 0-5) with the same basic diet throughout. From April to late October or early November, animals were fed on pasture, with individual concentrate supplementation (2 to 3 kg/day) given from August onwards. Prior to estrus synchronization and up to pregnancy Day-62, animals were fed indoors with concentrate (3 to 5 kg/day) from programmable, automated dispensers, and barley or oats and hay ad libitum. The indoor ration was also given from late October / early November until March or April to all animals and, at any time of year, from 10-15 days prior to birth. The concentrate contained 14.5% protein, >7% crude fiber, >3.5% fat,

leading to >12% metabolizable energy (MJ kg/DM), and an appropriate mixture of minerals and vitamins. The minimum amounts of raw foods in concentrate were 33% corn grain, 11% soybean and 14% oats. Recipients were synchronized in estrus with an intravaginal progestagen device (PRID Alpha, CEVA, Barcelona, Spain) for 8-11 days, followed by a prostaglandin F2α analogue (Dynolitic, Pfizer, Madrid, Spain) injected 48 h before progestagen removal. Recipients selected for transfer were those observed in standing estrus by experienced caretakers (2–3 times per day checks), and/or monitored in heat with an automated sensor system (Heatphone-Medria, Humeco, Huesca, Spain). In the absence of clear estrous signs, progesterone (P4) concentration was used to select recipients, with P4 fold change (FCh) Day-7/Day-0 >8 and Day-7 P4 values >3.5 ng/mL.

2. Blood plasma collection and processing

Blood was collected in ethylenediaminetetraacetic acid (EDTA)-vacuum tubes from coccygeal vein puncture. Blood tubes were refrigerated at 4°C and centrifuged at 2000 g. Supernatant plasma was aliquoted and stored at -150°C until NMR analysis.

Plasma P4 was measured on Day 0 and Day 7 at fixed times (10 AM on Day-0 –expected estrus, and Day-7, 4 to 6 h prior to ET); such times were informative of recipient fertility by metabolic fingerprint and metabolite biomarkers in earlier studies (Muñoz et al., 2014a; Muñoz et al., 2014b; Gomez et al., 2020a; Gómez et al 2020b). An enzyme-linked immunosorbent assay (ELISA) test (EIA-1561, DRG Diagnostics, Springfield, NJ, USA) was used for blood P4 measurement. The test was sensitive beginning at 0.5 ng/mL, showing <1% cross-reactivity from steroids other than P4. Inter- and intra-assay variation coefficients were 7% and 6% respectively.

3. Embryo transfer to recipients

Embryos were transferred non-surgically, under epidural anesthesia, at a fixed time 9 days + 4-6 h after progestagen removal. Before ET, all recipients were examined for detection of a healthy corpus luteum in one ovary by ultrasonography. Pregnancy was diagnosed by ultrasound scanning on days 40, 62, and monitored until birth.

Table 1 summarizes the numbers of ETs performed, pregnancy rates per recipient breed, and embryo cryopreservation status in this study (N1= 45 fresh+ 63 F/T embryos; Total N1=108). From a previous study (Gimeno et al., 2021), we obtained the metabolome of the cognate culture medium of the embryo transferred, available in a number of cases (N2=70). Recipients were transferred up to 4-times if non-pregnant in previous ETs, as we found that metabolites associated to pregnancy prediction were independent of ET repeats (Gómez et al., 2020c). A description of each sample ET and covariate factors used in this study is shown in Supplementary Table 1 (https://doi.org/10.17632/sc2vnbfrw7.1; Gimeno et al., 2022). Holstein heifers (1.64 years old on average at the time of 1st ET) represented the most abundant ET dataset, while AV breed and their crosses were 1.75 years old on average at first ET. The assignment of type of embryo transferred to a recipient at each ET and fertilizing bull did not follow pre-defined patterns. ETs were repeated after 30 to 60 days from the former non-pregnant diagnosis (on Day-40 or Day-62). ETs were preferentially performed in rounds of 3 to 8 recipients, with embryos sired by one (habitually) or two bulls per round and one or two embryo culture conditions.

Table 1. Recipients (AV: Asturiana de los Valles; AM: Asturiana de la Montaña; and Holstein) used and pregnancy rates (%) obtained after transfer of fresh and frozen in vitro produced embryos at gestational endpoints Day-40, Day-62 and birth.

Embryo type	Recipient breed	N1[1]	N2[2]	Gestational endpoints[3]		
				Day-40	Day-62	Birth
Fresh	AV	6	5			
	AM	5	1			
	Holstein	34	19			
Total		45		29/45 (64%)	26/45 (58%)	25/44[4] (57%)
Total			25	18/25 (72%)	17/25 (68%)	15/25 (60%)
Frozen	AV	15	13			
	Crossbred	5	2			
	Holstein	43	30			
Total		63		37/63 (59%)	35/63 (55%)	29/63 (46%)
Total			45	28/45 (62%)	27/45 (60%)	21/45 (47%)

[1]N1: All recipients used, which provided N=108 and N=107 plasma samples collected on Day-0 (estrus) and Day-7 (hours before the ET time), respectively.

[2]N2: Recipients that had available the cognate culture medium metabolome of the embryo transferred (a subset from N1), obtained in a previous study (Gimeno et al, 2021).

[3] Day: age of the cultured embryo counted from the onset of in vitro fertilization.

[4] One recipient deceased after pregnancy Day-62.

Nuclear Magnetic Resonance ($1H^+$NMR) analysis of blood plasma

Blood plasma samples were thawed at room temperature. A volume of 300 µl plasma was mixed with 300 µl chilled methanol and 300 µl chilled chloroform. The mixture was vortexed and then kept at -20°C for 30 min. After centrifugation (15000 g, 10 min, +4°C) to separate the polar phase, the sample was evaporated in a Speedvac (ThermoScientific) at +35°C. Samples were stored at -20°C before analysis.

$1H^+$NMR samples were prepared using extracted, dried blood plasma with the addition of 200 µL of 0.2 M potassium phosphate buffer in deuterium oxide (D2O) (pH 7.4 ± 0.5) and 10 µl of 3,2 mM trimethylsilylpropanoic acid (TSP). D2O provided a field frequency lock and TSP a chemical shift reference. The resulting solution was transferred to conventional 3-mm NMR tubes.

Briefly, $1H^+$NMR spectra were recorded at 298 K on a Bruker Ascend 600 MHz spectrometer (Bruker, Sadis, Wissembourg, France), equipped with a TCI cryoprobe. Standard $1H^+$NMR spectra were obtained using a NOESY pulse sequence with a 90° pulse, a relaxation delay of 20 seconds, and 64 scans in a time domain of 64 K data points. Data were processed with 0.2 Hz of line broadening for the exponential decay function using TopSpin version 3.2 software (Bruker Daltonik, Karlsruhe, Germany).

Spectral assignments were performed using the free version of ChenomX 7.1 software (ChenomX, Edmonton, Canada), in-house database, and Livestock Metabolome Database (LMDB http://lmdb.ca/). Metabolite quantification was carried out using TSP signal with a known concentration, as reference ([TSP] = 152 µM).

Experimental Design and Statistical Analysis

This study analyzed the metabolite concentrations in Day-0 and Day-7 plasma of recipients diagnosed as pregnant or open at specific time endpoints (Day-40, Day-62 and Birth). The experimental sequence is shown in Figure 1. Analyses included separate studies with embryos transferred fresh and after freezing/thawing. Metabolites that differed in concentration were identified, and, among them, candidate metabolite biomarkers were obtained, as well as altered underlying metabolic pathways where possible. For validation, metabolites in this study were compared with fresh controls

made on purpose in this experiment, and with independent samples corresponding to other fresh and V/W embryos transferred to recipients in our experimental herd in previous studies (Gómez et al., 2020a; Gómez et al., 2020b).

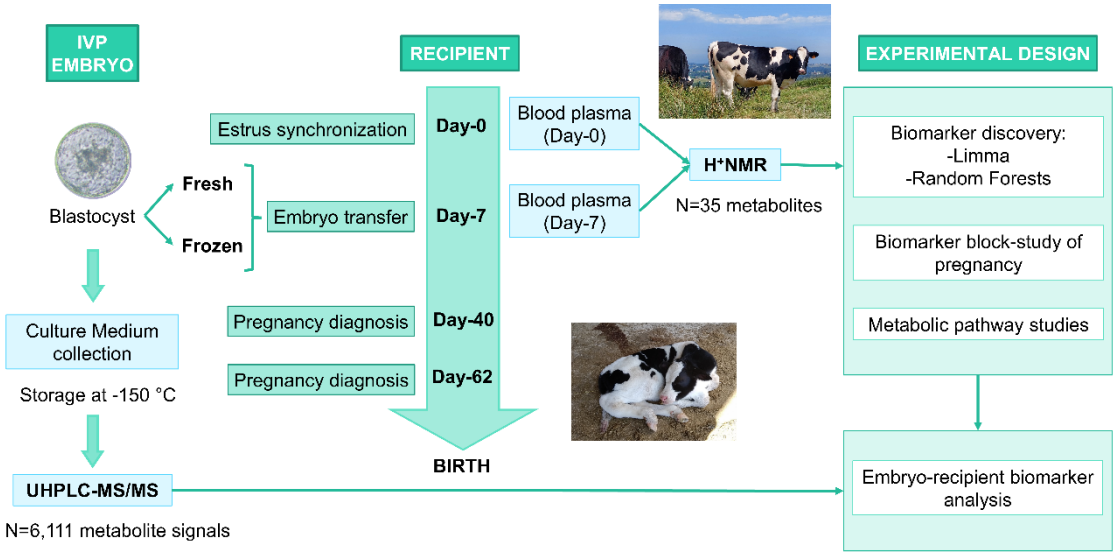


Figure 1. Workflow of experiments conducted in this study.

Furthermore, since pregnancy is based on competent embryos and competent recipients, we performed a study to increase the predictive power of biomarkers in accordance with the pregnancy competence of the cognate embryo, which was obtained by metanalysis of data from a previous study (Gimeno et al., 2021). Ultimately, to overcome individual factors that could lead to distorted results, we introduced experimental randomness using high variability in recipients and in embryos. Thus, we ensured sample variability in recipient females from different batches over a 5-year period (2016-2020) from Holsteins and AV cattle. From the embryonic perspective, since biomarker adjustment for each existing embryo production condition is unfeasible, we included two culture conditions i.e., fetal calf serum (FCS) and/or albumin (BSA) within group culture from Day-0 to Day-6, combined with fresh and F/T embryos, as it can be considered “randomized”. Variation in oocyte origin included blind slaughterhouse ovary collection. Variation in fertilizing bulls included N=7 individual bulls from 2 breeds used for IVF in the laboratory.

The NMR analysis in the recipient dataset identified 46 metabolites in plasma, of which 35 were also quantified (detailed in Supplementary Table 2; https://doi.org/10.17632/sc2vnbfrw7.1; Gimeno et al., 2022). Statistical analyses were performed with Metaboanalyst 5.0 (Chong et al., 2018) and Graphpad Prism software. Metabolite concentration data were scaled to adjust each variable/feature by a scaling factor based on the dispersion of the variable. Auto scaling consisted of mean-centering and division by the standard deviation of each variable, which was sufficient to reach a near-to-normal distribution (Figure 2).

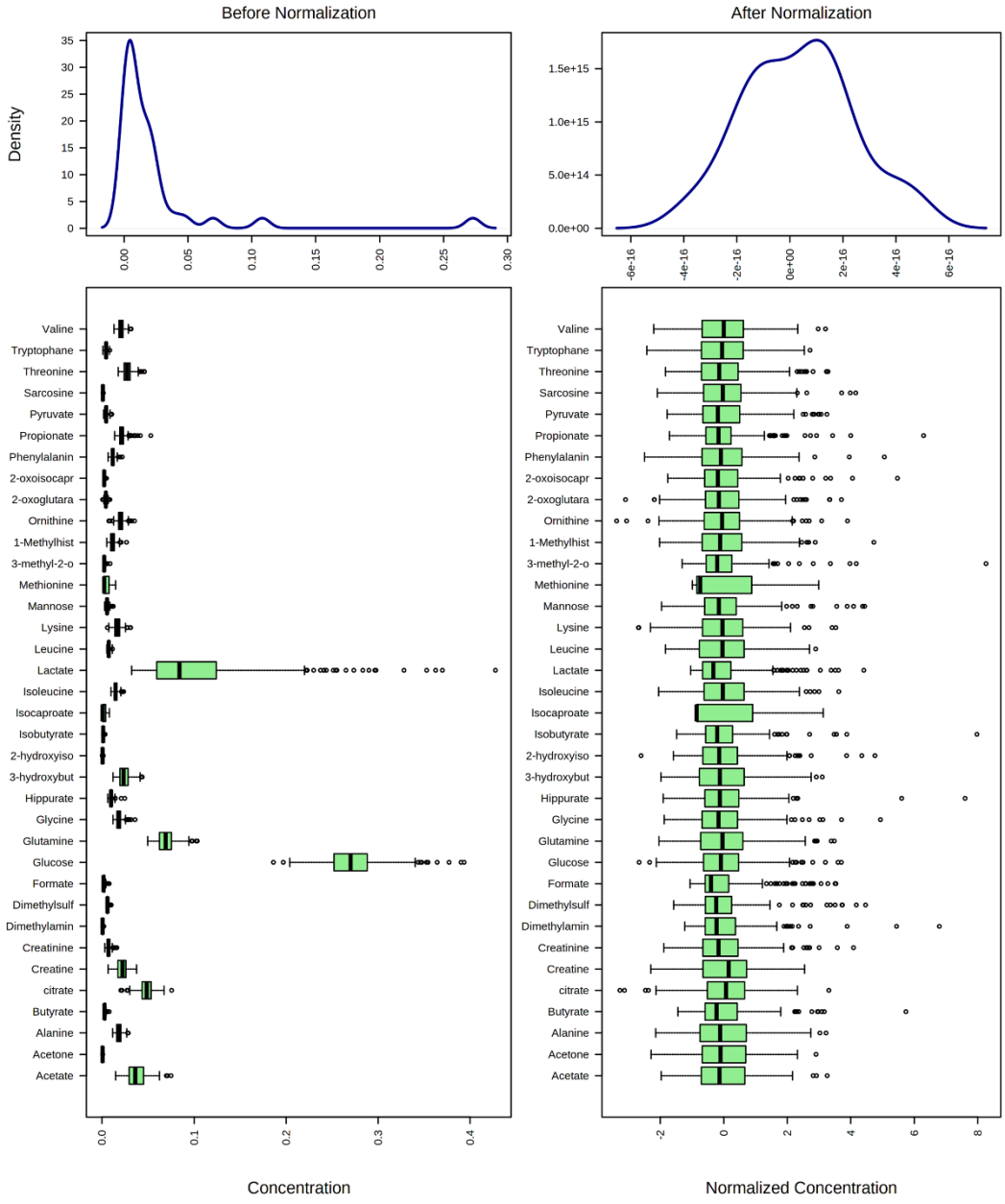


Figure 2. Normalization of metabolite concentration data.

Subsequently, metabolites identified and quantified were analyzed by univariate and multivariate statistics to detect confident biomarkers and pathways involved in pregnancy competence and their differences between recipients of F/T vs. fresh embryos. The analytical procedures were as follows:

1. Main metabolites in the entire dataset

We analyzed metabolites identified in Day-0 (N=108) and Day-7 (N=107) plasma samples as a function of pregnancy diagnosis at Day-40, Day-62 and birth.

Covariates used in these calculations were recipient age (linear and quadratic) difference with the mean herd average; embryo cryopreservation; embryo culture; recipient; breed; NMR analysis; and individual bull.

1.1. Univariate analysis: Linear models with covariate adjustments (Limma)

The overall datasets on Day-0 and Day-7 were analyzed by Limma. This approach uses linear models to perform significance t-testing with covariate adjustments. The study included relevant metabolites as candidates with pregnancy predictive potential independent of cryopreservation.

1.2. Multivariate analysis: Random Forests

A predictive model was built with metabolite features only as a function of the three pregnancy endpoints studied. The analysis was based on the machine learning classification algorithm Random Forests (RF). The mean decrease accuracy (MDA) tool from RF allowed obtention of rankings based on feature contributions to the classification accuracy by permutation. The parameters used were N=500 trees, N=7 predictors (covariates) and positive randomness.

2. Main metabolites: Biomarker block-study of pregnancy

Plasma samples (N=108 from Day-0 and N=107 from Day-7) were used in a block study in accordance with their controllable fixed factors (i.e., embryo cryopreservation, recipient breed, and day of blood collection) analyzed as a function of pregnancy endpoints. Blocks yielded more uniform datasets and increased the predictive power of metabolites (Gimeno et al., 2021). Once divided into blocks, metabolite concentrations were doubly analyzed with Wilcoxon test (univariate study) and T-test (for biomarker candidates with ROC-AUC>0.65). P values were <0.05, and 0.05>P<0.10 for tendencies. Some metabolites with non-significant ROC-AUC>0.65 were considered merely to illustrate specific blocks (marked in red in Supplementary Table 3; https://doi.org/10.17632/sc2vnbfrw7.1; Gimeno et al., 2022), and only when the same metabolite was significant in other blocks and/or previously identified as a main metabolite in the general dataset.

3. Metabolic pathway studies

In accordance with sufficiency of sample availability, two pathway studies were carried out in Metaboanalyst 5.0 tools with samples from animals diagnosed for pregnancy at Day-40:

3.1/ Day-40 pregnant vs. Day-40 open recipients transferred with F/T embryos: Metabolic pathway analysis.

The largest breed-homogeneous block in samples (Holsteins; N=23 pregnant and N=20 open recipients) was selected and analyzed on Day-0 and Day-7.

3.2/ Day-40, only pregnant recipients from fresh vs. F/T embryos: Metabolite set enrichment analysis (MSEA).

This study responded to the hypothesis that pregnant recipients of fresh (N=29) and F/T (N=37) embryos would have different metabolic requirements for pregnancy in response

to different embryos. MSEA directly investigated a set of functionally related metabolites (SMPDB; Human) without the need to preselect compounds based on an arbitrary cut-off threshold.

4. Improving the pregnancy predictive power of the recipient biomarkers by ROC curve-based models supported by embryonic spectral metabolite features obtained by UHPLC

Pregnancy depends on viable embryos and competent recipients. Thus, a viable embryo transferred to a non-competent recipient, and vice versa, leads to non-pregnancy and false negative samples. On the contrary, if the pregnancy capability is defined in the embryo and the recipient, a more exact pregnancy prediction can be estimated (Gómez et al., 2021).

Among the recipients used in this work, a group of N=70 recipient plasma samples had available complementary analysis of the embryo culture medium (CM) obtained in our previous study (Gimeno et al., 2021) (see Table 1). Such analysis, performed by UHPLC/MS-MS metabolomics, led to obtain 6,111 confident spectral metabolite features with which we calculated a confident embryonic competence value as shown below (see 4.1/ Model development). The matched groups were recipients with fresh embryos (N=25) and recipients with frozen embryos (N=45).

4.1. Model development

To refine birth expectations of recipient plasma in this study, we used a strategy to identify false negatives within paired embryos and recipients, as modified from Gómez et al. (2021).

For sample imputation, recipients were divided into four predictive groups by breed (Holsteins, including 2 Holstein crossbred; and AV) in a 2x2 factorial design with the status of the embryo transferred (fresh or F/T). The optimal results for analyses in these groups of recipients combined 3-5 metabolites per group. Embryos were divided into four predictive groups by fertilizing bull breed (Holstein and AV) in a 2x2 factorial design with

the cryopreservation of the embryo (fresh or F/T). The analyses within embryos combined up to 20 feature signals.

Combinations of recipients (metabolites) and embryos (feature signals) in each pregnancy predictive group were independently analyzed with the algorithm support vector machine (SVM), PLS-DA and RF to create pregnancy predictive biomarker models (ROC-AUC empirical P value <0.01 by permutation). For training and testing, ROC-AUC curves generated by Monte-Carlo cross validation were used with balanced sub-sampling to evaluate the feature importance. Classification models were performed with selected top features and validated on one third of the samples that were left out. To avoid overfitting, K-means (KM) clustering was used to detect and avoid features with similar behavior to minimize the redundancy in biomarkers (i.e., features in the same cluster that behave more similarly) (Tester tool; Metaboanalyst 5.0). Thus, metabolite features from >3 clusters were selected in the recipient group, and 5-10 clusters in the embryo group. Among the tested algorithms SVM gave the highest ROC curve classification. Confusion matrixes were obtained with actual and predicted values for birth / no birth both for embryos and recipients.

4.2. Predictions and iterations

Confusion matrixes between matched samples from recipients and embryos were compared. Samples were judged as misclassified when the predicted pregnancy status was not coincident with the actual pregnancy status. Criteria for corrections through iterations were based on the following four possible cases:

1) Actual “No birth” with recipient judged as “Birth” and embryo judged as “No birth”: reclassification of the recipient as “Birth” for testing in a subsequent iteration.

2) Actual “No birth” with recipient judged as “No birth” and embryo judged as “Birth”: reclassification of the embryo as “Birth” for testing in a subsequent iteration.

3) Actual “No birth” with recipient and embryo judged as “No birth”: no re-classification for testing in a subsequent iteration.

4) Actual “Birth” with recipient and embryo judged as “No birth”: based on the evidence, re-calculation as “Birth” of both embryo and recipient in a subsequent iteration.

Through successive iterations with the Tester tool, samples that became matched in accordance with the actual pregnancy status were considered true, while samples that do not fit with their reclassification were considered false (error).

4.3. Re-calculation of birth biomarker values in recipients

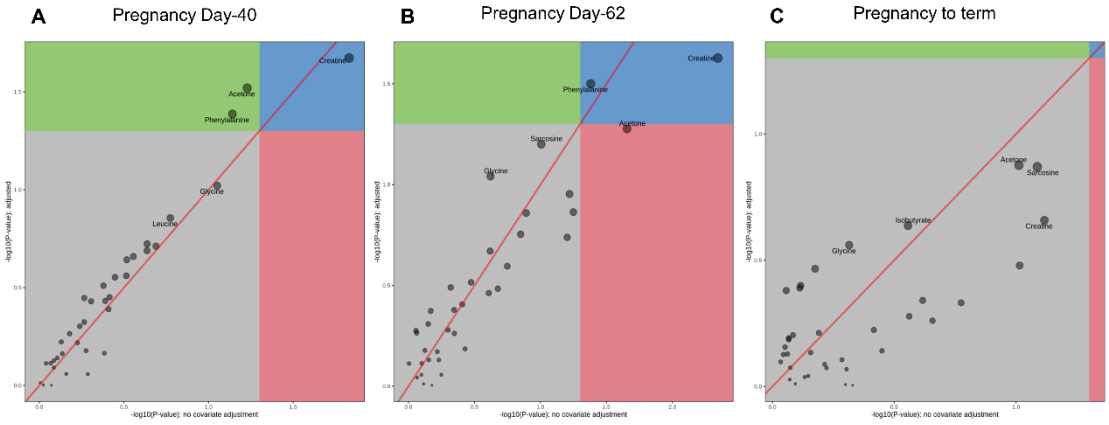
Iterations based on multivariate SVM analysis combining the embryo and recipient data led to a more accurate recipient classification for birth potential. Therefore, we hypothesized that the original ROC-AUC value of certain predictive metabolites would equally increase after iterations. Metabolite ROC-AUC classification and t-test of recipients were recalculated after iterative identification of recipients identified as “no birth” with predicted “birth” potential supported by embryo confusion matrix.

1. Main metabolites in the entire dataset

1.1. Univariate analysis: Linear models with covariate adjustments (Limma)

1.1.1/ Limma Day-0 analysis (N=108 samples)

Creatine was the most relevant metabolite on Day-0, appearing at higher concentration in pregnant recipients when analyzed for Day-40 and Day-62 endpoints, both as adjusted and non-adjusted by covariates (A/A) (Figure 3). Acetone and L-phenylalanine were also A/A metabolites on Day-40 and Day-62, respectively. However, L-phenylalanine at Day-40 was significant only when adjusted, while acetone on Day-62 was significant only if non-adjusted. No metabolite changing significantly on Day-0 was detected as a function of birth.



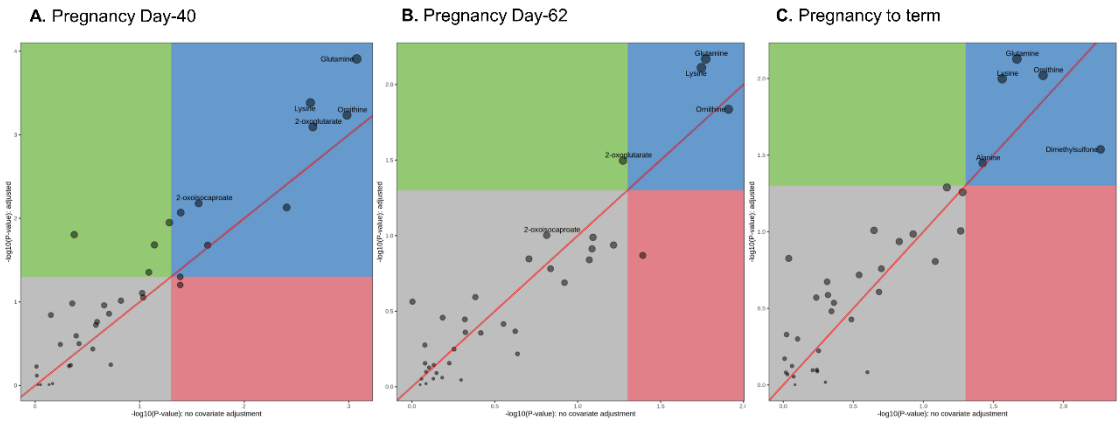
	Pregnancy Day-40				Pregnancy Day-62				Pregnancy to term			
	Adjusted		Not adjusted		Adjusted		Not adjusted		Adjusted		Not adjusted	
Metabolite	Log FCh	P value	Log FCh	P value	Log FCh	P value	Log FCh	P value	Log FCh	P value	Log FCh	P value
Creatine	0.4067	0.0212	0.4750	0.0118	0.3932	0.0235	0.5503	0.0045				
Acetone	0.4117	0.0302	0.3640	0.0487			0.4536	0.0221				
L-Phenylalanine	0.3894	0.0410			0.4297	0.0316	0.3949	0.0416				

Figure 3. Covariate boxplot showing P-values for metabolites quantified in Day-0 plasma of recipients transferred with fresh and frozen embryos that differ as a function of pregnancy on Day-40 (A), Day-62 (B), with and without covariate adjustment (covariates adjusted for each pregnancy stage were recipient age –linear and quadratic difference with the mean herd average-, embryo cryopreservation, embryo culture, recipient breed, NMR analysis, and individual bull). No metabolites fulfilling the required conditions were identified to term (C). The plot compares

p-values for each metabolite both before (x-axis) and after (y-axis) covariate adjustment. Green section: features significant (P<0.05) only after adjustment; red section: features significant only before adjustment; blue section: features significant in both cases; grey section: nonsignificant features. Log FCh: logarithm of Fold Change pregnant / non-pregnant.

1.1.2/ Limma Day-7 analysis (N=107 samples)

Diagnosis of pregnancy at Day-40 led to significantly different FCh between 12 metabolites (i.e., L-glutamine, L-lysine, ornithine, 2-oxoglutarate, 2-oxoisocaproate, L-phenylalanine, 3-methyl-2-oxovalerate, butyrate, L-methionine, propionate, L-alanine and L-isoleucine) (Figure 4). Of these, all were A/A but propionate and L-isoleucine, which were significant only when adjusted. Four of the above A/A metabolites also diagnosed pregnancy on Day-62 (L-glutamine, L-lysine, ornithine and 2-oxoglutarate), and at birth, with L-glutamine, L-lysine and ornithine being A/A and 2-oxoglutarate being significant only when adjusted. Furthermore, birth revealed a Day-40 A/A metabolite (L-phenylalanine) and a new A/A metabolite not recorded at Day-40 and Day-62 (dimethyl sulfone).



	Pregnancy Day-40				Pregnancy Day-62				Pregnancy to term			
	Adjusted		Not adjusted		Adjusted		Not adjusted		Adjusted		Not adjusted	
Metabolite	Log FCh	P value	Log FCh	P value	Log FCh	P value	Log FCh	P value	Log FCh	P value	Log FCh	P value
L-glutamine	-0.7792	0.0001	-0.6632	0.0006	-0.5609	0.0068	-0.4742	0.0141	-0.4742	0.0141	-0.4563	0.0175
L-lysine	-0.7156	0.0004	-0.5957	0.0019	-0.5480	0.0077	-0.4454	0.0190	-0.4459	0.0190	-0.4050	0.0325
Ornithine	-0.6971	0.0005	-0.6423	0.0007	-0.5032	0.0146	-0.4728	0.0128	-0.4728	0.0128	-0.4542	0.0162
Dimethylsulfone									-0.4181	0.0290	-0.5589	0.0032
2-oxoglutarate	-0.6697	0.0008	-0.6098	0.0016	-0.4369	0.0318	-0.3891	0.0440	-0.3891	0.0440		
2-oxoisocaproate	-0.5152	0.0066	-0.4498	0.0139								
L-phenylalanine	-0.5392	0.0074	-0.5815	0.0017					-0.3655	0.0480	-0.3947	0.0318
3-methyl-2-oxovalerate	-0.5078	0.0085	-0.4176	0.0241								
Butyrate	-0.4746	0.0112	-0.3962	0.0333								
L-methionine	-0.2191	0.0156	-0.1907	0.0234								
Propionate	-0.4398	0.0208										
L-alanine	-0.4688	0.0210	-0.4457	0.0217								
L-isoleucine	-0.4197	0.0442										

Figure 4. Covariate boxplot showing P-values for metabolites quantified in Day-7 plasma of recipients transferred with fresh and frozen embryos that differ as a function of pregnancy on Day-40 (A), Day-62 (B) and to term (C) with and without covariate adjustment (covariates adjusted for each pregnancy stage were recipient age – linear and quadratic difference with the mean herd average-, embryo cryopreservation, embryo culture, recipient breed, NMR analysis, and individual bull). The plot compares p-values for each metabolite both before (x-axis) and after (y-axis) covariate adjustment. Green section: features significant ($P<0.05$) only after adjustment; red section: features significant only before adjustment; blue section: features significant in both cases; grey section: non-significant features. Log FCh: logarithm of Fold Change pregnant / nonpregnant.

1.2. Multivariate analysis: Random Forests

Results of the RF analysis are presented in Figure 5, showing similar MDA predictive values between Day-40 and Day-62 for Day-0 and Day-7, but lower MDA values on Day-0 at birth.

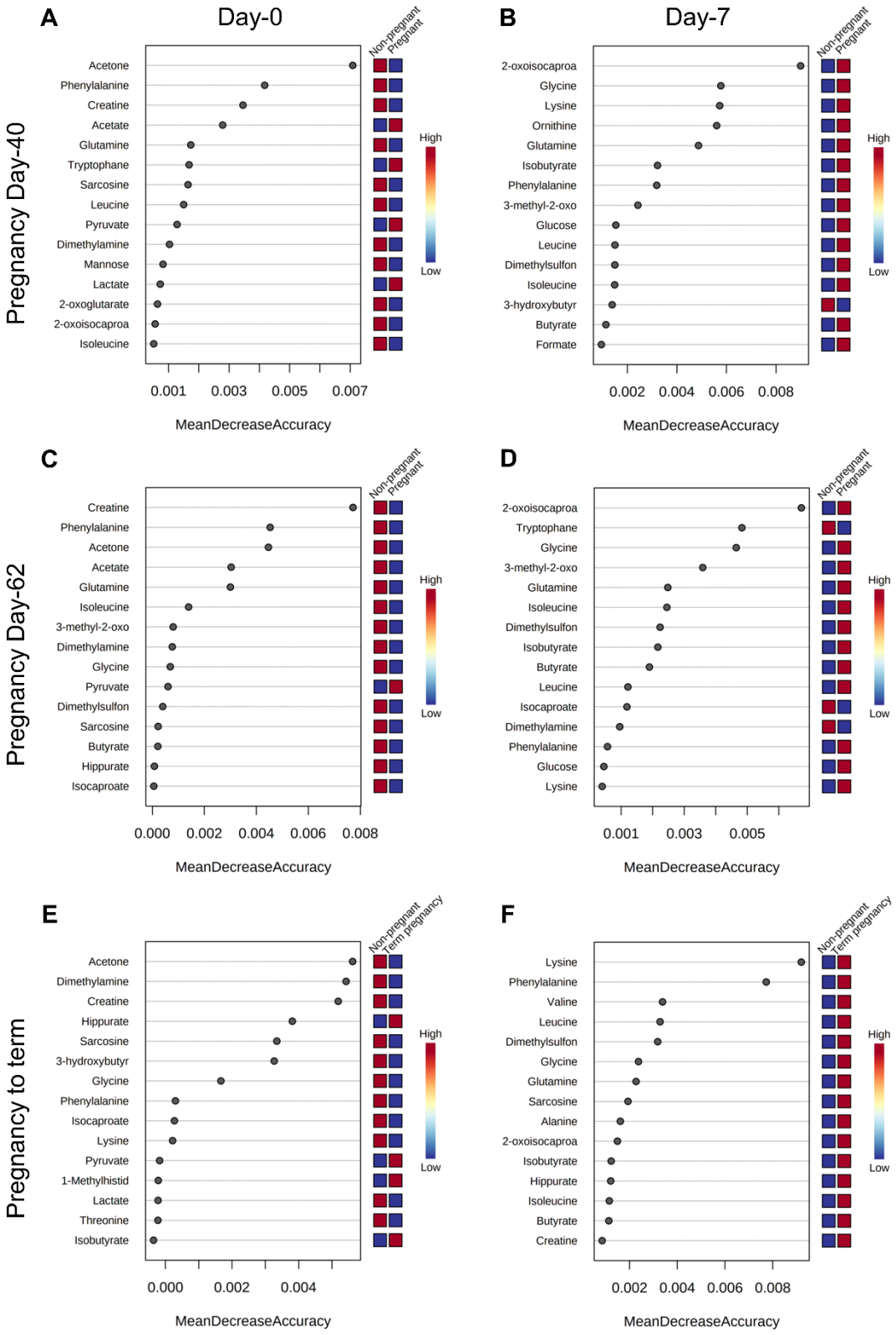


Figure 5. Metabolites identified in Day-0 (A, C, E) and Day-7 (B, D, F) plasma from recipients transferred with frozen and fresh embryos ranked by their contributions to classification accuracy to predict pregnancy (mean decrease accuracy; random forest classification algorithm) on Day-40 (A, B), Day-62 (C, D), and birth (E, F). Red: higher concentration. Blue: lower concentration.

Interestingly, on Day-0 the consistency of the top MDA values creatine, acetone and L-phenylalanine at Day-40 (Fig 5A) and Day-62 (Fig 5C) with the univariate study was total, while at birth (Fig 5E) acetone and creatine were also in the top, but with a poorer MDA value than at the earliest end-points.

On Day-7, the highest consistency with the univariate study was shown by metabolites classified at the pregnancy Day-40 (Fig 5B), with L-glutamine, L-lysine, ornithine, 2-oxoisocaproate, L-phenylalanine, 3-methyl-2-oxovalerate being included. In contrast, glycine ranked at the top by MDA but not in the univariate study. On Day-62 (Fig 5D), the top MDA metabolites were 2-oxoisocaproate, tryptophan, glycine, 3-methyl-2-oxovalerate and L-isoleucine, while L-glutamine was also consistent with the univariate study, together with L-lysine, although this last showed a low MDA. At birth (Fig 5F), again L-lysine and L-phenylalanine shared relevance with the univariate study, together with L-glutamine and dimethyl sulfone, although these latter with much lower MDA scores. Valine and L-leucine were new metabolites ranked at birth.

2. Main metabolites: Biomarker block-study of pregnancy

A total of 190 pregnancy predictive blocks were obtained (Supplementary Table 3), distributed as 65 for Day-0 and 125 for Day-7. The numbers of blocks accounting for each pregnancy endpoint were similar (65, 64, and 61 for Day-40, Day-62 and birth, respectively), although they differed with cryopreservation (i.e., 33 for frozen embryos, 86 for fresh embryos and 71 cryopreservation independent blocks). Among them, 71 blocks were independent from breed, 67 from Holsteins, 20 from non-Holsteins and 32 from AV. The numbers of blocks were associated with the most represented breed (Holstein), but not with the most represented embryos (i.e., frozen). Thus, representative blocks for frozen embryos were fewer on Day-0 plasma (9 in total, 3 at each endpoint) than in Day-7 plasma (24 in total, 13 at Day-40, 7 at Day-62 and 4 at birth). The number of blocks at each gestational endpoint decreased as the pregnancy proceeded both within frozen embryos and independent of cryopreservation (16 and 26 blocks for Day-40, 10 and 26 for Day-62, and 7 and 19 at birth, respectively). In contrast, within fresh embryos, the number of blocks increased at each endpoint until birth (23, 28 and 35 blocks for Day-

40, Day-62 and birth) (Figure 6). Blocks with up-regulated metabolites in pregnant samples were 63, while 126 blocks contained down-regulated metabolites.

	Blocks	Plasma		Day-40			Day-62			Birth			By breed					
Metabolite	Total	0	7	Fresh	Frozen	Both	Fresh	Frozen	Both	Fresh	Frozen	Both	All	H	No H	AV	avAUC	Block-AUC
Creatine	20	7	13	2	4	1	2	2	2	2	2	3	7	6	6	1	0.7182	14.3640
L-Lysine	12	3	9	2	0	3	2	0	2	2	0	1	4	6	0	2	0.7409	8.8908
L-Valine	12	3	9	2	0	1	2	0	1	3	0	3	4	3	1	4	0.7137	8.5644
L-Phenylalanine	12	4	8	0	3	2	1	2	1	1	2	0	4	8	0	0	0.6765	8.1180
L-Glutamine	11	2	9	2	1	2	2	0	1	2	0	1	4	7	0	0	0.7035	7.7385
L-Leucine	10	4	6	2	1	1	2	1	0	2	0	1	3	6	0	1	0.7681	7.6812
L-Ornithine	10	3	7	2	0	2	2	0	1	2	0	1	4	6	0	0	0.7479	7.4790
2-oxoglutarate	10	1	9	2	0	2	2	0	1	2	1	0	4	5	1	0	0.6966	6.9660
L-alanine	9	3	6	1	0	2	2	0	1	2	0	1	4	1	1	3	0.7173	6.4557
L-Glycine	9	0	9	1	0	0	2	0	2	2	0	2	4	4	1	0	0.6930	6.2370
Dimethylsulfone	8	2	6	2	0	1	2	0	1	2	0	0	3	3	0	2	0.6998	5.5984
1-Methylhistidine	7	1	6	0	0	1	1	0	2	1	0	2	3	0	3	1	0.7006	4.9042
Acetone	6	4	2	0	2	1	0	1	2	0	0	0	1	0	3	2	0.7438	4.4628
Creatinine	5	2	3	0	0	1	0	0	1	2	1	0	2	0	1	2	0.7504	3.7520
2-hydroxyisobutyrate	5	4	1	0	1	0	0	1	0	2	1	0	2	0	3	0	0.7119	3.5595
L-Isoleucine	5	3	2	0	0	1	1	0	1	1	0	1	2	0	0	3	0.6964	3.4820
Butyrate	5	4	1	1	0	1	1	0	1	1	0	0	3	0	0	2	0.6936	3.4680
Propionate	5	3	2	1	0	1	1	0	1	1	0	0	3	0	0	2	0.6877	3.4385
Sarcosine	4	4	0	1	0	1	0	0	1	0	0	1	1	0	0	3	0.6963	2.7852
L-Threonine	4	3	1	2	1	0	1	0	0	0	0	0	2	2	0	0	0.6858	2.7432
2-oxoisocaproate	4	0	4	0	1	1	0	1	1	0	0	0	0	4	0	0	0.6747	2.6988
L-Tryptophane	3	2	1	0	0	0	1	0	0	0	0	2	1	0	0	2	0.6951	2.0853
Glucose	3	1	2	0	0	0	0	0	2	1	0	0	2	1	0	0	0.6800	2.0400
3-methyl-2-oxovalerate	3	0	3	0	1	0	0	1	0	1	0	0	1	2	0	0	0.6726	2.0178
Citrate	2	0	2	0	0	1	0	0	1	0	0	0	0	0	0	2	0.8133	1.6266
Dimethylamine	2	1	1	0	0	0	0	0	0	2	0	0	1	1	0	0	0.7146	1.4292
Hippurate	2	1	1	0	0	0	1	0	0	1	0	0	2	0	0	0	0.7076	1.4152
Isobutyrate	2	0	2	0	1	0	0	1	0	0	0	0	0	2	0	0	0.6747	1.3494
TOTAL	**190**	**65**	**125**	**23**	**16**	**26**	**28**	**10**	**26**	**35**	**7**	**19**	**71**	**67**	**20**	**32**		

Figure 6. Heatmap representative of abundance of blocks within metabolites according to the day of the sample (i.e., Day-0 or Day-7 plasma), pregnancy endpoints (i.e., Day-40, Day-62 and birth) and transferred embryo status (i.e., frozen, fresh or both), and recipient breed (all, H: Holstein, No H: No-Holstein, or AV: Asturiana de los Valles). The ROC-AUC average (avAUC) of each metabolite is also shown. Block*avAUC value considers the number of conditions into which the metabolite is discriminant for pregnancy and their avAUC values and represents the importance of each metabolite.

Among 35 metabolites analyzed, 28 were considered biomarker candidates acting as pregnancy predictive in specific sample blocks. Metabolites not represented were acetate, formate, 3-hydroxybutyrate, isocaproate, lactate, mannose, L-methionine, and pyruvate. Metabolites were ranked by the number of blocks in which they participated according to pregnancy endpoints, recipient breed and transferred embryo status (Figure 6). Creatine was an outstanding metabolite (20 blocks), showing the widest and most uniform distribution over pregnancy endpoints (7, 6 and 7 blocks for Day-40, Day-62 and birth, respectively), and cryopreservation (8 frozen, 6 fresh and 6 non-cryopreservation dependent). Creatine was a pregnancy biomarker associated with Holsteins (6 blocks), cattle groups with Holstein representation (i.e., non-Holsteins, through crossbred, 6 blocks) and breed independent (7 blocks; higher proportion of Holsteins). In contrast, the

pure AV group with the double muscled AV cattle showed just 1 block for creatine. The other metabolites that completed the top-ten ranking, bearing 115/190 blocks, were mainly amino-acids and one tri-carboxylic acid: L-lysine, L-valine, L-phenylalanine, L-glutamine, L-leucine, L-ornithine, 2-oxoglutarate, L-alanine and L-glycine. The ten metabolites ranked by their average ROC-AUC (avAUC) (0.8133 to 0.7137) were citrate, L-leucine, creatinine, L-ornithine, acetone, L-lysine, creatine, L-alanine, dimethylamine and L-valine. We estimated the importance of each metabolite with the Block*avAUC value ranking, which considers the number of conditions in which the metabolite is discriminant for pregnancy and their avAUC values. Once again, creatine (Block*avAUC= 14.3640) ranked first followed by L-lysine, L-valine, L-phenylalanine, L-glutamine, L-leucine, L-ornithine, 2-oxoglutarate, L-alanine, L-glycine (Block*avAUC=6.2370).

For practical purposes and identification of the best metabolites that can be used as biomarkers in each condition, Table 2 shows the top four ROC-AUC candidate biomarkers identified in recipient plasma per block. Abundance of these top biomarkers was more prominent on Day-7 (53) than Day-0 (20), and biomarkers were more predictive for Day-40 and Day-62 than at birth. Lower abundance of biomarkers that predicted birth is observed mainly in recipients transferred with frozen embryos, which had just one predictive metabolite to term pregnancy. In a lesser extent, such a reduction of birth biomarkers was also seen in blocks independent on cryopreservation, with the exception of Holsteins at birth, with 4 biomarkers in Table 2. In contrast, biomarkers of recipients that received fresh embryos did not show pronounced losses of their predictive ability to term.

Table 2. The top four ROC-AUC candidate biomarkers identified in recipient Day-7 and Day-0 (highlighted in blue) plasma for each block determined by breed (Holstein; AV: Asturiana de los Valles; No-H: non Holstein recipients consisting of AV, Asturiana de la Montaña -AM- and crossbred recipients; --: recipient from all breeds), cryopreservation (Cryo; Fz: recipients of frozen embryos; Fh: recipients of fresh embryos; -- : independent from embryo cryopreservation), and pregnancy endpoint.

			Biomarker 1		Biomarker 2		Biomarker 3		Biomarker 4	
Breed	Cryo	Endpoint	Metabolite-Day	AUC	Metabolite-Day	AUC	Metabolite-Day	AUC	Metabolite-Day	AUC
--	--	Day-40	Glutamine-7	0.677	Lysine-7	0.676	2-oxoglutarate-7	0.663	Phenylalanine-0	0.661
--	--	Day-62	1-Methylhistidine-7	**0.718**	Glycine-7	**0.703**	Creatine-0	0.668		
--	--	Birth	Glycine-7	**0.669**						
--	Fz	Day-40	Creatine-7	0.729	Phenylalanine-7	0.698				
--	Fz	Day-62	Creatine-7	0.732	Acetone-7	0.656				
--	Fz	Birth	Creatine-7	0.707						
--	Fh	Day-40	Lysine-0	0.804	Ornithine-0	0.804	Glutamine-0	0.774	2-oxoglutarate-7	0.763
--	Fh	Day-62	Valine-7	0.771	Lysine-7	0.769	Ornithine-7	0.751	Leucine-7	0.747
--	Fh	Birth	Ornithine-0	0.781	Lysine-7	0.768	Dimethylsulfone-7	0.764	Creatine-0	0.737
Holstein	--	Day-40	Ornithine-7	0.710	Glutamine-0	0.708	2-oxoglutarate-7	0.701	Lysine-7	0.701
Holstein	--	Day-62	2-oxoglutarate-7	0.671	Ornithine-0	0.671	Lysine-7	0.668	Glutamine-7	0.650
Holstein	--	Birth	Lysine-7	0.682	Ornithine-7	0.659	Creatine-7	0.655	Glutamine-7	0.653
Holstein	Fz	Day-40	Phenylalanine-7	0.744	Leucine-7	0.717	2-oxoisocaproate-7	0.700	Creatine-7	**0.698**
Holstein	Fz	Day-62	Creatine-7	**0.691**	Phenylalanine-7	**0.669**				
Holstein	Fz	Birth	**Phenylalanine-7**	**0.662**						
Holstein	Fh	Day-40	Ornithine-7	0.830	Lysine-7	0.826	Creatine-0	0.758	Leucine-0	0.758
Holstein	Fh	Day-62	Lysine-0	0.806	Ornithine-7	0.791	Leucine-0	**0.747**	Valine-7	0.747
Holstein	Fh	Birth	Lysine-0	0.831	Ornithine-7	0.801	Creatine-7	0.733	Glutamine-7	0.707
AV	--	Day-40	Citrate-7	0.813	Acetone-0	0.800	Alanine-7	0.787		
AV	--	Day-62	Citrate-7	0.813	Acetone-0	0.800	Alanine-7	0.787		
AV	--	Birth	Glycine-7	0.669						
No-H	--	Day-40	Creatine-7	**0.695**						

Table 2. *Cont.*

			Biomarker 1		Biomarker 2		Biomarker 3		Biomarker 4	
Breed	Cryo	Endpoint	Metabolite-Day	AUC	Metabolite-Day	AUC	Metabolite-Day	AUC	Metabolite-Day	AUC
No-H	--	Day-62	Creatine-0	0.776	Acetone-0	0.754	1-Methylhistidine-7	**0.718**	Glycine-7	**0.703**
No-H	--	Birth	1-Methylhistidine-7	0.751	Creatine-7	0.692				
No-H	Fz	Day-40	Creatine-0	**0.750**	Creatine-7	**0.750**				
No-H	Fz	Day-62	**2-hydroxyisobutyrate-0**	**0.743**						
No-H	Fz	Birth	Creatine-7	0.780						

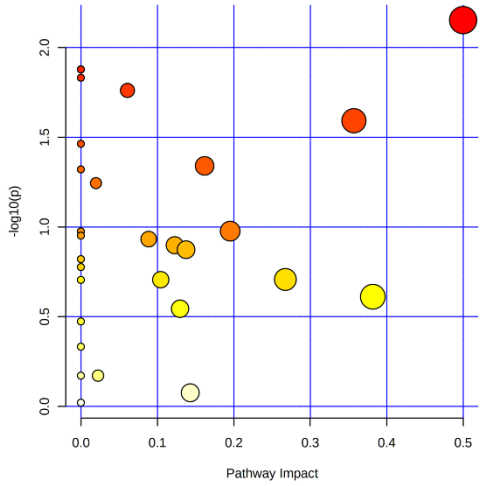
Blocks not represented due to low numbers of recipients: AV-Frozen; AV-Fresh; crossbred, AM, pooled as "No Holstein" recipients (No-H).

Bold AUC: tendencies in either Wilcoxon test or T-test or both (P<0.10); **Bold in metabolite and AUC**: AUC>0.65 but non-significant.

3. Metabolic pathway studies

3.1. Day-40 pregnant vs. Day-40 open recipients transferred with F/T embryos: Metabolic pathway analysis.

In correspondence with the higher number of regulated metabolites identified, Day-7 pathway analysis led to the discovery of pathways that significantly differed between pregnant and open Holstein recipients as diagnosed on Day-40 (Figure 7). The following pathways (Bos Taurus) stood out due to their impact: Phenylalanine, tyrosine and tryptophan biosynthesis; Phenylalanine metabolism; Alanine, aspartate and glutamate metabolism; and Arginine biosynthesis.



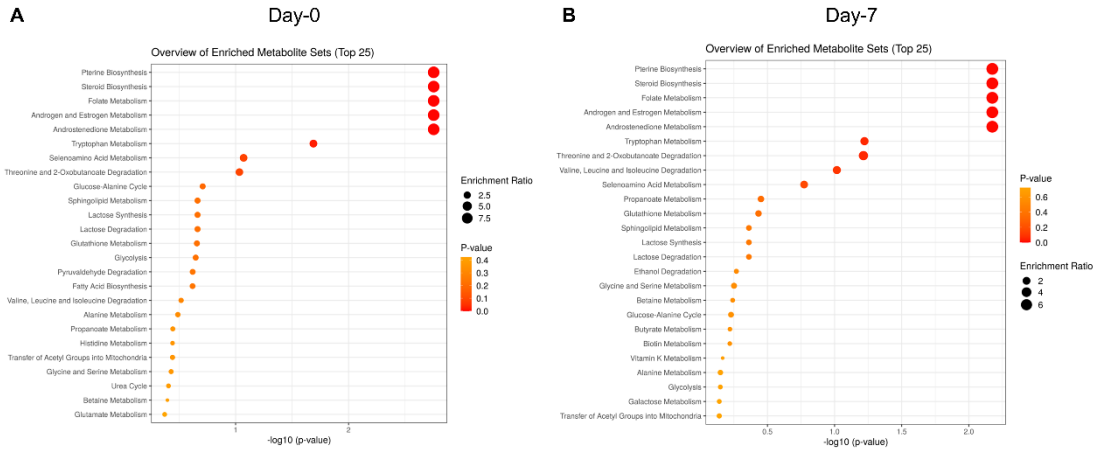
Pathway Name	P value	FDR	Impact
Phenylalanine, tyrosine and tryptophan biosynthesis	0.007	0.089	0.5
Purine metabolism	0.013	0.089	0
Pyrimidine metabolism	0.013	0.089	0
Nitrogen metabolism	0.013	0.089	0
D-glutamine and D-glutamate metabolism	0.015	0.089	0
Arginine biosynthesis	0.017	0.089	0.061
Phenylalanine metabolism	0.026	0.113	0.357
Aminoacyl-tRNA biosynthesis	0.034	0.113	0
Alanine, aspartate and glutamate metabolism	0.046	0.148	0.162
Valine, leucine and isoleucine biosynthesis	0.048	0.148	0

Figure 7. Metabolome view showing all matched pathways (Bos Taurus) differentially regulated between Day-40 pregnant and open Holstein recipients according to the p values (color gradient) from the pathway enrichment analysis and pathway impact values (dot sizes) from the pathway topology analysis. P values, false discovery rate (FDR) and impact value of the top regulated pathways (pregnant / non-pregnant ratio) are detailed.

3.2. Day-40, only pregnant recipients from fresh vs. F/T embryos: Metabolite set enrichment analysis (MSEA).

All pregnant recipients were used with separate analysis for Day-0 and Day-7 (Figure 8). Interestingly, the same top six metabolically enriched sets were identified on Day-0 (Fig 8A) and Day-7 (Fig 8B): Pterine Biosynthesis, Steroid Biosynthesis, Folate Metabolism,

Androgen and Estrogen Metabolism, Androstenedione Metabolism, and Tryptophan Metabolism. Enrichment and significance were more impacted on Day-0 than on Day-7.



Pathway Name	P value	FDR
Pterine biosynthesis	0.002	0.020
Steroid biosynthesis	0.002	0.020
Folate metabolism	0.002	0.020
Androgen and estrogen metabolism	0.002	0.020
Androstenedione metabolism	0.002	0.020
Tryptophan metabolism	0.020	0.194
Selenoamino acid metabolism	0.085	0.659

Pathway Name	P value	FDR
Pterine biosynthesis	0.007	0.076
Steroid biosynthesis	0.007	0.076
Folate metabolism	0.007	0.076
Androgen and estrogen metabolism	0.007	0.076
Androstenedione metabolism	0.007	0.076
Tryptophan metabolism	0.060	0.496
Threonine and 2-oxobutanoate degradation	0.061	0.496
Valine, leucine and isoleucine degradation	0.096	0.684

Figure 8. Enrichment of metabolite set pathways in pregnant fresh vs pregnant frozen on Day-0 (A) and Day-7 (B). The size of the circles per metabolite set represents the Enrichment Ratio and the color represents the pvalue. Tables down each plot describe the top metabolic pathway enriched, P value and False discovery rate (FDR).

4. Improving the pregnancy predictive power of the recipient biomarkers by ROC curve-based models supported by embryonic spectral features

4.1. Predictions and iterations

Table 3 shows the results of the combined pregnancy prediction.

Table 3. Transferred embryos (Fresh -Fh- or Frozen -Fz-) matched with their recipients showing the actual (*italic*) gestational status at birth and the ROC-AUC value (ROC) predicted for birth after iterations (ITER-) calculated with the algorithm support vector machine (SVM) within specific groups of embryos and recipients (AV: Asturiana de los Valles -includes one recipient Asturiana de la Montaña: AM; H: Holstein; Cb: crossbred). Divergencies between the actual status and the predicted status in an iteration are marked in **bold**. Significant ROC-AUC values calculated with P value <0.01 by permutation

Embryo			Recipient			*Actual*	Predicted status in embryos				Predicted status in recipients					
Sample	Group	Bull	Sample	Group	Breed	*Status*	ITER-1	ROC	ITER-2	ROC	ITER-1	ROC	ITER-2	ROC	ITER-3	ROC
1487	AV H Fh	AV	5111	H Fh	H	*Birth*	Birth	1.000			Birth	0.887	Birth	0.993		
1509	AV H Fh	AV	7278	H Fh	H	*Birth*	Birth	1.000			Birth	0.887	Birth	0.993		
1572	AV H Fh	AV	7277	H Fh	H	*Birth*	Birth	1.000			**No birth**	0.887	Birth	0.993		
2504	AV H Fh	AV	4290	H Fh	H	*Birth*	Birth	1.000			Birth	0.887	Birth	0.993		
2384	AV H Fh	H	7531	H Fh	H	*Birth*	Birth	0.983			Birth	0.887	Birth	0.993		
2437	AV H Fh	H	8447	H Fh	H	*Birth*	Birth	0.983			Birth	0.887	Birth	0.993		
2438	AV H Fh	H	2363	H Fh	H	*Birth*	Birth	0.983			Birth	0.887	Birth	0.993		
2439	AV H Fh	H	3613	H Fh	H	*Birth*	Birth	0.983			Birth	0.887	Birth	0.993		
2454	AV H Fh	H	6094	H Fh	H	*Birth*	Birth	0.983			Birth	0.887	Birth	0.993		
2575	AV H Fh	H	4557	H Fh	H	*Birth*	Birth	0.983			**No birth**	0.887	Birth	0.993		
2576	AV H Fh	H	7550	H Fh	H	*Birth*	Birth	0.983			Birth	0.887	Birth	0.993		
1757	AV H Fh	H	3955	H Fh	H	*Birth*	Birth	0.983			Birth	0.887	Birth	0.993		
1758	AV H Fh	H	1959	H Fh	H	*Birth*	Birth	0.983			Birth	0.887	Birth	0.993		
1571	AV Fh	AV	5140	H Fh	H	*No birth*	No birth	1.000			**Birth**	0.887	**Birth**	0.993		
2142	AV H Fh	H	4290	H Fh	H	*No birth*	No birth	0.983			**Birth**	0.887	No birth	0.993		
1488	AV Fh	AV	3844	H Fh	H	*No birth*	No birth	1.000			No birth	0.887	No birth	0.993		
2308	AV Fh	AV	4290	H Fh	H	*No birth*	No birth	1.000			No birth	0.887	No birth	0.993		
2309	AV Fh	AV	7530	H Fh	H	*No birth*	No birth	1.000			No birth	0.887	No birth	0.993		
1993	AV Fh	AV	3613	H Fh	H	*No birth*	No birth	1.000			No birth	0.887	No birth	0.993		
2505	AV Fh	AV	7235	AV H Fh	AV	*Birth*	Birth	1.000			Birth	0.925				
2143	H Fh	H	4943	AV H Fh	AV	*Birth*	Birth	0.997			Birth	0.925				

Table 3. *Cont.*

Embryo			Recipient			*Actual*	Predicted status in embryos				Predicted status in recipients					
Sample	Group	Bull	Sample	Group	Breed	*Status*	ITER-1	ROC	ITER-2	ROC	ITER-1	ROC	ITER-2	ROC	ITER-3	ROC
2385	AV H Fh	H	1381	AV H Fh	AV	*No birth*	No birth	0.983			No birth	0.925				
2386	AV H Fh	H	7237	AV H Fh	AV	*No birth*	No birth	0.983			No birth	0.925				
2456	AV H Fh	H	1344	AV H Fh	AV	*No birth*	No birth	0.983			No birth	0.925				
1473	AV Fh	AV	6022	AV H Fh	AM	*No birth*	No birth	1.000			No birth	0.925				
1520	AV Fz	AV	6887	H Fz	H	*Birth*	Birth	0.948	Birth	0.948	Birth	0.737	Birth	0.840	Birth	0.855
1521	AV Fz	AV	6886	H Fz	H	*Birth*	Birth	0.948	Birth	0.948	Birth	0.737	Birth	0.840	Birth	0.855
2175	AV Fz	AV	7922	H Fz	H	*Birth*	**No birth**	0.948	**No birth**	0.948	Birth	0.737	Birth	0.840	Birth	0.855
1782	AV Fz	AV	6810	H Fz	H	*Birth*	Birth	0.948	Birth	0.948	Birth	0.737	Birth	0.840	Birth	0.855
1846	AV Fz	AV	7540	H Fz	H	*Birth*	Birth	0.948	Birth	0.948	Birth	0.737	Birth	0.840	Birth	0.855
1848	AV Fz	AV	7541	H Fz	H	*Birth*	Birth	0.948	Birth	0.948	Birth	0.737	Birth	0.840	Birth	0.855
1680	H Fz	H	4710	H Fz	H	*Birth*	Birth	0.973	Birth	0.981	Birth	0.737	Birth	0.840	Birth	0.855
2242	H Fz	H	7530	H Fz	H	*Birth*	Birth	0.973	Birth	0.981	**No birth**	0.737	Birth	0.840	Birth	0.855
2319	H Fz	H	1356	H Fz	H	*Birth*	Birth	0.973	Birth	0.981	Birth	0.737	Birth	0.840	Birth	0.855
1900	H Fz	H	5988	H Fz	H	*Birth*	Birth	0.973	Birth	0.981	Birth	0.737	**No birth**	0.840	Birth	0.855
1522	AV Fz	AV	6480	H+Cb Fz	Cb	*Birth*	Birth	0.948	Birth	0.948	**No birth**	0.740	Birth	0.840	Birth	0.855
1523	AV Fz	AV	6885	H+Cb Fz	Cb	*Birth*	Birth	0.948	Birth	0.948	Birth	0.740	Birth	0.840	Birth	0.855
2489	AV Fz	AV	7530	H Fz	H	*No birth*	No birth	0.948	No birth	0.948	**Birth**	0.737	**Birth**	0.840	**Birth**	0.855
1842	AV Fz	AV	7530	H Fz	H	*No birth*	No birth	0.948	No birth	0.948	**Birth**	0.737	**Birth**	0.840	**Birth**	0.855
1675	H Fz	H	2398	H Fz	H	*No birth*	No birth	0.973	No birth	0.981	**Birth**	0.737	**Birth**	0.840	**Birth**	0.855
1678	H Fz	H	4787	H Fz	H	*No birth*	No birth	0.973	No birth	0.981	**Birth**	0.737	**Birth**	0.840	**Birth**	0.855
2178	AV Fz	AV	7531	H Fz	H	*No birth*	No birth	0.948	No birth	0.948	**Birth**	0.737	**Birth**	0.840	**Birth**	0.855
2285	H Fz	H	713755	H Fz	H	*No birth*	No birth	0.973	No birth	0.981	No birth	0.737	**Birth**	0.840	**Birth**	0.855
2041	H Fz	H	4557	H Fz	H	*No birth*	No birth	0.973	No birth	0.981	**Birth**	0.737	**Birth**	0.840	**Birth**	0.855
1901	H Fz	H	2398	H Fz	H	*No birth*	No birth	0.973	No birth	0.981	**Birth**	0.737	No birth	0.840	No birth	0.855
2516	AV Fz	AV	880	H Fz	H	*No birth*	**Birth**	0.948	**Birth**	0.948	**Birth**	0.737	No birth	0.840	**Birth**	0.855
1613	H Fz	H	5140	H Fz	H	*No birth*	**Birth**	0.973	**Birth**	0.981	**Birth**	0.737	No birth	0.840	**Birth**	0.855

Table 3. *Cont.*

Embryo			Recipient			*Actual*	Predicted status in embryos				Predicted status in recipients					
Sample	Group	Bull	Sample	Group	Breed	*Status*	ITER-1	ROC	ITER-2	ROC	ITER-1	ROC	ITER-2	ROC	ITER-3	ROC
2177	AV Fz	AV	713755	H Fz	H	*No birth*	No birth	0.948	No birth	0.948	No birth	0.737	No birth	0.840	No birth	0.855
1679	H Fz	H	7136	H Fz	H	*No birth*	No birth	0.973	No birth	0.981	No birth	0.737	No birth	0.840	No birth	0.855
1725	H Fz	H	2476	H Fz	H	*No birth*	No birth	0.973	No birth	0.981	No birth	0.737	No birth	0.840	No birth	0.855
1799	H Fz	H	7922	H Fz	H	*No birth*	No birth	0.973	No birth	0.981	No birth	0.737	No birth	0.840	No birth	0.855
2284	H Fz	H	1487	H Fz	H	*No birth*	No birth	0.973	No birth	0.981	No birth	0.737	No birth	0.840	No birth	0.855
2297	H Fz	H	9925	H Fz	H	*No birth*	No birth	0.973	No birth	0.981	No birth	0.737	No birth	0.840	No birth	0.855
2298	H Fz	H	9925	H Fz	H	*No birth*	No birth	0.973	No birth	0.981	No birth	0.737	No birth	0.840	No birth	0.855
2557	H Fz	H	9925	H Fz	H	*No birth*	No birth	0.973	No birth	0.981	No birth	0.737	No birth	0.840	No birth	0.855
1732	H Fz	H	2032	H Fz	H	*No birth*	No birth	0.973	No birth	0.981	No birth	0.737	No birth	0.840	No birth	0.855
1790	H Fz	H	2032	H Fz	H	*No birth*	No birth	0.973	No birth	0.981	No birth	0.737	No birth	0.840	No birth	0.855
1519	AV Fz	AV	6203	AV Fz	AV	*Birth*	Birth	0.948	Birth	0.948	**No birth**	0.911				
2517	AV Fz	AV	7234	AV Fz	AV	*Birth*	Birth	0.948	Birth	0.948	Birth	0.911				
2518	AV Fz	AV	1382	AV Fz	AV	*Birth*	**No birth**	0.948	**No birth**	0.948	Birth	0.911				
1847	AV Fz	AV	7230	AV Fz	AV	*Birth*	Birth	0.948	Birth	0.948	Birth	0.911				
1614	H Fz	H	4758	AV Fz	AV	*Birth*	Birth	0.973	Birth	0.981	Birth	0.911				
1615	H Fz	H	3598	AV Fz	AV	*Birth*	Birth	0.973	Birth	0.981	Birth	0.911				
2294	H Fz	H	7239	AV Fz	AV	*Birth*	Birth	0.973	Birth	0.981	Birth	0.911				
2296	H Fz	H	7237	AV Fz	AV	*Birth*	Birth	0.973	Birth	0.981	Birth	0.911				
1783	AV Fz	AV	7235	AV Fz	AV	*No birth*	No birth	0.948	No birth	0.948	No birth	0.911				
1905	AV Fz	AV	4943	AV Fz	AV	*No birth*	No birth	0.948	No birth	0.981	No birth	0.911				
2283	H Fz	H	1382	AV Fz	AV	*No birth*	No birth	0.973	No birth	0.981	No birth	0.911				
2295	H Fz	H	7237	AV Fz	AV	*No birth*	No birth	0.973	No birth	0.981	No birth	0.911				
2551	H Fz	H	1275	AV Fz	AV	*No birth*	No birth	0.973	No birth	0.981	No birth	0.911				

Embryos showed the highest ROC-AUC values in the sample classification models [i.e., ranging between 0.948 – 1.000 (Iteration 1) and 0.948 – 0.981 (Iteration 2)], with Iteration 2 being not necessary for fresh embryo samples and no misclassification (i.e., 25/25 correctly classified samples). Unsolvable, erroneous classification of some samples occurred in the frozen embryo group with 2 "birth" misclassified as "no birth", and 2 "no birth" classified as "birth" coinciding with the same classification error in the recipient, which precluded the identification of which -the embryo or the recipient- was a candidate to correct for the next iteration; such misclassifications remained after 3 iterations, leading to 41/45 correctly identified samples in frozen embryos.

Recipients of fresh embryos were correct for 24/25 samples. Holsteins needed two iterations, and only one "no birth" sample remained misclassified as "birth". Recipients from AV were correctly classified with no iteration. Within frozen embryos, the Holstein recipients (the largest embryo-recipient matched sample dataset with 32 ETs, including 2 crossbred), interestingly, had 9 "no birth" cases initially classified as "birth"; of them, after three iteration rounds, the ROC-AUC value grew from 0.737 to 0.855, and 8 of these recipients were definitely classified as "birth" as matched with non-competent embryos. AV recipients of frozen embryos showed one case of unsolvable classification from "birth" to "no birth", with only one iteration needed.

4.2. Re-calculation of birth biomarkers in recipients

The original Holstein dataset containing 49 samples was fed with the new predicted status of "birth" in 9 "no birth" recipients (see Table 3) and a new ROC-AUC for single biomarkers was calculated (Table 4).

Table 4. Biomarkers predictive of birth identified by H^+NMR in Day-7 plasma of Holstein recipients of frozen embryos after recalculation of metabolite concentration values by iterative identification of actual "no birth" recipients with predicted "birth" potential supported by embryo confusion matrix (*). Many of these biomarkers did not predict birth prior to iteration.

Metabolite	ROC-AUC	T-Test	Log FCh
Creatine	0.85117	0.00000588	0.46652
Butyrate	0.75251	0.0030875	0.218
1-Methylhistidine	0.72575	0.035673	0.27461
L-Glutamine	0.71572	0.013677	0.12309
D-Glucose	0.70736	0.0079813	-0.11701
L-Glycine	0.70736	0.022528	0.17702
L-Isoleucine	0.68896	0.014258	0.14392
L-Leucine	0.67726	0.038238	0.14411
L-Phenylalanine	0.67559	0.02963	0.1214
Propionate	0.67391	**0.052078**	0.13442
Hippurate	0.66890	0.033148	0.12401
L-Alanine	0.66555	0.025607	0.14095
2-oxoisocaproate	0.66388	0.022209	0.12641

(*) Predictive values of recipients calculated by multivariate support vector machine (SVM) calculations (empirical value P<0.01 by permutation).

Tendencies 0.05>P<0.10 shown in **bold**.

Interestingly, together with L-phenylalanine, 12 new biomarkers predicted birth with significant ROC-AUC>0.65 regarding the original recipient dataset, with a particular reinforcement of the predictive role for creatine (ROC-AUC = 0.851) (Figure 9 shows the ROC-AUC (8A) and boxplot (8B) for creatine). Biomarkers predictive of earlier pregnancy stages (Day 40 / Day 62) in the original dataset appeared again at birth after iteration (i.e., Phenylalanine, L-Leucine, 2-oxoisocaproate and creatine), while 5 new biomarkers with ROC-AUC>0.700 were identified (Butyrate, 1-Methylhistidine, L-Glutamine, D-Glucose, L-Glycine).

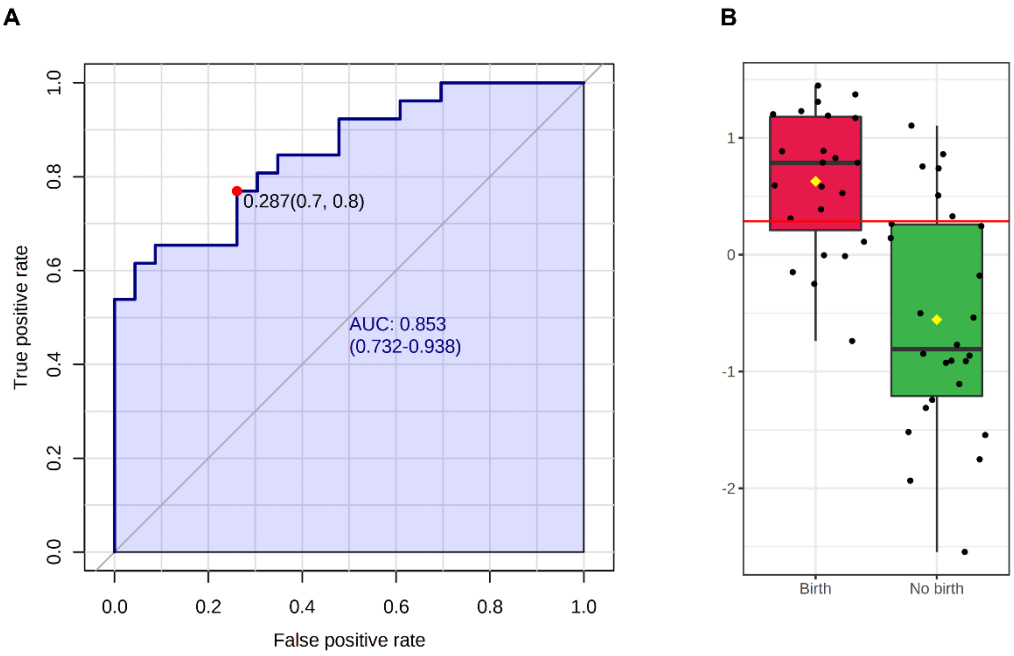


Figure 9. ROC-AUC (A) and boxplot (B) obtained after iterations for creatine concentrations that differ between recipients that reached or not birth. Black dots represent the concentrations of all samples of each metabolite scaled as Log Fold Change values. The notch indicates the 95% confidence interval around the median. The mean concentration of each group is shown by a red diamond. The optimal cutoff is indicated as a horizontal red line (i.e., the closest to the left-hand corner in the corresponding ROC-AUC).

DISCUSSION

In this work, for the first time, we identified biomarkers and metabolic pathways representative and predictive of pregnancy and birth in bovine recipients of IVP F/T embryos. In previous works we used Fourier transform infrared spectroscopy (FTIR) to predict pregnancy and birth in IVP (Muñoz et al., 2014a) and in vivo cultured (Muñoz et al., 2014b) embryos, as well as in the plasma of their recipients. However, the identification of molecules involved in pregnancy was not possible until more recent work with NMR with recipients of V/W and fresh embryos (Gómez 2020b; Gómez 2020c). Variability in fertilizing bulls, oocyte origins, culture systems and cryopreservation techniques increase the value of identified biomarkers in our studies. Likewise, the large number of samples and their use in different animal cohorts and breeds represents a proof of cross-validation often absent in livestock metabolomics studies (Goldansaz et al., 2017). Showing the same relationship between a studied phenotype and a biomarker in independent populations increases the value of the biomarker even if no obvious mechanistic relationship can be observed (Mayeux, 2004; Agakov et al 2011; Te Pas, et al; 2017).

Thus, the pregnancy and birth biomarkers identified here were both specific of F/T embryos and shared with V/W embryos and fresh embryos (Gómez et al., 2020a; Gómez et al., 2020b; Gómez et al., 2021). The presence of different metabolite biomarkers led to identification of regulatory pathways that differed not only between open and pregnant recipients, but also between recipients of cryopreserved and fresh embryos (Gómez 2020a; Gómez 2020b).

Profiling embryonic competence improves evaluation of recipient competence

The biomarkers we discovered in recipients of F/T embryos were not observed in recipients of V/W embryos in a previous report (Gómez et al., 2020a). However, after iterative pregnancy prediction combining information from recipient plasma and embryo CM, we here identified L-glutamine and L-glycine as early pregnancy biomarkers (i.e., Day-40 and Day-62) for Holstein recipients, as shown before within V/W embryos

(Gómez et al., 2020a). The iteration procedure detected misidentified recipients with a high ROC-AUC probability value. Previously we hypothesized and showed that absence of reciprocal information from embryos and recipients holds up the identification of pregnancy biomarkers (Muñoz 2014a; Muñoz 2014b; Gómez et al., 2021). The rationale can be explained from the information of the embryonic side, which is long term predictive since pregnancy rates to term greatly change with embryos produced with or undergoing different technologies, such as cloning, cryopreservation and certain culture conditions (e.g., serum) (Liu et al., 2013; Carrillo-González and Maldonado-Estrada, 2020; Gómez et al., 2020d; Valente et al., 2022). These technologies share similar early pregnancy rates, followed by a cascade of pregnancy losses, more pronounced with extremely altered embryos (i.e., clones). The similar early pregnancy rates between IVP embryos and more competent embryos (e.g., fresh in vivo), endorse our findings. Thus, the embryo, as responsible for most miscarriages, hides the true recipient competence to term as shown in a pioneer work modelling pregnancy failure after Day-60 (McMillan, 1998). In the present work we observed misclassification due to the pregnancy losses experienced by frozen embryos, interestingly, whose competence was accurately identified in the large data set once supported by embryonic metabolite signals.

In our study, more embryos than recipients were identified as non-viable for pregnancy; thus, independent recipient classification led to non-predictive results (i.e., more recipients were judged as viable than effectively pregnant). In contrast, recipient classification weighed with viability of embryos, half of which were qualified as non-viable, improved pregnancy prediction of recipients. Furthermore, the specificity of biomarkers is consistent with the type of embryo and its cryopreservation status, which could also imply, in a certain extent, a special capacity of the recipient to carry one or another embryo type to term. At the same time, complementary information from the embryo and the recipient predicts better to term and makes it possible to mate viable embryos with competent recipients, thus optimizing biomarker identification, leading to improved birth rates. We also emphasize that, in healthy recipients, the embryo, as responsible for most of miscarriages, shows an early metabolic fingerprint of such pregnancy losses that translate into measurable changes in culture medium.

Metabolic differences between recipients of fresh and F/T embryos

Recipients of fresh and cryopreserved (i.e., V/W) embryos show distinct metabolic profiles in Day-0 and Day-7 plasmas (Gomez et al., 2020a; Gomez et al., 2020b; Gomez et al., 2021), in agreement with the present study with frozen embryos. The rationale of metabolic differences between recipients of F/T and fresh embryos must be explored in the consequences of embryo freezing. Thus, F/T and fresh embryos that lead to pregnancy and birth exhibit distinct metabolite concentrations in their surrounding CM; such differences are particularly relevant for amino-acids and lipids (Gimeno et al., 2021).

In previous studies, lipid metabolism was related with pregnancy success both in IVP embryos (Gimeno et al., 2021) and recipients (Gomez et al., 2020a; Gomez et al., 2021). The involvement of lipid metabolism has also been reported in live embryos collected from Jersey cows, which have higher, different lipid content than Holsteins (Baldoceda et al., 2015). Such embryos show poorer survival to cryopreservation (Gilbert et al., 2022), and lower pregnancy rates after transfer once F/T (Steel and Hasler, 2004). Not only the total lipid contents, but certain lipids are responsible for decreased survival to cryopreservation (de Andrade Melo-Sterza and Poehland, 2021). Thus, in the embryonic cell, the freezing damage reduces membrane contents in lipids such as Lysophosphatidylcholine (Janati Idrissi et al., 2021), while the best freezing-survivors, in vivo embryos, have membranes enriched in phospholipids like phosphatidylcholines (Sudano et al., 2012; Janati Idrissi et al., 2021). Phosphatidylcholines are in turn methylated phosphatidylethanolamines, a lipid family we detected as acting as pregnancy biomarkers in F/T embryos (Gimeno et al., 2021). In accordance with a previous work with V/W and fresh embryos (Gómez et al., 2020a), we propose that recipients of cryopreserved embryos that become pregnant should have the intrinsic uterine ability to counteract at least the cryopreservation damage in the embryo, facilitating immediate embryo recomposition in the uterus upon transfer (e.g., membrane damage). Such a short-term effect would allow implantation of long-term damaged embryos, i.e., likely carrying serious epigenetic damage from cryopreservation, including cytogenetic alteration (Maldonado et al., 2015; Inaba et al., 2016; Hayashi et al., 2019), which would be prone to undergo late miscarriage, as explained by some specific biomarkers that differed between birth and earlier pregnancy stages (i.e., Day-40) identified in the embryo CM (Gimeno et al., 2021; Gomez et al., 2021). As an example, the miscarriage biomarker dimethyl adipate also acts as a robust pregnancy biomarker mainly in F/T embryos, but

at early pregnancy stages (Gimeno et al., 2021). In contrast, fresh embryos would not show such specific damages, and therefore the restorative ability exerted by the histotroph over the embryo could be a reason for differences shown in pregnancy biomarkers between recipients of fresh and cryopreserved embryos, provided that a proportion of plasma metabolites is reflected in the uterine fluid (UF) (Hugentobler et al., 2007). Thus, we identified six differentially enriched pathways in Day-40 pregnant recipients from fresh and F/T embryos, both within plasma samples on Day-0 and Day-7, which were pterine biosynthesis, steroid biosynthesis, folate metabolism, androgen and estrogen metabolism, androstenedione metabolism, and tryptophan metabolism. Furthermore, the fact that up to 60% losses of IVP embryos take place between Day-8 and Day-17 (Mamo et al., 2011; Hue et al., 2019) suggests that the environment that the embryo challenges just after ET greatly conditions its fate. Furthermore, the uterus responds to embryos bearing distinct competence with changes in the amino-acid concentrations in the UF (Groebner et al., 2011). Differences in histotroph amino-acids between cows (less fertile) and heifers (more fertile) remain at least until gestational Day-19 (Forde et al., 2017).

Further evidence of differences between recipients of F/T and fresh embryos are defined by creatine. In our case, creatine was a consistent pregnancy and birth biomarker, mainly with F/T but also with fresh embryos; and mainly on Day-7, but also on Day-0. Creatine showed positive FCh in recipients of F/T embryos (8 blocks; higher in pregnant) while the recipients of fresh embryos showed negative FCh (6 blocks; lower in pregnant). Interestingly, proteins of creatine metabolism were upregulated in UF from cows yielding poor quality in vivo embryos (Aranciaga et al., 2021), which is consistent with our results for fresh embryos. To a lesser extent, the same reversed FChs were observed for creatinine (contrary to creatine, 1 block negative in frozen, positive in fresh) and 2-hydroxyisobutyrate (3 frozen blocks and 2 fresh blocks higher and lower in pregnant recipients, respectively). The upregulation of general and amino-acid metabolism in cows carrying poor quality embryos described by Aranciaga and co-workers (2021) is also consistent with our distribution of blocks and with the multiple amino-acid pathway activation we recorded in recipients of F/T embryos. In this way, the number of blocks that had lower metabolite concentrations in pregnant recipients accounted for double the quantity than non-pregnant ones.

Relevant metabolite biomarkers of pregnancy

Collectively, embryonic biomarkers would predict better birth than earlier stages, while recipients -in general- show more predictive power in the beginning of pregnancy (Muñoz et al 2014a; Gomez et al., 2020a; Gomez et al., 2020b). In contrast, in vivo collected embryos -the highest viability standard- transferred fresh after a 24h culture step to analyze CM by FTIR in a simultaneous trial performed in two independent laboratories (France and Spain), showed higher predictive ability on Day-60 than at birth (Muñoz et al., 2014b), while recipients of such embryos showed similar predictive capacity through gestational endpoints.

Ornithine, L-Lysine and L-glutamine were relevant biomarkers in our study. Such metabolite concentrations increase on Day-0 in females later diagnosed as pregnant by AI (Phillips et al., 2018). Ornithine was a consistent biomarker for pregnancy at Day-40, mainly among fresh embryos produced under a variety of culture conditions and in all different breeds examined in previous studies (Gómez et al., 2020a; Gómez et al 2020b) and in the present study, suggesting robustness as a confident biomarker. Recently, ornithine, was identified as one of two important metabolite hubs in Day-0 plasma of artificially inseminated (AI) pregnant and non-pregnant recipients, as well as glutamine in non-pregnant recipients (Banerjee et al., 2022). Ornithine is involved in the arginine biosynthesis pathway, which was upregulated in open vs pregnant F/T Holsteins diagnosed on Day-40, together with phenylalanine, tyrosine and tryptophan biosynthesis, phenylalanine metabolism, alanine, aspartate and glutamate metabolism pathways. Moreover, in the non-pregnant group, the ART3 gene (ADP-Ribosyltransferase-3, an arginine-specific ADP-ribosyltransferase) was downregulated and positively correlated with ornithine (Banerjee et al., 2022). Ornithine and arginine are precursors for polyamine synthesis. Ornithine decarboxylase 1 (ODC1) converts arginine into ornithine which in turn forms putrescine (Lenis et al., 2017). Polyamines are essential for early embryonic development and successful pregnancy establishment (Lenis et al., 2017; Pendeville et al., 2001; Fozard et al., 1980). Interestingly, expression of ODC1 was found to be significantly lower in biopsies from blastocysts resulting in resorption compared with those resulting in calf delivery (El-Sayed et al., 2006). In porcine parthenotes, addition of polyamines to the culture medium increased the blastocyst rate, total cell number, and decreased apoptosis (Cui and Kim, 2005). L-arginine and ornithine are regulated throughout pregnancy and, interestingly, hold their concentrations in serum, uterine fluid

and fetal-annex fluids under nutritional restriction (Crouse et al., 2019), which suggest they can be suitable biomarkers. Whatever the reproductive technique used to reach pregnancy, there is a widely supported association between ornithine and fertility.

The pregnancy predictive ability of biomarkers in recipients was lower with frozen embryos, particularly at birth, in contrast with both fresh and early pregnancy stages of F/T (summarized in Table 2). We reported similar findings with recipients of V/W embryos (Gómez et al., 2020a), with one single biomarker being predictive both at birth (L-Glycine) and at earlier pregnancy stages. In the present study, L-Glycine also predicted at earlier stages and, with a lesser capability, at birth, but independent of breed. However, combining the information provided by embryos improves the capacity to predict birth in recipients, as shown with L-Glycine herein. Another interesting biomarker, acetone, was altered in all groups except in pure Holstein, and mainly in recipients transferred with F/T embryos. In a previous work we identified acetone as a birth biomarker in AV recipients transferred with V/W embryos, but not with fresh embryos (Gómez et al., 2020b). Therefore, acetone seems to be a biomarker associated with cryopreservation. Similar to the present work with F/T embryos in AV and no-Holstein breeds, 2-hydroxyisobutyrate predicted birth in Day-7 plasma of AV recipients transferred with V/W embryos (i.e., AV, AM and crossbred) (Gómez et al., 2020b).

Supplementation to CM with specific metabolites identified in this study could improve the metabolic status of the embryo. However, as the study was carried out in an experimental herd, results can be constrained by specific nutrition, environmental conditions, and embryo culture systems; therefore, the extent of our results to other scenarios need caution to be made. Although the combined methodology to detect and mate competent embryos and recipients led to upgraded biomarkers in recipients and embryos consistent in part with other studies, further proofs of validation are necessary yet.

CONCLUSION

Generally, embryos were more predictive of birth than of earlier pregnancy stages; on the contrary, recipients of frozen embryos showed more predictive power at the beginning of pregnancy, while recipients of fresh embryos were more predictive at birth. However, used together, such complementary information leads to better pregnancy prediction to term. Cryopreservation, although showing not significantly more miscarriage rates in our sample set, led to obtaining some specific biomarkers which were different from recipients of fresh embryos. Furthermore, more biomarkers differed between initial (i.e., Day-40 and Day-62) pregnancy endpoints and birth among recipients of F/T embryos, confirming the drop of biomarker abundance observed at birth in previous studies with V/W embryos. We also observed that pregnancy competence is lower in a high proportion of our IVP embryos than in recipients, making embryo studies more reliable than investigating recipient competence, which we can estimate over 80-85% of ET cycles, in agreement with previous findings. Therefore, the expected gain of pregnancy prediction is much greater with embryos (45-55%) than with recipients. Predicting on Day-0, although less efficient because more biomarkers appear on Day-7, entails advantages in terms of decision making (one week before ET). The use of recipient biomarkers combined with embryo biomarkers (which were obtained in a non-invasive way) provides a method both to improve the reliability and accuracy of single biomarkers and, used together, to highly increase the pregnancy expectation with F/T embryos in cattle.

SUPPLEMENTARY INFORMATION

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6| CAPÍTULO IV

FITNESS OF CALVES BORN FROM IN VITRO PRODUCED FRESH AND CRYOPRESERVED EMBRYOS

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Fitness of calves born from *in vitro*-produced fresh and cryopreserved embryos

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In cattle, vitrified/warmed (V/W) and frozen/thawed (F/T), *in vitro*-produced (IVP) embryos, differ in their physiology and survival from fresh embryos. In this study, we analyzed the effects of embryo cryopreservation techniques on the offspring. IVP embryos cultured with albumin and with or without 0.1% serum until Day 6, and thereafter in single culture without protein, were transferred to recipients on Day 7 as F/T, V/W, or fresh, resulting in $N = 24$, 14, and 13 calves, respectively. Calves were clinically examined at birth, and blood was analyzed before and after colostrum intake (Day 0), and subsequently on Day 15 and Day 30. On Day 0, calves from V/W and F/T embryos showed increased creatinine and capillary refill time (CRT) and reduced heartbeats. Calves from F/T embryos showed lower PCO_2, hemoglobin, and packed cell volume than calves from V/W embryos while V/W embryos led to calves with increased Na^+ levels. Colostrum effects did not differ between calves from fresh and cryopreserved embryos, indicating similar adaptive ability among calves. However, PCO_2 did not decrease in calves from V/W embryos after colostrum intake. Serum in culture led to calves with affected ($P < 0.05$) temperature, CRT, HCO_3^-, base excess (BE), TCO_2, creatinine, urea, and anion gap. On Day 15, the effects of embryo cryopreservation disappeared among calves. In contrast, Day 30 values were influenced by diarrhea appearance, mainly in calves from V/W embryos (i.e., lower values of TCO_2, HCO_3^-, and BE; and increased glucose, anion gap, and lactate), although with no more clinical compromise than calves from fresh and F/T embryos. Diarrhea affected PCO_2 and Na^+ in all groups. Embryo cryopreservation, and/or culture, yield metabolically different calves, including effects on protein and acid–base metabolism.

KEYWORDS

bovine, embryo-freezing, calves, embryo-vitrification, creatinine, acid-base

Introduction

In vitro assisted reproductive technologies are sustained by cryopreservation of gametes and embryos. Cryopreservation suppresses the necessity to have recipients available at the time of embryo production, thus facilitating worldwide exchanges of genetic material. In cattle, cryopreservation of *in vitro*-produced (IVP) embryos for

embryo transfer (ET) takes place normally after 7 ± 1 days of culture. Embryo cryopreservation (covering slow freezing and vitrification) induces damage such as morphological alteration (1), DNA fragmentation (2), decreased cell numbers and increased apoptotic cell ratio (3–5), abnormal gene expression (6–8) and histone modifications (9), and changes in embryo metabolism (10). Adaptation of embryos to divergent conditions may trigger changes in post-natal phenotypes that may persist until adulthood, as observed in rats (11, 12). In cattle and other mammalian species, *in vitro* embryo production technologies lead to offspring phenotypes deviating from those naturally conceived or resulting from the transfer of *in vivo* collected embryos (13–15). An extreme example occurs among calves born from somatic cell nuclear transfer (SCNT) (16–18).

Generally, IVP cryopreserved embryos are less able to reach term pregnancy upon transfer than their fresh counterparts and *in vivo*-produced embryos (4, 19). Cryopreservation reduces embryo survival rates and cell numbers *in vitro* (3, 4, 20, 21) and causes a series of functional and structural damage in embryonic cells [reviewed by Mogas (22)]. At the transcriptomics level, vitrification itself alters the expression of genes relevant for development in IVP embryos surviving cryopreservation (3, 23–25). Thus, IVP embryos show altered cell differentiation, lipid metabolism, and cell adhesion, with the intensity of such changes being higher in vitrified than in frozen embryos (6). Moreover, embryonic genes associated with organogenesis, immune response, and regulation of cognitive functions are altered after vitrification, compared to fresh embryos (25). Extensive transcriptomic changes remain within vitrified/warmed embryos exposed to the uterus and collected on Day 14, which show alteration in cell proliferation, cellular stress response, mitochondrial dysfunction, control of translation initiation, and DNA repair (23).

However, *in vitro* experiments often do not accurately represent embryonic survival *in vivo*, and, while *in vitro* quality parameters are sometimes superior in vitrified/warmed (V/W) over frozen/thawed (F/T) embryos, such differences are not observed in pregnancy rates (4, 26). Indeed, ET reports with large numbers of V/W and F/T IVP embryos indicate that both cryopreservation systems perform equally in pregnancies reaching term, sometimes with rates close to fresh IVP embryos (4, 13, 19, 27–30). Such studies focused on pregnancy and/or birth rates, sometimes combined with calf morphometry and survival; nevertheless, how V/W and F/T affect calf phenotypes and basic hematological parameters is presently unknown.

A proportion of calves born from IVP embryos are prone to show larger birth weight (BW) and perinatal mortality (14), under a phenotype known as large offspring syndrome (LOS) or abnormal offspring syndrome (AOS) (31–33) that courses with placental overgrowth (34, 35) and compromised vascular development (18, 36, 37). However, subtle alterations may remain within animals derived from IVF procedures (38), even without obvious phenotypic abnormality (33, 38); thus, such animals are normally incorporated into the productive process in farms. Clinical studies detected differences between calves born from IVP embryos compared to calves born from artificial insemination (AI) (39, 40), and between these and clones (41). Furthermore, male IVP calves show activation of the hypothalamus–pituitary–gonadal axis earlier than *in vivo* developed calves produced by multi-ovulation and embryo transfer (MOET) (38).

Neonates experience a striking metabolic challenge at birth. The placental nutrition ceases abruptly and is replaced by a cyclic enteral food supply to conserve homeostasis (42). Such a change requires progressive adaptation to adulthood with adrenal, pancreatic, and thyroid hormones driving from fetal setpoints to anabolic oxidative metabolism, energy storage, and tissue accretion (43). The welfare of calves has been often analyzed by measuring biochemical and hematological parameters (39–41, 44–46). Such studies are crucial because of their contribution to defining normalcy and alteration intervals. In contrast, limited knowledge of the effects of cryopreservation on calf fitness was documented.

In the present study, we hypothesized that cryopreservation, as one of the hallmarks that trigger alterations and reduce the viability of IVP embryos, may underlie effects on calf fitness that cannot be obvious in offspring. For this purpose, we used embryo cryopreservation procedures that yielded gestation length (GL), BW, and daily gain weight of the fetus that did not differ between fresh, F/T, and V/W calves (4). This way, the absence of labor difficulties derived from heavier calves and/or extended gestation may facilitate the evaluation of the effects of cryopreservation as is or as a specific technique in itself (i.e., F/T or V/W).

Materials and methods

The study was conducted following the guidelines of the Declaration of Helsinki and approved by the Animal Research Ethics Committees of SERIDA and the University of Oviedo (PROAE 33/2020; Resolución de 13 de Noviembre de la Consejería de Medio Rural y Recursos Naturales), in accordance with European Community Directive 86/609/EC.

All reagents were purchased from SIGMA (Madrid, Spain) unless otherwise stated.

Calves used in this study were born in a 3-year period (May 2017-July 2020) from IVP embryos transferred to recipients following procedures that did not induce differences between fresh, frozen/thawed (F/T), and vitrified/warmed (V/W) embryos in calf BW and GL (4) An update of animals born in our herd confirmed no differences in BW average, although 5/35 F/T and 8/33 V/W cases showed BW > 50 kg, compared with 1/26 in fresh embryos (3, 3, and 1 of them, respectively, used in this study; unpublished data). However, only two calves born from V/W and fresh

TABLE 1 Calves sampled according to their embryonic origin (i.e., cryopreservation) and timing before or after colostrum intake, and calves deceased during the sampling period (30 days from birth).

	Colostrum intake				Diarrhea		Non-Diarrhea	
Embryo	Before	After	Male	Female	Treated	Untreated	Dead	Dead
Fresh	12	13	5	8	2	1	0	0
Vitrified	10	14	7	7	2	2	0	1
Frozen	19	23	17	7	5	4	2	2

Diarrhea cases before Day-15 ($N = 3$) and after Day-15 ($N = 13$; mostly affecting Day-30 measurements by diarrhea/or its treatment).
Deaths: 1 vitrified (non-diarrhea: Day-2); 2 frozen (non-diarrhea: 1 Day-2 and 1 Day-7; diarrhea: 1 Day-10 and 1 Day-12).
Total calves: 24 Frozen; 13 Fresh; 14 Vitrified.

TABLE 2 Update of Day 40 and Day 62 pregnancy rates, pregnancy loss rates, birth rates, and calving ease after transfer of Day 7 and Day 8 vitrified, frozen, and fresh embryos cultured from Day 0 to Day 6 in groups with either BSA (0.6%) or FCS (0.1%) + BSA (0.6%), and subsequently in individual culture without protein supplements (0.5 mg/ml PVA) from Day 6 to Day 7 in the experimental herd.

				Pregnancy rates (%)		Pregnancy loss >Day 40		Calving ease (*N*) %			
Embryo	Culture	Day	*N*	Day 40	Day 62		Birth rates (%)	1 and 2	3 and 4	5	Calves analyzed
Fresh	BSA	7	32	(22/32) 68.7	(18/32) 56.2	21.8 (7)	(15/32) 46.9	(15) 100	-	-	7
	FCS+BSA	7	17	(11/17) 64.7	(10/17) 58.6	27.3 (3)	*(8/16) 50.0	(7) 87.5	-	(1) 12.5	5
Vitrified	BSA	7	50	(32/50) 64.0	(31/50) 62.0	25.0 (8)	(24/44) 54.5	(21) 87.5	(3) 12.5	-	10
	BSA	8	2	(1/2) 50.0	(1/2) 50.0	-	(1/2) 50.0	(1) 100	-	-	-
	FCS+BSA	7	10	(5/10) 50.0	(5/10) 50.0	20.0 (1)	*(4/9) 44.4	(4) 100	-	-	4
Frozen	BSA	7	65	(36/65) 55.4	(34/65) 52.3	13.8 (9)	(27/59) 45.8	(23) 85.2	(4) 14.8	-	18
	BSA	8	17	(5/17) 29.4	(5/17) 29.4	40.0 (2)	(4/17) 26.7	(4) 100	-	-	2
	FCS+BSA	7	17	(9/17) 52.9	(8/17) 47.0	29.4 (5)	(4/17) 26.7	(3) 75	(1) 25	-	4

*Two recipients dead after fresh ET (1 open and 1 pregnant after Day 62). Birth rates are unmatched with pregnancy rates reflecting that some pregnancies are ongoing. Of the total of calves born at term, five died 24 h after birth, four corresponding to a transfer of a vitrified embryo cultured with BSA, and one to a fresh embryo cultured with BSA. Calving ease, 1 (no assistance required); 2 (soft traction without manipulation); 3 (hard traction); 4 (manipulation and traction); and 5 (cesarean section).
Distribution of samples used per bull, Bull A, $N = 12$; Bull B, $N = 9$; Bull C, $N = 10$; Bull D, $N = 4$; Bull E, $N = 7$; Bull F, $N = 7$; Bull G, $N = 2$.
The calves analyzed in the present study are detailed.

embryos and pregnancies >290 days showed ≥60 kg at birth (4). For these experimental purposes, the calves available for analysis at Day 0 were $N = 13$ (fresh), $N = 14$ (V/W), and $N = 24$ (F/T) (refer to Table 1, for details). The readers are referred to our articles (4, 24, 47) for a complete description of oocyte collection from slaughterhouse ovaries, *in vitro* maturation (IVM) and *in vitro* fertilization (IVF), which was performed with frozen/thawed semen from single seven individual bulls [three Holstein and four Asturiana de los Valles (AV)]. Pregnancy rates from each procedure were also described (4, 24). An update of embryo transfers performed in our experimental herd, pregnancy, pregnancy loss, birth rates, and calving ease for fresh, F/T, and V/W embryos cultured with or without serum is shown in Table 2.

In vitro embryo production

Extended *in vitro* culture (IVC) procedures were described (47). Briefly, after IVF, presumed zygotes were loaded into the embryo culture medium (CM) which consisted of modified synthetic oviduct fluid (mSOF) with MEM non-essential amino acids (M7145), BME amino acids (B6766), citrate (0.1 µg/ml), myo-inositol (0.5 µg/ml), and Bovine serum albumin (BSA) (A3311) (6 mg/ml) with or without 0.1% (v/v) FCS (SIGMA F4135), under mineral oil at 38.7°C, 5% CO_2, 5% O_2, 90% N_2, and saturated humidity. Embryos were cultured in groups until Day 6. On Day 6 (143 h after IVF onset) morulae, early blastocysts and blastocysts were cultured individually in 12-µl protein-free mSOF with polyvinyl-alcohol (P8136) under mineral oil for 24 h. Thereafter, Day 7 embryos at the expanding

blastocyst stage (ExB) and fully expanded blastocysts (FEB) were transferred fresh or cryopreserved (F/T or V/W) to recipients synchronized on cycle Day 7. As an exception, one calf was derived from one Day 7 embryo which was re-cultured for 24 h with new protein-free CM and transferred as an F/T Day 8 embryo.

Embryo freezing and thawing

Freezing and thawing followed a described procedure (4). Only ExB and FEB were collected from single culture drops and washed three times in phosphate buffered saline (PBS) + 4-g/L BSA and loaded in a freezing medium containing PBS (P4417), 1.5-M EG, and 20% CRYO3 (5617, Stem Alpha, France) for 10 min. Embryos were loaded into a French straw between two columns with PBS + 0.75-M EG + 20% CRYO3, and two further columns PBS + 0.75-M EG + 20% CRYO3 separated by air. The straw was sealed with a plug and loaded into a programmable freezer (Crysalis, Cryocontroller PTC-9500) at −6°C for 2 min and seeded once with supercooled forceps. Straws remained for 8 min at −6°C and were subsequently dehydrated at a −0.5°C/min rate up to reach −35°C. Finally, the straws were stored in LN_2 until used for ET. Thawing was performed by holding the straws for 10 s on air and 30 s in at 35°C (water bath) and drying with 70% ethanol. Each thawed straw containing a single embryo was mounted in an ET catheter and non-surgically transferred to recipients deeply in the uterine horn ipsilateral to the corpus luteum of synchronized recipients.

Embryo vitrification

Vitrification procedures have been previously described (48). Only ExB and FEB blastocysts were vitrified in two steps with fibreplugs (CryoLogic Vitrification Method; CVM), working on a heated surface (41°C) in a warm room (25°C). Embryos were handled in a basic vitrification medium (VM: TCM 199-HEPES + 20% (v/v) FCS). Each blastocyst was exposed to VM with 7.5% ethylene-glycol (EG, 102466-M), 7.5% dimethyl sulfoxide (DMSO) (D2650, vitrification solution-1) for 3 min, and then moved into a drop containing VM with 16.5% EG, 16.5% DMSO and 0.5-M sucrose (vitrification solution-2; VS2). The time spent by the embryos in VS2 (including loading) did not exceed 25 s. Sample vitrification was induced by touching the surface of a supercooled block placed in LN_2 with a hook. Fibreplugs with the vitrified embryos were stored in closed straws in LN_2 until warming previous to ET. Embryos were warmed in single-step by directly immersing the fibreplug end in 800 µl of 0.25 M sucrose in VM, where the embryo was kept for 5 min and subsequently washed twice in VM and twice in mSOF containing 6-mg/ml BSA and 10% FCS before preparing for ET.

Recipient management, embryo transfer, and pregnancy diagnosis

All recipients were managed, fed, transferred, and housed during gestation; calving; and perinatal period (30 days) in the experimental herd. Such procedures have been described in depth (49, 50) and performed to minimize environmental differences. Embryos were transferred to AV, Holstein, and crossbred recipients synchronized in estrus with a progestagen-releasing device (PRID Alpha; CEVA, Barcelona, Spain), loaded intravaginally for 8–11 days, and removed 48 h after prostaglandin F2α analog (Dynolitic, Pfizer, Madrid, Spain) injection. Estrus appearance was observed by experienced caregivers 2–3 times per day and/or monitored with an automated sensor system (Heatphone, Medria, Humeco, Huesca, Spain). In the absence of clear estrous signs, progesterone concentrations were measured to select recipients, with P4 fold change Day 7/Day 0 >8 and Day 7 P4 values >3.5 ng/ml. An enzyme-linked immunosorbent assay (ELISA) test (EIA-1561, DRG Diagnostics, USA) was used for progesterone measurement. Before ET, all recipients were clinically explored by ultrasonographic scanning for detection of a corpus luteum in one ovary and transferred at a fixed time. ETs were performed non-surgically under epidural anesthesia. Fresh embryos were washed twice in Embryo Holding Media (019449, IMV Technologies) and mounted in straw in the same medium. Vitrified embryos were warmed, examined in their morphology, and mounted as fresh embryos for transfer. Frozen/thawed embryos were directly transferred in straw and not examined. Pregnancy was diagnosed on Day 40 and Day 62 by ultrasonography and birth rates were monitored.

Rationale

We planned a study based on sample variability to reach experimental randomness. The variability in the experimental conditions and individual bulls is intended to add degrees of freedom to our study and avoid linkage between any particular condition (i.e., a specific embryo culture, a single bull, etc.) and the parameters we analyzed among calves from fresh, F/T, and V/W embryos. Gestation was allowed to end naturally in the experimental herd without calving induction. Calving and birth time were monitored with an intravaginal sensor (Vel'Phone, Medria, Humeco, Huesca, Spain). After birth, mothers were adjusted in milk production by feeding 3 kgs concentrate/day, given by automated dispenser; supervised manual milking was performed when necessary to reduce possible excess of milk production in dairy mothers. Calves were kept with mothers in free stalls and suckled colostrum and milk ad libitum in order to satisfy milk amounts >20% of calf body weight (51). Mothers and calves were held in the same shared environment

until calves were aged 1 month. The health status of calves was assessed daily by experienced caregivers who monitored behavior changes, suckling and appetite, fatigue, diarrhea (presence/ absence), cough, eye, and/or nasal discharges. At birth (Day 0), blood samples were collected before colostrum intake and 1–4 h after colostrum intake (52). Hematological and biochemical analysis were performed on blood samples taken subsequently at fixed times (10 a.m. on Days 15 ± 1 and 30 ± 1 of life), together with clinical examination. Blood was collected in vacuum tubes (containing lithium heparin) from the jugular vein, shaking 10 times, and directly analyzed in a Vetscan i-STAT One analyzer (Scil Animal Care, Madrid, Spain; CG4+ and CHEM8+ modules). The analyzer was loaded in a room in the experimental farm kept at 25°C, and blood tubes were collected independently to minimize time spent until analysis. Parameters measured in calves and taken into account in embryos for analysis are shown in Table 3. Parameters measured by Vetscan i-STAT One were previously subjected to a time-course validation to check consistency and, eventually, apply corrections (which were not necessary as all measures were performed in blood collected in time). Within the measured parameters, acid–base status was estimated through the followings: partial pressure of CO_2 (PCO_2), which represents the respiration fraction of acid–base balance (i.e., Cellular production of CO_2 and ventilatory removal of CO_2); HCO_3^-, which is the metabolic component of acid–base balance; total CO_2 (TCO_2) is a measure of carbon dioxide in several states: CO_2 in physical solution or loosely bound to proteins, HCO_3^- or CO_3 ions and carbonic acid (H_2CO_3); anion gap (AG), which detects organic acidosis by measuring differences between the cations Na^+ and K^+ and the anions Cl^- and HCO_3^-; PO_2, i.e., partial pressure of oxygen dissolved in blood; oxygen saturation (sO_2), which represents the total amount of hemoglobin (Hb) able to bind oxygen—oxyhemoglobin plus deoxyhemoglobin; base excess (BE), which is the non-respiratory component of pH; and pH.

TABLE 3 Variables controlled in embryos and parameters measured in calves on Days 0, 15, and 30 after birth.

Parameter	Details
Recipient Breed	AV / Holstein / Crossbred
Mother weight at birth	Kg
Calving easy	AU: 1-5 (no intervention to Caesaran section)
Gestation length	Days
Calf breed	AV / Holstein / Crossbred
Calf sex	Male / Female
Colostrum intake	Yes / No
Suckling reflex	Yes / No
Capillary refill time	Seconds: 1-5 (time to recover color after gum pressure)
Rectal temperature	°C
Conjunctival appearance	AU: 1-Anemic; 2-Pale; 3-congestive; 4 icteric
Nasal mucosa appearance	AU: 1-No discharge; 2-Discharge; 3-Colored discharge
Ganglion size	Palpation: 0-not-increased; 1-increased
Ganglion pain	Digital pressure: No / Yes
Calf weight	Kg
Body Size	Cm
Chest perimeter	Cm
Heartbeats	Beats / min (auscultation)
Respiration	Breathings /min (auscultation)
pH	Vetscan i-STAT One (CG4+)
CO_2 partial pressure (PCO_2)	mm Hg (Vetscan i-STAT One; CG4+)
O_2 partial pressure (PO_2)	mm Hg (Vetscan i-STAT One; CG4+)
Base excess (BE)	mmol/L (Vetscan i-STAT One; CG4+)
HCO_3^-	mmol/L (Vetscan i-STAT One; CG4+)
Total CO_2 (TCO_2)	mmol/L (Vetscan i-STAT One; CG4+)
O_2 saturation (sO_2)	% (Vetscan i-STAT One; CG4+)
Lactic acid	mmol/L (Vetscan i-STAT One; CG4+)
Na^+	mmol/L (Vetscan i-STAT One; Chem8+)
K^+	mmol/L (Vetscan i-STAT One; Chem8+)
Cl^-	mmol/L (Vetscan i-STAT One; Chem8+)
Ca^{2+}	mmol/L (Vetscan i-STAT One; Chem8+)
Glucose	mg/dL (Vetscan i-STAT One; Chem8+)
Urea	mg/dL (Vetscan i-STAT One; Chem8+)
Creatinine	mg/dL (Vetscan i-STAT One; Chem8+)
Hematocrit	% PCV[(a)] (Vetscan i-STAT One; Chem8+)
Hemoglobin	g/dL (Vetscan i-STAT One; Chem8+)
Anion gap	mmol/L (Vetscan i-STAT One; Chem8+)

[(a)] Packed cell volume. AV: Asturiana de los Valles. AU, Arbitrary units.

Statistical analysis

Statistical analysis was performed with SAS/STAT package (Version 9.2; SAS Institute, Inc.) using GLM models. The biochemical and hematological parameters analyzed were first submitted to a time-course validation test for consistency to obtain appropriate time intervals for chemometric analysis. Subsequently, for their study, data were divided into two sets (Day 0, with and without colostrum intake; and a time-course Day 0, Day 15, and Day 30 analysis, excluding Day 0 samples obtained prior to colostrum intake). This second analysis included the effects of diarrhea, which appeared at specific time points in some calves (refer to the footnote in Table 1). Major effects alone and/or combined with pre-planned interactions were studied in both datasets. First, the effect of cryopreservation systems on calf fitness on Day 0 was analyzed in combination with or without colostrum intake. The following major effects were considered and weighed in the GLM model: the origin of the calf based on embryo cryopreservation (fresh, frozen, and vitrified); colostrum intake; embryo culture medium prior to Day 6 (i.e., with BSA or BSA+FCS); calf sex; calf breed (Holstein, AV, and crossbred); and individual bull and recipient breed (random effects). Second, the time-course effects of

cryopreservation on the original embryos were analyzed in their interaction with blood sampling times on Day 0 (after colostrum intake), Day 15, and Day 30. The major effects included in the models were as above, except those made of colostrum intake and included diarrhea as a random effect. Parameters that did not show significant interactions between cryopreservation and sampling day were analyzed singly in the time course (i.e., Day 0, Day 15, and Day 30). Data were expressed as LSmeans ± SEM. The *P*-values of <0.05 were considered significant for variable values analyzed within each model. Subsequently, the predicted least square mean difference value (PDIFF) was calculated as a *post-hoc* test to identify significant differences ($P < 0.05$) between least square means.

Results

Analytical validation

The stability of hematological and biochemical parameters was tested in samples through a time-course experiment (Supplementary Tables S1A, B). Within the CG4+ analytical module, no parameter showed significant deviations in measured concentrations before an average reading time of 10.75 min (range: 10–11 min). Only lactate showed significant ($P < 0.01$) differences with the precedent times at a reading time of 14.75 min (range: 14–16 min). Parameters analyzed by the CHEM8+ module did not differ at any analytical time (top time: 13.2 min, range: 12–14 min). In accordance with these validation studies, all samples were analyzed and read <6 min after blood collection.

Studies on Day 0

Day 0 samples analyzed were $N = 91$ ($N = 41$ before and $N = 50$ after colostrum intake), taken from $N = 51$ calves (frozen: 24; fresh: 13; vitrified: 14) (refer to Table 1). Within such a dataset, BW was affected by embryo culture and sex but not by embryo cryopreservation; chest perimeter by embryo culture, and calf size by sex (Table 4). In the main effects shown in Table 4, the bull (individual random effect) had the largest influence, with 17 parameters being affected, most of them with strong significance ($P < 0.01$). Colostrum intake affected eight parameters, while cryopreservation and embryo culture influenced 7 and 10 values measured in calves, respectively. Recipient breed affected four parameters, calf sex seven parameters, and calf breed had no effect. Lactate, respiration rates, PO_2, and sO_2 were independent of all variables analyzed.

Interactions between embryo cryopreservation systems and colostrum on Day 0 are shown in Figures 1, 2. Notably, relevant effects of both embryo cryopreservation systems on clinical traits in calves (Figure 1) were observed for capillary refill time (CRT) and differed significantly ($P < 0.001$) from the value in calves of fresh embryos (calves of F/T: 3.613 ± 0.232 s and calves of V/W embryos: 3.235 ± 0.247 s; vs. calves of fresh embryos: 2.394 ± 0.252 s) (Figure 1A) and heartbeat rate (calves from fresh: 154.6 ± 5.6 vs. F/T: 143.9 ± 5.2 and from V/W embryos: 138.8 ± 5.5; $P < 0.034$) (Figure 1B). Within acid–base equilibrium and blood gases (Figure 1), differences between cryopreservation systems were also recorded for PCO_2 ($P < 0.01$) (Figure 1C), between calves derived from F/T embryos (49.55 ± 1.86 mm Hg) vs. calves from fresh embryos (56.80 ± 2.02) but not within calves from V/W embryos (52.71 ± 1.97); at the same time, colostrum intake did not reduce PCO_2 in calves of V/W embryos, contrary to in calves from F/T and fresh embryos. Base excess (Figure 1D) and pH (Figure 1E) were not affected by cryopreservation but they showed a significant restorative effect of colostrum ($P = 0.005$ and $P < 0.001$, respectively). The concentration of Na^+ (mmol/L) also differed in calves from V/W embryos vs. calves from fresh and F/T embryos ($P < 0.05$) (140.3 ± 0.6 vs. 139.0 ± 0.6 and 139.0 ± 0.5, respectively) (Figure 1F), and K^+ (mmol/L) tended to increase in calves when the original embryo was F/T vs. fresh ($P = 0.089$; pdiff = 0.0356). Among the metabolites analyzed (Figure 2), creatinine in calves was clearly affected ($P = 0.004$) by both embryo cryopreservation systems (Figure 2A; F/T: 3.875 ± 0.306 mg/dl and V/W: 3.881 ± 0.326; vs. fresh: 2.770 ± 0.333), as well as by culture medium and calf sex, tending to decrease after colostrum intake ($P = 0.097$; not shown in tables). Urea (Figure 2B) did not differ with embryo cryopreservation but showed a significant dependence on the embryo culture system ($P = 0.0046$). Both hematological parameters (Figure 2), Hb (g/dl) (Figure 2C) and PCV (%) (Figure 2D), differed ($P < 0.05$) between calves born from F/T and V/W embryos, but they did not differ from calves of fresh ones (Hb: 8.565 ± 0.470 vs. 9.844 ± 0.500, and 9.233 ± 0.511; PCV: 25.22 ± 1.38 vs. 28.95 ± 1.47, and 27.12 ± 1.50, respectively). The remainder of Day 0 interactions colostrum*embryo cryopreservation did not significantly differ and are shown in Supplementary Table S2.

Time-course studies in the perinatal period

These studies used $N = 138$ samples, corresponding to Day 0 ($N = 50$), Day 15 ($N = 44$), and Day 30 ($N = 44$). Five calves died before Day 15. On Day 15, two samples were not taken,and, on Day 30, other two samples from different calves were not taken either. Table 1 shows further calf details. We first analyzed the interaction between embryo cryopreservation and calf age at three time points. The sample distribution per groups for F/T, fresh, and V/W resulted in Day 0: 23, 13, and 14; Day 15: 20, 13, and 11; Day 30: 19, 13, and 12, respectively.

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TABLE 4 Main effects from clinical signs and blood parameters in calves born from fresh, vitrified, and frozen embryos measured on Day 0 prior to and after colostrum intake.

Parameter	Colostrum	Cryo[a]	Embryo Culture	Calf Sex	Calf Breed	Recipient breed	Bull
CRT		<0.001	0.010				0.010
Birth weight			<0.001	0.003			0.005
Chest perimeter			<0.001				0.001
Size				0.012			0.001
Temperature	0.010		<0.001				
Conjunctival	0.047					0.016	
Nasal flux						<0.001	
Heartbeats	**0.051**	0.034					<0.001
Respiration							
pH	<0.001						0.002
PCO_2	<0.001	0.008					0.015
Base excess	0.005		0.033				0.004
HCO_3^-	0.037		0.011				
TCO_2			0.012				0.015
Lactate							
Na^+		0.040		0.012		<0.001	<0.001
K^+							0.027
Cl^-				0.003		<0.001	
Ca^{2+}	0.027						<0.001
Glucose							0.010
Urea			0.023				0.001
Creatinine		0.004	0.030	0.004			
PCV		0.046		0.005			<0.001
Hemoglobin		0.044		0.005			<0.001
Anion gap			0.027				<0.001
PO_2							
sO_2							

[a]Cryo: embryo fresh or cryopreserved (vitrified or frozen). CRT, capillary refill time. Tendency values ($0.05 > P < 0.06$) are shown in bold.

Values measured on a temporal scale were mostly independent of embryo cryopreservation and showed changes regarding Day 0 values. However, on Day 30, several parameters were influenced by the previous appearance of diarrhea. Mild diarrhea was either untreated or corrected with diet. Moderate-to-severe diarrhea was treated with diet and nutritional and electrolyte replacement (Calf Lyte Plus, Vetoquinol, Spain), with or without injected sulfadoxinetrimethoprim therapy (Borgal; Virbac, Esplugues de Llobregat, Barcelona, Spain).

Figures 3–5 show values for the day effect and the interaction day*cryopreservation. Within acid–base equilibrium and blood gas concentrations parameters (Figure 3), PCO_2 decreased in calves from fresh and V/W embryos, but remained constant in calves from F/T embryos (Figure 3A), while overall TCO_2 decreased on Day 30, an effect more marked within calves born after transfer of V/W embryos (Figure 3B) which was also shown by ion bicarbonate (Figure 3C) and BE (Figure 3D). On the contrary, PO_2 and sO_2 increased throughout with no incidence of cryopreservation effects (Figures 3E,F respectively). The anion gap also showed an increase in Day 30 over Day 15 values within calves from V/W embryos, while the other time points and groups remained without changes (Figure 3G). Temperature increased in all groups from Day 0 to a plateau on Day 15 and Day 30 (Figure 3H), with a more pronounced, significant rise in calves from fresh embryos. Among calf electrolytes (Figure 4), none was affected by cryopreservation, with Na^+ decreasing abruptly from Day 0 until Day 15 and Day 30 (Figure 4A), while Cl^- showed a transient decrease only on Day 15 (Figure 4B), K^+ a transient increase on Day 15 (Figure 4C) and Ca^{2+} rose on Day 15 to remain stable up to Day 30 (Figure 4D). Among metabolites analyzed (Figure 5), carbohydrates showed contrary profiles between them, with glucose rising from Day 0 to remain stable on Day 15 and Day 30 (with a significant peak for calves from V/W embryos on

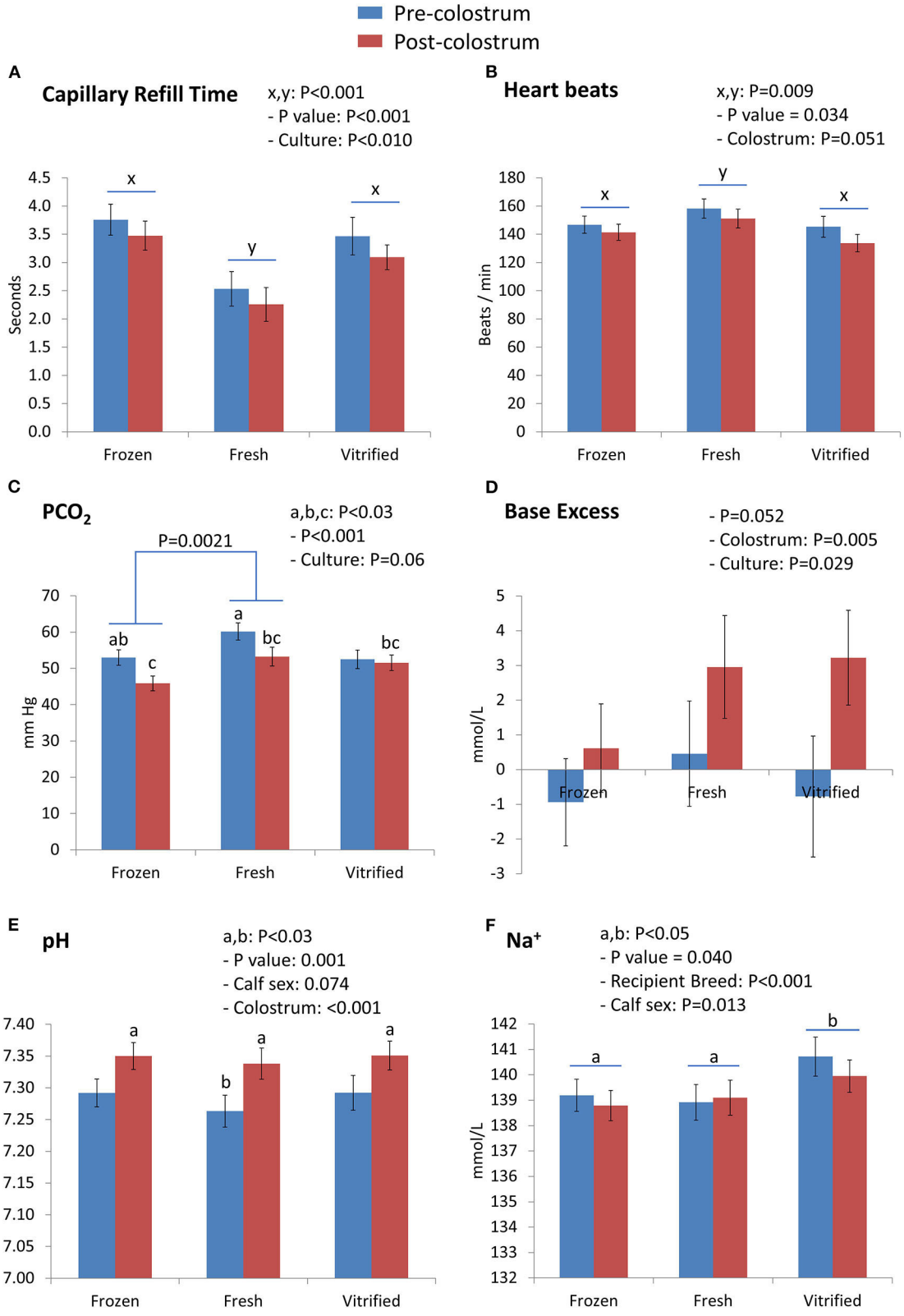

FIGURE 1
Clinical traits [capillary refill time **(A)** and heartbeats **(B)**] and acid–base equilibrium and blood gases parameters [PCO_2 **(C)**, base excess **(D)**, pH **(E),** and Na^+ **(F)**] differed on Day 0 between groups of calves (from frozen/thawed, fresh, and vitrified/warmed embryos). Values are LSM ± SEM. Samples were $N = 42$ pre-colostrum and $N = 50$ post-colostrum, corresponding to $N = 24$ for the frozen group, $N = 13$ for the fresh group, and $N = 14$ for the vitrified group of calves. Underlined superscripts indicate the cryopreservation effect, and not-underlined superscripts indicate the interaction between the cryopreservation system and colostrum intake.

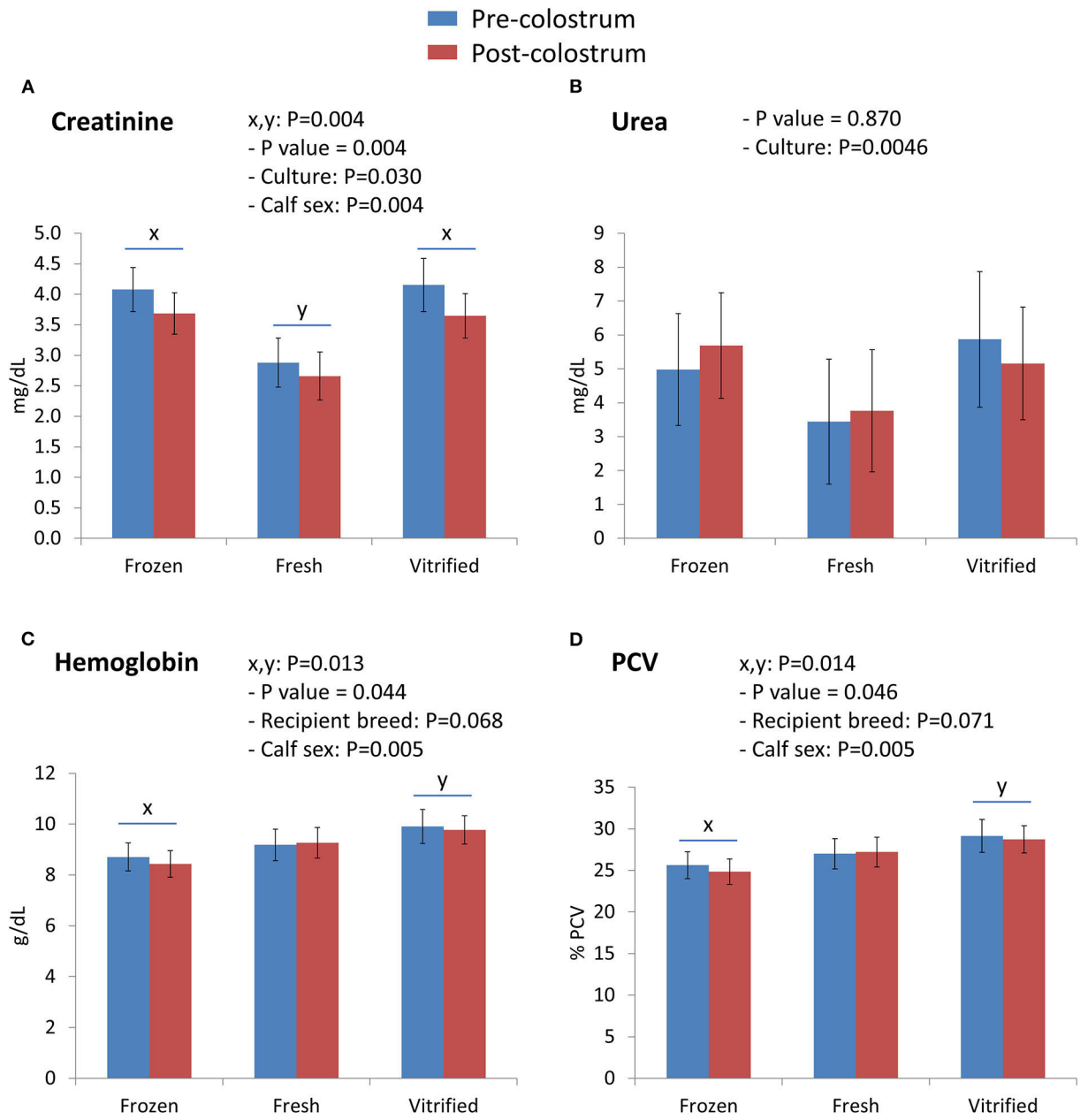

FIGURE 2
Metabolite parameters [creatinine **(A)** and urea **(B)**] and hematological variables [hemoglobin **(C)** and packed cell volume **(D)**] differed on Day 0 between calves born from frozen/thawed, fresh, and vitrified/warmed embryos. Data are expressed as LSM ± SEM. Underlined superscripts (x,y) indicate the cryopreservation effect.

Day 30) (Figure 5A), in contrast with lactate, which decreased from Day 0 (Figure 5B). Creatinine showed on Day 0 -with the post-prandial samples—the same changes described above due to embryo cryopreservation, and thereafter decreased to a basal level on Day 15 and Day 30 (Figure 5C). Hematological parameters (Figure 5), Hb (Figure 5D), and PCV (Figure 5E) showed parallel decreases from Day 0 with comparable values between Day 15 and Day 30.

The diarrhea influence on parameters was exerted clearly on Day 30 and particularly in calves from V/W embryos; despite this, such calves did not show a higher incidence of the illness than calves from F/T and fresh embryos (refer to Table 1). Parameters influenced by diarrhea on Day 30 were temperature, PCO_2, TCO_2, HCO_3^-, BE, and Na^+. Parameters that did not show diarrhea influence on Day 30 were PO_2, sO_2, AG, Cl^-, glucose, lactate, creatinine, PCV, and Hb. Culture

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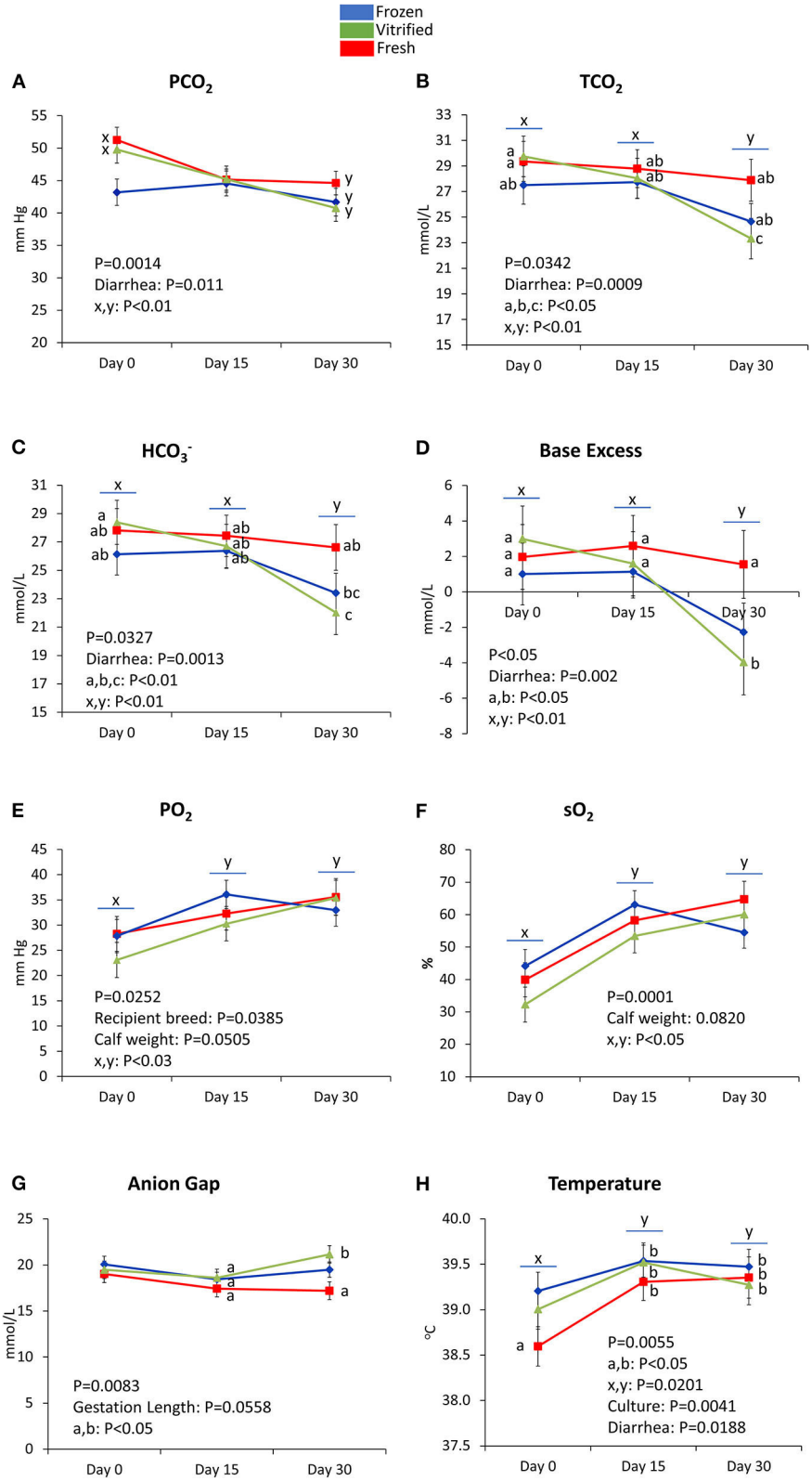

FIGURE 3
Acid–base equilibrium and blood gas parameters differed on Day 0, Day 15, and/or Day 30 between groups of calves (from frozen/thawed, fresh, and vitrified/warmed original embryos): PCO_2 **(A)**, TCO_2 **(B)**, HCO_3^- **(C)**, base excess **(D)**, PO_2 **(E)**, sO_2 **(F)**, anion gap **(G),** and temperature **(H)**. Data are expressed as LSM ± SEM. Underlined superscripts indicate the day effect, and not-underlined superscripts indicate the interaction between days and cryopreservation.

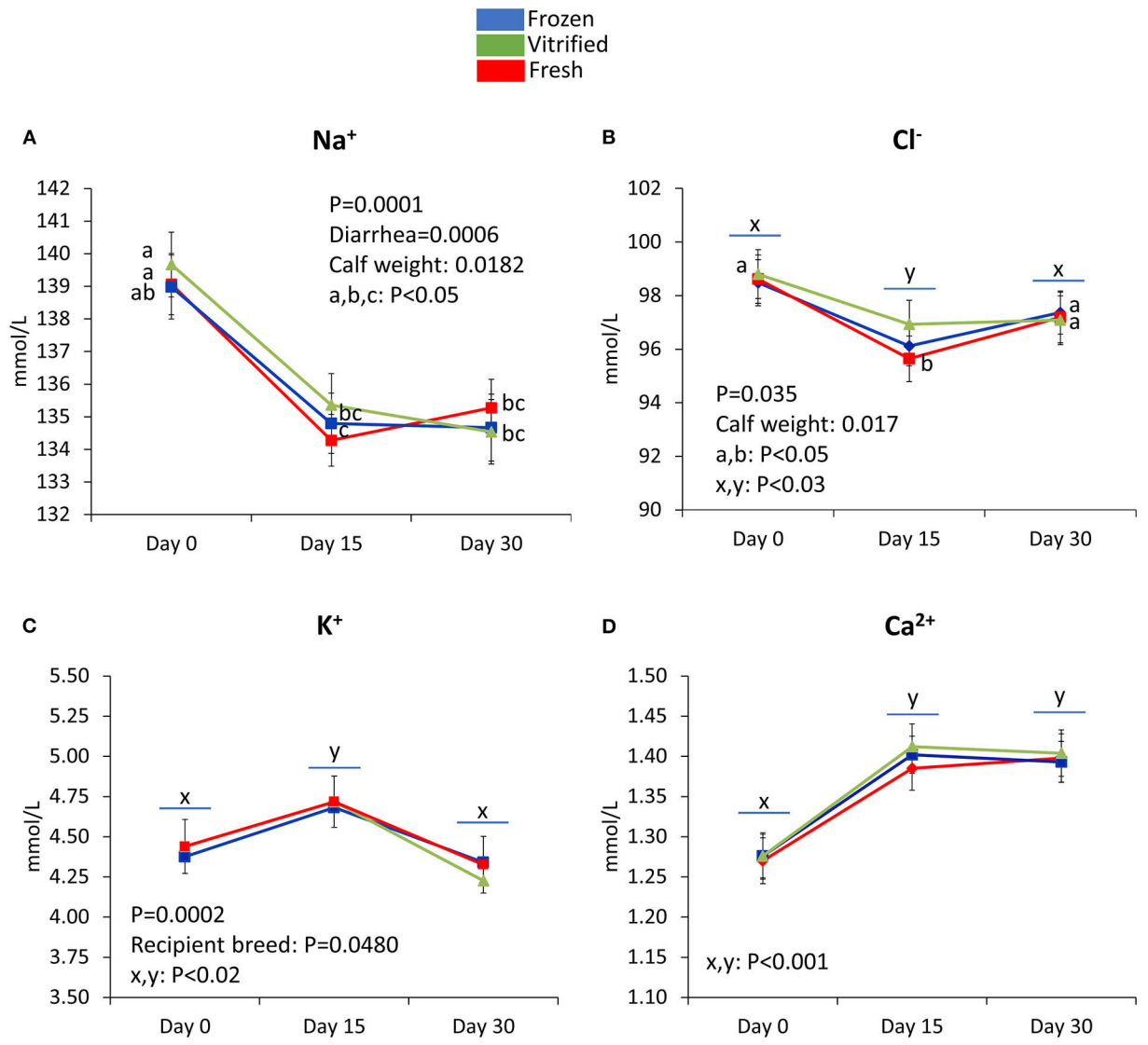

FIGURE 4
Electrolytes that differ in the three groups of calves in the temporal study (Day 0, Day 15, and Day 30). Na^+ **(A)**, Cl^- **(B)**, K^+ **(C)**, and Ca^{2+} **(D)** concentrations are shown. Data are expressed as LSM ± SEM. Underlined superscripts indicate the day effect, and not-underlined superscripts indicate the interaction between days and the cryopreservation system.

conditions (i.e., the presence of 0.1% FCS in culture prior to Day 6) affected calf temperature (not shown in figures), while lactate, creatinine, PCV, and Hb were significantly different between male and female calves. Calf weight (repeatedly measured on days of sampling) affected concentrations of PO_2, sO_2, Na^+, and Cl^-. Gestation length affected AG, creatinine, PCV, and Hb. The remaining parameters measured did not show time-dependent changes or interaction with the embryo cryopreservation origin of the calves; such values are described in Supplementary Table S3.

Creatinine showed low or no correlation with parameters that can be altered by dehydration (i.e., CRT: R = 0.26752, P = 0.0371; PCV: R = 0.18547, P = 0.1524; Hb: R = 0.1485, P = 0.18721) showing only correlations with PCO_2 (R = −0.30673; P = 0.0241), GL (R = 0.37304; P = 0.0031), and chest perimeter (R = 0.30860; P = 0.0155) but no correlation with calf weight and size at birth; nor did any other parameter measured. This indicates that cryopreservation accounted for most of the creatinine variation.

A comparison between the values of parameters obtained in our study on Day 0 (before and after colostrum intake), Day 15, and Day 30 and reference values reported in other studies is shown in Supplementary Table S4. Total consistency of our values with a given list of reference values was not observed; in

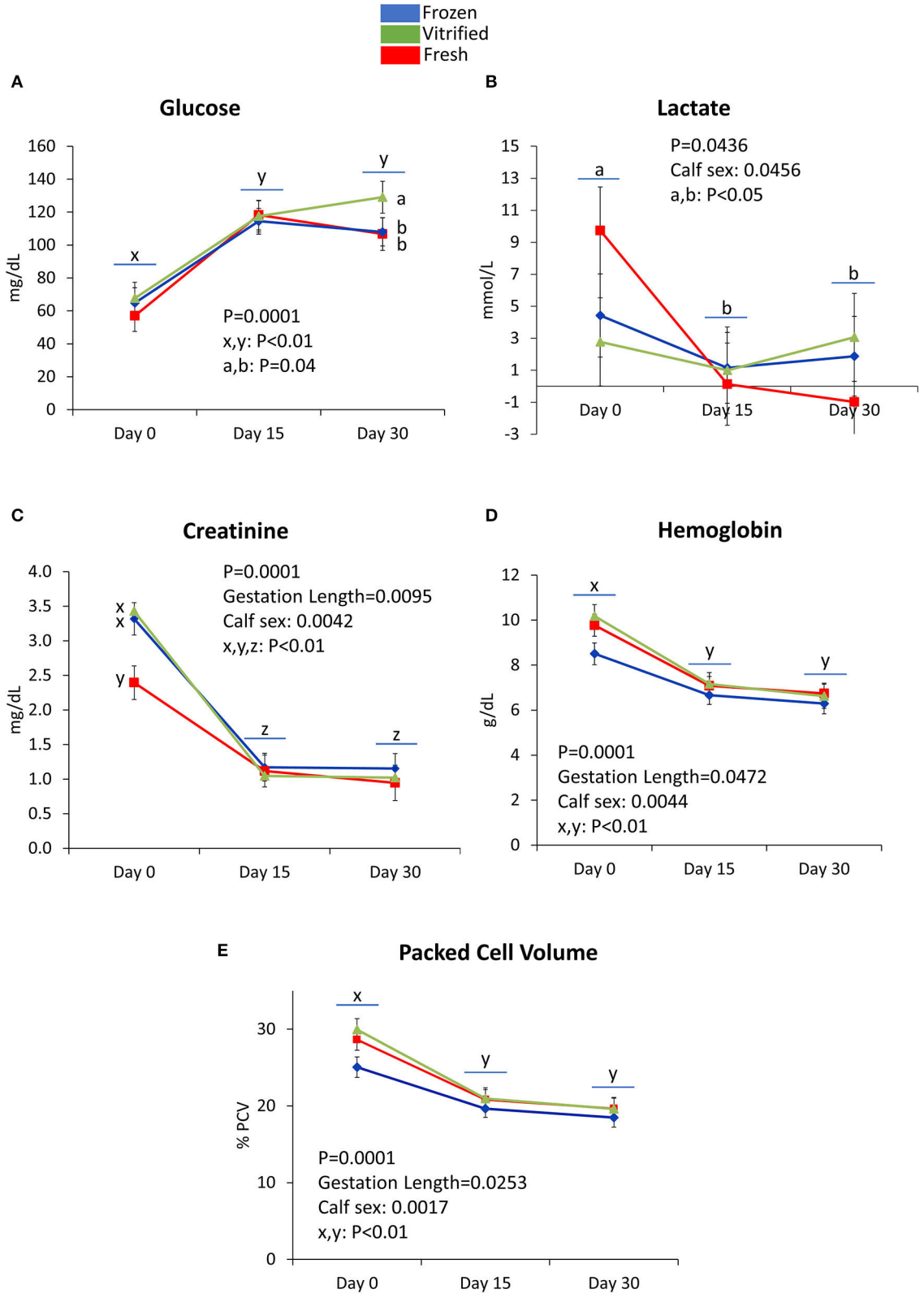

FIGURE 5
Metabolite concentration [glucose **(A)**, lactate **(B),** and creatinine **(C)**] and hematological parameters [hemoglobin **(D)** and packed cell volume **(E)**] measured on Day 0, Day 15, and Day 30 differed ($P < 0.05$) between calves derived from frozen/thawed, fresh, and vitrified/warmed embryos. Data are expressed as LSM ± SEM. Underlined superscripts indicate the day effect, and not-underlined superscripts indicate the interaction between days and the cryopreservation system.

addition, no reference value list showed complete consistency with each other. However, all our specific parameters showed adjustment to at least one reference value at any time, with the exception of urea (lower concentration in our study) and TCO_2 (which was not measured in the referred studies).

Discussion

We evaluated calf fitness on Day 0 (before and after colostrum intake) and on Days 15 and 30 after birth to monitor adaptive changes. Part of the parameters measured on Day 30, but not before, was significantly affected by the appearance of diarrhea, which was of mild-to-moderate intensity in most cases, including two deaths. Although the focus of our study was investigating the effects of embryo cryopreservation among IVP calves, we compared our results with recommended values (Cornell University), and values obtained in similar studies (40, 41, 46, 53–58). Overall, parameters measured in our study fitted well with parameters given by other authors in an independent way, but reliable and linear comparisons must be cautiously interpreted because the age and conditions of animals in literature were not always the same as our sampling times, and studies were generally conducted under different nutritional and management diets. Therefore, accounting for the effects of other environmental variables (farm, feeding time and composition, type of food, reproductive methods, such as AI, cloning, other *in vitro* production systems, analytical platforms, and undetermined residual factors) is not feasible (46). The analyzer we used has been positively evaluated in calves (40, 59–61), and although some minor biases were noted between our and other analyzers, they did not interfere with the clinical value of data (62).

Embryo technologies, calf performance, and growth

In vitro-produced calves often come from a prolonged gestation (40, 63), which contributes to increased BW and puts mother and calf at risk and may include a proportion of calves with LOS/AOS phenotype [reviewed by Rivera et al. (64)]. However, in our experimental herd, there were no BW differences between fresh, F/T, and V/W calves. Beef recipients, like AV, have shorter GL than dairy cattle (65) and lower milk yield. For this reason, the recipient breed (Holstein, AV, crossbred) was a factor to correct in our study, which affected nasal flux, conjunctival appearance, Na^+, and Cl^- values at birth. Instead, in our time-course study, the recipient breed only affected PO_2 and K^+. Although the transfer of V/W embryos leads to reduced pregnancy and birth rates and increased GL (14), embryo cryopreservation as a source of alteration in newborn calves has been not studied in depth. This is in contrast with data obtained from technologies of embryo production (12, 15). For example, PCV, Hb, urea, and creatinine differ between clones and calves from AI (41), and in our study with cryopreservation. Clones and calves showing AOS present hypoglycemia (41), contrary to our study, where neither glucose nor lactate at birth was affected by cryopreservation. Glucose did not increase after colostrum intake, in accordance with (66), but contrary to Guo and Tao (52), and increased on Day 15, as shown by others (66–68). This is opposed to lactate, which decreases with age (52, 66, 67). However, we do not know the reason for higher glucose in calves from V/W embryos on Day 30, as an isolated finding, although temporary glucose increases have been reported in calves obtained from IVP embryos vs. AI (39). Alterations induced in calves by reproductive techniques may help to identify parameters "sensitive" to injuries. Thus, the type and sense of changes reported between calves from IVP and/or cloned embryos vs. calves from AI could have parallel effects due to cryopreservation in our IVP embryos.

Acid–base equilibrium and blood gases

The combination of metabolic and respiratory acidosis, due to altered gas exchange between the fetus and the mother, is a major cause of perinatal calf mortality (69, 70). Deviation in acid–base balance entailed lower values of hematic HCO_3^-, pH, and BE in heavier calves compared with those with more reduced BW (71). In our study, calves from fresh, V/W, and F/T embryos did not differ in BW and GL. Calf overgrowth leads to labor difficulties, which affect at least pH, lactate, and PCO_2 at birth (72). Gas values, pH, lactate, and BE are early predictors of respiratory compromise and their values correlate with blood levels of lung injury proteins (73). However, in our study, parameters involved in the acid–base equilibrium on Day 0 reflected only slight differences between cryopreserved and fresh embryos, without pathological damage.

Reduced PCO_2 concentration in F/T calves suggests primary respiratory alkalosis due to hypoxia, pointing to hyperventilation during labor as a probable cause. After colostrum intake, PCO_2 decreased in F/T and fresh calves but not within V/W. All differences in PCO_2 disappeared on Day 15 and Day 30, with a PCO_2 reduction over the days. In contrast, the concentration of HCO_3^- was steady on Day 0 and Day 15, being reduced by diarrhea on Day 30. Such a decrease in HCO_3^- was more pronounced in V/W calves, perhaps suggesting a higher sensitivity to diarrhea and/or more compromised immune status, which should be investigated in future studies. TCO_2 and BE, not affected by cryopreservation at birth, were however affected by diarrhea, and in calves from V/W embryos on Day 30. AG, an indicator of diarrhea and metabolic acidosis, showed a slight increase on Day 30 in calves from V/W vs. fresh embryos, which is also consistent with the timing for diarrhea. Interestingly, calves from F/T embryos

showed intermediate values between calves from V/W and fresh embryos on Day 30 in TCO_2, HCO_3^-, BE, and AG, reflecting that the possible metabolic alteration by diarrhea in F/T did not occur to the greater extent showed by V/W.

Cryopreservation had a limited or no effect on PO_2 and sO_2, parameters associated with oxidative stress. Interestingly, sO_2 does not account for fetal Hb or dysfunctional Hbs, by which sO_2 is seen here as a measure of the temporal replacement of fetal calf Hb by adult Hb, a process that ends at 13 weeks of age (74, 75). Embryo cryopreservation did not interfere with this normal adaptation to adult life. Consistent with sO_2, PCV values decreased throughout, reflecting the physiological replacement of large fetal erythrocytes by the lower volume adult cells (76). At the end of pregnancy, the fetus responds to hypoxia with a transient higher concentration of Hb and PCV. Thus, colostrum intake, a rapid erythrocyte replacement, and a decrease in fetal Hb trigger changes in hematological parameters in the first weeks after birth (77–79). Colostrum had the expected restorative effects on calf physiology, and there were no major significant interactions between colostrum and the cryopreserved or fresh status of the original embryos.

Oxidative stress is prevalent in calves from IVP and cloned embryos, which show higher concentrations of free radicals and less counteracting glutathione in the blood than in adult cattle (80, 81). Such higher levels of free radicals were attributed to the onset of breathing after birth, which enhances the contact between lung epithelia and oxygen (80). However, we identified lower PO_2 and sO_2 at birth than later on in our three types of calves, and oxygen-based parameters did not vary with embryo cryopreservation or any other effect at birth. We suggest that non-respiratory factors also underlie increased ROS levels in newborn calves.

Measures of acid–base balance (pH, PCO_2, PO_2, BE, and HCO_3^-), PCV, Hb concentration, and blood cells did not differ between calves born from IVP embryos and AI aged 1 and 7 days (40). However, Sangild et al. (68) observed calves from IVP embryos with higher pH, Hb, oxygen contents, and temperature. Electrolytes can in turn increase in calves from IVP embryos at birth [K^+: (68)] and on Day 7 [K^+ and Na^+: (40)] or decrease at birth [K^+: (40); Na^+ and Cl^-: (68)]. We noted a tendency toward elevated K^+ in calves from fresh over F/T embryos, and we agree with other authors regarding the decrease in Na^+ and Cl^- over time as explained by colostrum and water intake leading to hemodilution (40). In parallel, the Ca^{2+} supply in milk would explain its rise until Day 15, as observed by Sangild et al. (68) although not seen by Rerat et al. (40). Embryo cryopreservation seemed to impose more changes on calves than reported for IVP vs. AI.

Calf temperature at birth did not vary with cryopreservation and decreased with colostrum intake. Nor temperature at birth does differ between calves from IVP and AI calves, despite the fact that the regulating plasmatic 3,5,3′-triiodothyronine (T3) and thyroxine (T4) hormones at birth are lower in calves derived from IVP embryos (40). However, cloned calves show higher temperatures than AI calves until 50 days of age (41) and the expression of genes related to thermogenesis differs in the hypothalamus of young male calves obtained from IVP vs. MOET embryos (38). Collectively, the above suggests that temperature regulatory mechanisms are not specific targets for embryo cryopreservation.

Protein metabolism: Urea and creatinine

Creatinine concentration was higher at birth in calves from V/W and F/T vs. fresh embryos, indicating a consistent alteration induced by cryopreservation, whatever the cryopreservation technique used. Creatinine also increased in umbilical cord plasma (82) and calf venous blood (39) of IVP vs. AI fetuses. Subsequently, creatinine decreases (78, 83–85) as found in our study. The endogenous metabolism in muscles generates creatinine as waste, in a direct proportion of muscle mass, and blood creatinine concentration does not depend on nutrition. The responsiveness of our calves to the colostrum intake by decreasing creatinine, and the disappearance of differences observed shortly after, indicate reversibility and no obvious damage induced by cryopreservation. The creatinine alteration is in contrast with the steady concentrations of urea among groups on Day 0. Urea concentration depends on nutrition, and its increase in blood indicates protein catabolism (83), although we did not observe any decrease in urea concentration with time. In the ewe, high urea levels in the uterus and oviduct reduce embryo development rates and enhance fetal growth (86, 87), as occurs in culture with serum (88), and our study.

Sex affected Day 0 concentrations of Na^+ and Cl^- (increased in men), but such differences disappeared afterward. However, sex differences in creatinine, PCV, and Hb observed on Day 0 remained throughout. Creatinine concentration was higher in males at birth (40), which can be explained by the larger muscle mass in these calves (40, 89). Our results are consistent with observations of Dillane et al. (46) for Cl^-, PCV, and pH (which showed a tendency), are contrary for Na^+, and are not coincident for glucose, HCO_3^-, PCO_2, AG, and K^+ (not affected by sex in our study). Other studies did not find differences attributable to calf sex for the above and other parameters investigated (76, 90).

Adaptation to extrauterine life

As reported by Schäff et al. (67), we observed how parameters measured in the first month of an age change to adapt to mature life. CRT is a measure of mild-to-moderate dehydration in calves (91). In our story, calves from F/T and V/W embryos seemed to show this moderate dehydration,

as their CRT surpassed 3 but not 4 s both before and after colostrum intake. CRT $\geq$ 3 s reflects a 4.3% reduction in hydration in dairy calves compared with lower refill times (91). PCV is also typically elevated in dehydration (92), as shown in calves from V/W embryos compared with calves from F/T embryos but not within calves from not cryopreserved embryos. Rectal temperature and respiration rates decrease by dehydration (91), but such parameters did not change between calf types. Collectively, V/W embryos led to calves who showed more dehydration signs than calves from F/T embryos, although without reaching a clinical compromise, since CRT cannot be measured in calves with severe dehydration (91, 93). Thus, the pH values of all calf types at birth were higher than 7.2, a clinical limit to judge a calf as acidotic (93), and all types responded to colostrum intake with pH rises between 7.30 and 7.35. Animals over 7 days old should reflect values > 7.31 (94) or > 7.36 (90).

Metabolic adaptation in the calf starts during the third week of life when newly synthesized, calf proteins must replace colostrum-provided and *in utero*-synthesized proteins (83). Disrupting neonatal metabolic adaptation would make the calf susceptible to infectious- and non-infectious diseases. We observed here that calves from V/W embryos showed metabolic traits compatible with diarrhea despite not being clinically affected and/or no more affected than their counterparts.

With the few exceptions marked on Day 0, generally, colostrum intake and subsequent development led to similar calf adaptation of fresh, V/W, and F/T embryos until Day 15. The incidence of diarrhea led to differences on Day 30 within calves from V/W embryos (i.e., two cases treated and two cases not treated within 13 surviving calves). HCO_3^-, AG, BE, and TCO_2, differed on Day 30 between calves from V/W vs. fresh and F/T embryos, consistent with significant effects of diarrhea. Other parameters were affected by diarrhea (i.e., temperature, PCO_2, and Na^+) but without particular differences between calf groups.

Conclusion

In the present study, apparently normal calves from cryopreserved embryos show particular clinical and biochemical traits, more pronounced on Day 0 than afterward, as observed with CRT, creatinine, or heartbeat rate. Furthermore, V/W and F/T embryos also led to specific effects on calves, as occurred with PCO_2 or Na^+. However, differences from embryo cryopreservation disappeared in calves on Day 15 and Day 30. In contrast, on Day 30, diarrhea altered several parameters, and our results point to V/W embryos as making calves more susceptible to these effects, although overall mortality rates did not differ. The small concentration of serum we used in embryo culture was reflected in the protein metabolism (i.e., urea and creatinine) of calves, although no conclusive statement can be drawn without studying such changes at the cellular level. Anyhow, such differences observed on Day 0 disappeared over time, with the only temperature remaining affected. Colostrum was restorative in the three groups of calves, which indicates an initial similar adaptation to extrauterine life between calves from fresh and cryopreserved embryos. This is the first study to compare the clinical status of calves born from fresh vs. frozen and vitrified IVP embryos. The molecular basis of the observed differences, and whether they persist in progeny as subtle effects not approached herein, requires further investigation.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The animal study was reviewed and approved by the Animal Research Ethics Committees of SERIDA and University of Oviedo (PROAE 33/2020; Resolución de 13 de Noviembre de la Consejería de Medio Rural y Recursos Naturales), in accordance with European Community Directive 86/609/EC.

Author contributions

EG and JB contributed to the conceptualization and methodology. EG and IG contributed to data curation, software, formal analysis, and writing of the original draft. EG contributed to the funding acquisition and supervision. SC, DM-G, AM, JP-J, and IG contributed to the investigation and contributed to resources. EG, IG, SC, and DM-G contributed to the visualization. EG, IG, AM, JB, and JP-J contributed to the writing of the review and editing. All authors have read and agreed to the published version of the manuscript.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2022.1006995/full#supplementary-material

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7| DISCUSIÓN GENERAL

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El objetivo principal de la presente tesis es identificar biomarcadores en el MC de los embriones para diagnosticar el sexo y predecir el éxito de la gestación de manera no invasiva y previa a la transferencia del embrión, utilizando para ello la metabolómica. A tal efecto, hemos producido un conjunto de muestras que encierra una alta variabilidad, conseguida a través de la producción de embriones con ovocitos aislados de ovarios de donantes de razas indeterminadas, siete toros de dos razas y dos condiciones de cultivo *in vitro* hasta el día sexto del desarrollo. También hemos tenido en cuenta la influencia de cofactores fijos, como son los estadios del embrión al principio (día 6) y al final (día 7) del cultivo individual, y el uso de embriones frescos y congelados antes de su transferencia a receptoras. De esta manera se logró estimar con elevada certeza el sexo y la viabilidad del embrión (ROC-AUC>0.7). No obstante, al depender el éxito de la gestación del embrión y de la receptora, también identificamos biomarcadores de gestación en las receptoras utilizando muestras de plasma en día 0 (estro) y día 7 (pocas horas antes de realizar la transferencia). A continuación, usando la información emparejada de 70 embriones y receptoras, desarrollamos un modelo para predecir la gestación a término basado en aprendizaje e iteraciones. Este enfoque mejoró sustancialmente la predicción de la gestación, incrementando el valor ROC-AUC de los metabolitos marcadores. Finalmente, se examinó la influencia de la criopreservación del embrión, mediante congelación lenta y vitrificación, en la salud perinatal de los terneros nacidos.

Factores que afectan al metaboloma del embrión medido en el medio de cultivo

Hemos demostrado que un único metabolito no ha sido capaz de actuar como marcador válido en todas las condiciones analizadas, tanto para el diagnóstico del sexo como para la predicción de la gestación. Esto es debido a la influencia de diversos factores en el metaboloma medido en el MC del embrión.

El factor individual (el toro productor de los espermatozoides empleados en la FIV) ejerció la mayor influencia en la concentración de los metabolitos identificados en el MC

del embrión. Esta influencia sólo pudo ser compensada con la variabilidad que proporciona un número elevado de individuos (siete toros en nuestro estudio). Los espermatozoides de distintos toros difieren en su cinética y en el porcentaje de embriones que se desarrollan *in vitro*, en el sex ratio de tales embriones, y en sus índices de gestación (Alomar *et al*., 2008; Hansen *et al*., 2010). El espermatozoide contribuye al desarrollo del embrión con factores de transcripción, proteínas, marcas epigenéticas específicas y metabolitos (Morgan *et al*., 2020). La contribución paterna se refleja además notablemente en la duración de la gestación, el peso y la altura del ternero al nacimiento (Swali & Wathes, 2006, Fang *et al*., 2019; Coleman *et al*., 2021). Estos resultados coinciden con nuestro estudio, donde los parámetros clínicos, como el peso al nacimiento, el perímetro torácico y el tamaño, y los parámetros bioquímicos analizados en sangre de los terneros recién nacidos mostraron una fuerte influencia paterna en 17 de los 27 parámetros estudiados.

La raza del toro utilizado en la FIV también comporta cambios en el metabolismo del embrión. En los estudios que conforman esta tesis se usaron toros de dos razas: raza Holstein, mundialmente extendida, y raza Asturiana de los Valles. La característica más sobresaliente de la raza Asturiana de los Valles es su gran desarrollo muscular, con la característica grupa doble, comparable a otras razas comerciales de carne como la Azul Belga o la Piamontesa, lo que extiende el ámbito e interés de los resultados presentados. La Asturiana de los Valles presenta una mayor actividad metabólica que la raza Holstein (Gómez *et al*., 2020d). Esta característica puede ser debida a la hipertrofia muscular, causada por una mutación en el gen de la miostatina (McPherron & Lee, 1997), la cual incrementa el metabolismo energético y la demanda nutricional de los animales. Otros autores señalaron la influencia de la raza en el desarrollo y en el metabolismo del embrión. Así, los embriones de raza pura (Holstein y Pardo Alpina) y sus cruces difieren en los índices de desarrollo, en la expresión génica y en el metabolismo de aminoácidos (Lazzari *et al*., 2011). Los embriones de otras razas (como Jersey y Holstein) también difieren en su composición lipídica y en la expresión de genes relacionados con el metabolismo de lípidos (Baldoceda *et al*., 2015). Esto sugiere que los embriones de distintas razas podrían tener requerimientos nutricionales particulares y exhibir un metabolismo diferente. Por lo tanto, la raza es un factor que debe ser incluido en la búsqueda de metabolitos biomarcadores, como hemos hecho en nuestros estudios.

La raza de la receptora del embrión también afectó a la intensidad con que se mostraron en los terneros ciertos signos clínicos (flujo nasal y apariencia de las mucosas conjuntivas al nacimiento) y algunos parámetros bioquímicos (Na^+ y Cl^- en día 0, y presión parcial de O_2 (PO_2) y K^+ en los días 15 y 30 de vida). La duración de la gestación varía según la raza de la receptora, siendo más corta en las razas de carne que en las razas lecheras (Baruselli *et al*., 2021). En nuestro estudio, la duración de la gestación afectó a los valores de anión gap, creatinina, hematocrito y hemoglobina. Dillane *et al*. (2018) describieron la influencia de la raza del ternero sobre la concentración de ciertos parámetros bioquímicos en sangre, afectando a parámetros como HCO_3^-, pCO_2, exceso de base (BE, del inglés *base excess*), anión gap, glucosa y hemoglobina.

La presencia de suero durante el CIV alteró el metabolismo del embrión, a juzgar por el metaboloma identificado en el MC. En nuestros estudios utilizamos el medio de cultivo más frecuente (SOF), dotado con los suplementos más comunes en los laboratorios de producción de embriones, FCS y BSA, presumiendo que serían capaces de producir diferencias metabólicas constatables. La presencia de FCS provocó cambios notorios en el metabolismo del embrión y en la fisiología del ternero, aun cuando las últimas 24 horas de CIV se realizaron en un medio sin proteína. Este medio sin proteína, común a todos los embriones, permitió el análisis cromatográfico directo del MC, sin extracción, para evitar así alterar la composición del metaboloma.

El uso de suero en producción de embriones *in vitro* es controvertido. Así, mientras que algunos estudios han demostrado que el suero favorece el desarrollo *in vitro* (Thompson *et al*., 1998; Leivas *et al*., 2011; Murillo *et al*., 2017), otros autores han constatado un menor desarrollo, una baja supervivencia a la criopreservación, y alteraciones en la expresión génica de los embriones (Rizos *et al*., 2003; Heras *et al*., 2016). El suero también acentúa el dimorfismo sexual, aumentando el sex ratio (Camargo *et al*., 2010), como observamos en nuestro trabajo (Gimeno *et al*., 2022). Estos cambios pueden ser debidos a que los machos producidos con suero exhiben el triple de genes diferencialmente expresados que las hembras (1283 *vs.* 456) en comparación con embriones del mismo sexo desarrollados en el animal vivo (Heras *et al*., 2016). En nuestro estudio, 13 de los 31 metabolitos identificados relacionados con el sexo del embrión estuvieron marcadamente afectados por el tipo de cultivo, y otros 6 mostraron tendencias. La producción *in vitro* de embriones, especialmente en presencia de suero, se ha relacionado con alteraciones a largo plazo en la descendencia, como el LOS/AOS (Rivera

et al., 2021). En nuestro estudio, los parámetros clínicos como el tiempo de llenado capilar (CRT, del inglés *capillary refill time*), peso al nacimiento, perímetro torácico y temperatura, y bioquímicos como el BE, HCO_3^-, TCO_2, urea, creatinina y anión gap en terneros recién nacidos, se vieron afectados por la presencia de FCS en el cultivo (Gómez *et al*., 2022).

Biomarcadores que identifican el sexo del embrión

En el Capítulo I analizamos el MC de embriones para buscar metabolitos cuya concentración se asociase significativamente al sexo del embrión. Tras un análisis estadístico por bloques de complejidad creciente en cuanto al número de factores incluidos, y con el objetivo de discriminar aquellos factores que pudieran enmascarar el diagnóstico del sexo, obtuvimos un total de 31 metabolitos con presencia en 182 bloques. Entre los metabolitos identificados no se encontró un biomarcador único para diagnosticar el sexo en todas las condiciones del estudio. Sin embargo, cada uno de los metabolitos fue indicador específico del sexo para unas condiciones concretas (es decir, raza, medio de cultivo y estadio, o sus combinaciones). Esta característica permitió identificar con fiabilidad el sexo de todos y cada uno de los embriones presentes en un cultivo utilizando 2 ó 3 metabolitos, pero con tan sólo un metabolito por cada embrión.

Las clases de metabolitos más relevantes para la identificación del sexo del embrión fueron aminoácidos y lípidos. Entre los biomarcadores obtenidos, la prolina y la valina sirvieron para diagnosticar el sexo en un mayor número de casos de embriones de raza Asturiana de los Valles y en cultivo sin suero. Para esta misma raza, el MG(16:0/0:0/0:0) fue el biomarcador indicado para embriones cultivados con suero, mientras que el ácido 12-hidroxidodecanoico fue representativo del sexo con distintos rangos de valores en cultivo con BSA y con BSA+FCS. En el caso de la raza Holstein, destacó el ácido citramálico para embriones cultivados con BSA -y también con independencia del tipo de cultivo-, mientras que el único biomarcador fiable para embriones cultivados con suero fue el ácido benzoico.

Nuestros resultados respecto al *turnover* de aminoácidos fueron diferentes a los reportados por Sturmey *et al*. (2010), donde los embriones hembra consumieron más arginina, glutamato y metionina, y menos glicina, mientras que los machos consumieron

más fenilalanina, tirosina y valina. En nuestro estudio, ciñéndonos a embriones en estadio de mórula en día 6, los embriones hembra consumieron más ácido glutámico, fenilalanina, triptófano y tirosina, y los machos más arginina, prolina y valina, siendo estos consumos independientes de la raza y del medio de cultivo. En cambio, considerando los embriones de día 6 en fase de blastocisto (tanto blastocisto temprano como blastocisto propiamente dicho), las hembras consumieron más histidina, metionina, prolina, treonina, valina y ácido pipecólico, mientras que los machos mostraron predilección por el consumo de ácido glutámico y triptófano. Nuestros resultados y los de Sturmey *et al*. (2010) pueden reflejar diferencias en la composición del medio de cultivo (10% de FCS en Sturmey *et al*. (2010), *vs.* 0.1% en nuestro estudio), así como distintos períodos de cultivo estudiados (en Sturmey *et al*. (2010), el cultivo individual comenzó en día 7, sin diferenciar entre estadios embrionarios iniciales). El metabolismo de los embriones es cambiante a lo largo del desarrollo (Obeidat *et al*., 2019) y lo hace de manera diferente en función del sexo del embrión (Rubessa *et al*., 2018). No obstante, los valores de algunos aminoácidos fueron coincidentes con los resultados de Sturmey *et al*. (2010), como el mayor consumo de metionina en hembras, en lo que corresponde a nuestros estadios de blastocisto temprano y blastocisto (más avanzados). El mayor consumo de treonina en embriones hembra en fase de blastocisto temprano y blastocisto de día 6 se corresponde con la mayor expresión de genes propios del metabolismo de la glicina, serina y treonina en embriones hembra (Heras *et al*., 2016).

El adipato de dimetilo fue el único lípido cuya concentración relativa resultó afectada por el sexo del embrión dentro del conjunto total, altamente variable, de muestras (N=167 muestras). Sin embargo, tras desglosar los resultados por factores que ocultaban el efecto del sexo, y mediante el análisis por bloques, se identificaron 10 lípidos más como candidatos a biomarcadores. Entre estos factores, el cultivo afectó a 7 de los lípidos identificados (considerando resultados significativos y tendencias), de manera que los embriones de cada sexo mostraron un patrón lipídico diferente en función del tipo de cultivo (observación no publicada). En general, en presencia de suero los embriones hembra liberaron lípidos con mayor frecuencia (9 bloques FCh>0 *vs.* 25 bloques FCh<0), mientras que en ausencia de suero fueron los machos quienes segregaron lípidos en más ocasiones al MC (23 bloques FCh>0 *vs.* 13 bloques FCh<0). La acumulación de lípidos intracelulares en los embriones depende del medio de cultivo utilizado. El suero hace que se acumulen gotas lipídicas en mayor número y tamaño en los blastocistos (Sata *et al*.,

1999; Abe *et al*., 2002; Sudano *et al*., 2011; Murillo *et al*., 2017). No obstante, los embriones cultivados con suero no difieren en la expresión de genes del metabolismo lipídico de los embriones producidos *in vivo*, aunque sí lo hacen en muchas otras rutas metabólicas (Heras *et al*., 2016). Así, los embriones Holstein machos y hembras cultivados con 1% de suero de vaca en celo presentan un perfil lipídico intracelular similar (Janati Idrissi *et al*., 2021). En nuestro estudio, considerando únicamente los embriones Holstein en cultivo con suero, tampoco observamos diferencias en el contenido de lípidos en el MC entre embriones de distinto sexo.

Biomarcadores que predicen la gestación

En el Capítulo II se comparó el metaboloma del MC de embriones IVP transferidos frescos y congelados a receptoras. Una vez diagnosticada la gestación en día 40, día 62 y a término, se estudiaron las concentraciones relativas de metabolitos para predecir el éxito de la gestación en función del día en el cual se efectuó el diagnóstico. En general, el perfil metabólico difirió entre grupos de embriones que dieron lugar a gestación y aquellos que no, aunque tales diferencias fueron menos evidentes en embriones cultivados con suero. Nuestros resultados coinciden con otros estudios que han identificado un perfil metabólico distinto entre embriones que dan lugar a gestación y embriones que no se implantan, tanto en bovino (Muñoz *et al*., 2014a; Muñoz *et al*., 2014b; de Oliveira Fernandes *et al*., 2021; Gomez *et al*., 2021; de Oliveira Fernandes *et al*., 2023) como en humano (Seli *et al*., 2007; Scott *et al*., 2008; Seli *et al*., 2008; Marhuenda-Egea *et al*., 2011; Eldarov *et al*., 2022).

En un estudio previo se obtuvieron metabolitos biomarcadores para predecir el éxito de la gestación con embriones vitrificados a partir del análisis del MC por cromatografía de gases (GC-qTOF) (Gómez *et al*., 2021). Entre los 37 metabolitos identificados entonces, únicamente cuatro predijeron la gestación: el glicerol monoestearato, ácido cáprico y ácido palmítico (gestación a término), y el ácido esteárico (día 62 y a término). Las diferencias en el número de biomarcadores identificados respecto al presente estudio con embriones frescos y congelados pueden deberse no sólo a la técnica de análisis metabolómico utilizada, que puede llevar a identificar metabolitos diferentes, sino también al tratamiento de los datos. Además, aun cuando los embriones vitrificados se

generaron en las mismas condiciones que las del presente estudio, el análisis estadístico se realizó con todas las muestras juntas, sin un análisis de bloques, debido al tamaño muestral netamente inferior al de nuestro estudio actual (N=36 muestras de MC *vs*. N=84, respectivamente). De hecho, en el presente estudio, el análisis multivariante con todas las muestras de embriones transferidos frescos y congelados, y sin ajustes, tampoco consiguió separar los conjuntos de muestras en función del éxito de la gestación debido a la heterogeneidad de los factores fijos.

Los embriones frescos y congelados que no gestaron presentaron mayor concentración de ácido glutámico en el MC que los que dieron lugar a nacimientos (a excepción de 3 de los 10 bloques en embriones congelados). Cabe resaltar que altos niveles de ácido glutámico se han encontrado igualmente en el MC de embriones transferidos en fresco que no dieron lugar a gestación en bovino (de Oliveira Fernandes *et al*., 2021) y en humano (Seli *et al*., 2008). El *turnover* de aminoácidos de los embriones que gestaron mostró similitudes con el de embriones humanos que implantaron, aunque el MC analizado en humano corresponde al día 5 de desarrollo (Eldarov *et al*., 2022). Así, los embriones que implantaron consumieron más prolina, valina y triptófano, en coincidencia con nuestro análisis.

El MC de los embriones competentes para gestar contuvo menos lípidos, confirmando lo que observamos en embriones vitrificados que no gestaron, los cuales liberaron más ácidos grasos no esterificados, en particular, esteárico, cáprico y palmítico (Gómez *et al*., 2021). El metabolismo lipídico de los embriones también se ha correlacionado con la viabilidad de la gestación en bovino utilizando ESI-MS (de Oliveira Fernandes *et al*., 2023) y en humano mediante NMR (Marhuenda-Egea *et al*., 2011). Los lípidos son componentes esenciales de las membranas plasmática y de orgánulos celulares. Además, participan en la transducción de señales y en procesos biológicos como la proliferación y diferenciación celular y la reprogramación epigenética (Kang *et al*., 2014; Milazzotto *et al*., 2020). Los ácidos grasos pueden ser utilizados como fuente de energía a través de la β-oxidación, como precursores de la síntesis de prostaglandina y como elementos de anclaje de proteínas a la membrana celular (Sturmey *et al*., 2009; Dunning *et al*., 2014). En el presente trabajo, todos los lípidos identificados fueron ácidos grasos o derivados de éstos, con el ácido palmítico notablemente representado a través de compuestos como fosfatidiletanolamina (18:2/20:2), palmitamida y palmitoiletanolamida. Los embriones con un alto contenido lipídico sobreviven peor a la criopreservación (Amstislavsky *et al*.,

2019). El medio de cultivo influye en el perfil lipídico de los embriones, de manera que los blastocistos producidos con suero contienen mayores concentraciones de los ácidos grasos palmítico, esteárico, palmitoleico y oleico (Sata *et al*., 1999). En conjunto, resulta por tanto razonable que los biomarcadores lipídicos de gestación difieran en función del estado en el que se transfiera el embrión, fresco o criopreservado, y del medio de cultivo utilizado, como hemos demostrado en nuestro trabajo.

No hemos podido encontrar más estudios en bovino que hayan identificado metabolitos biomarcadores de la capacidad de gestación del embrión. Además, los estudios que han comparado los biomarcadores de gestación en embriones transferidos frescos y tras la criopreservación son escasos. En cualquier caso, nuestro trabajo proporciona información relevante, ya que indica que los biomarcadores que predicen la gestación pueden ser diferentes en función del estado en que se transfiera el embrión (fresco, vitrificado o congelado).

El éxito de la gestación no depende únicamente del embrión, sino que se necesita una correcta sincronía entre un embrión viable y una receptora competente. Los estudios de biomarcadores para predecir el éxito de la gestación generalmente han analizado solo el embrión (de Oliveira Fernandes *et al*., 2021; Gimeno *et al*., 2021a; de Oliveira Fernandes *et al*., 2023) o solo la receptora (Gómez *et al*., 2020b, Gómez *et al*., 2020c). Al carecer de información precisa sobre la capacidad de la parte complementaria (la viabilidad del embrión o la capacidad de la receptora, en su caso) para llevar adelante la gestación, se producen falsos negativos. Es decir, en un estudio que analice solamente receptoras, la transferencia de un embrión no viable a una receptora competente daría lugar a una receptora no gestante que, en realidad, es una falsa negativa; y lo mismo, pero a la inversa, sucedería si el estudio considerase únicamente embriones. Este problema fue abordado en un estudio anterior donde el plasma de la receptora y el MC del embrión se analizaron mediante GC-qTOF. La combinación de resultados del embrión y la receptora se realizó con métrica F1 score. El F1 score evalúa la calidad de un modelo de clasificación combinando la precisión y la exhaustividad (recall) en una única medida, utilizando cada metabolito de la receptora y del embrión para crear una matriz de confusión. El uso de F1 score mejoró significativamente la predicción de la gestación tras la transferencia de embriones vitrificados e identificó nuevos biomarcadores que, individualmente, carecían de significación estadística antes de realizar el análisis conjunto embrión-receptora (Gómez *et al*., 2021).

Basándonos en la estrategia anterior (Gómez *et al*., 2021), en el Capítulo III se diseñó una estrategia de mejora mediante algoritmos de aprendizaje. A tal efecto, se usaron los metabolitos identificados en el plasma (analizado por NMR) de receptoras transferidas con embriones frescos y congelados, y las señales espectrales obtenidas en el MC (analizado por UHPLC-MS/MS) de su embrión correspondiente. La combinación de la información de la receptora y del embrión se realizó utilizando el algoritmo de aprendizaje S*upport Vector Machine* (SVM). Los embriones y receptoras mal clasificados, aquellos en que la predicción del éxito de la gestación a término no coincidió con el estado de gestación real, consiguieron en su mayor parte ser correctamente reclasificados mediante iteraciones. Esta es una de las principales diferencias metodológicas con el estudio con embriones vitrificados, en el cual no se pudieron realizar iteraciones válidas debido al bajo número de muestras. A partir de iteraciones sucesivas, se detectaron receptoras erróneamente clasificadas (falsas negativas), especialmente entre las transferidas con embriones congelados. Esto puede ser debido a que el embrión proporciona información importante de la gestación a largo plazo, siendo más responsable que las receptoras de las pérdidas gestacionales tardías, al igual que hemos visto con la mejor capacidad de predicción a término mostrada por el embrión en Gómez *et al*. (2021). Así, aunque los embriones producidos por distintas tecnologías como la SCNT, la congelación, la vitrificación o el tipo de cultivo (como el suero), se mantienen en porcentajes de gestación temprana similares, los porcentajes de aborto tardío (entre día 62 y gestación a término) difieren según la tecnología empleada (Liu *et al*., 2013; Gómez *et al*.., 2020a). Esto podría explicar que en nuestro estudio se identificaran más embriones que receptoras como no viables. Una vez combinada la información de la receptora con el embrión, la competencia de la receptora se asignó correctamente, excepto en 2 receptoras de embriones congelados, donde no fue posible identificar si el falso positivo era el embrión, la receptora, o ambos.

Algunos biomarcadores plasmáticos fueron únicos para receptoras de embriones congelados, pero otros biomarcadores fueron los mismos que se identificaron antes en receptoras de embriones vitrificados (Gómez *et al*., 2020b; Gómez *et al*., 2020c; Gómez *et al*., 2021). La ornitina, lisina y glutamina destacaron como marcadores de gestación en muestras de plasma de día 7 en nuestro estudio, tanto para embriones transferidos frescos como tras la congelación. Curiosamente, las hembras preñadas por IA presentan concentraciones más elevadas de estos tres metabolitos en día 0 en comparación con las

que no logran gestar (Phillips *et al*., 2018). La ornitina se identificó en estudios anteriores como biomarcador de gestación en día 40, día 62 y a término, principalmente en embriones frescos producidos en diferentes condiciones de cultivo y en distintas razas (Gómez *et al*., 2020b; Gómez *et al*., 2020c). La fiabilidad de la ornitina como biomarcador de gestación resulta pues contrastada en varios escenarios. La glutamina plasmática predijo eficazmente la gestación en día 40 y día 62 con embriones frescos y vitrificados (Gómez *et al*., 2020b; Gómez *et al*., 2020c). En Holstein, la lisina se identificó como biomarcador de gestación en día 40, día 62 y a término en embriones vitrificados (Gómez *et al*., 2020c; Gómez *et al*., 2021). La información combinada del embrión y de la receptora identificó la fenilalanina, leucina y creatina como biomarcadores de gestación temprana (día 40 y día 62) y también de la gestación a término cuando los embriones se congelaron, mientras que la glutamina y la glicina se identificaron *de novo*. Cabe destacar que glutamina y glicina fueron también biomarcadores de embriones vitrificados (Gómez *et al*., 2020c).

Las concentraciones de algunos aminoácidos de los fluidos oviductal y uterino presentan correlaciones con sus niveles plasmáticos (Hugentobler *et al*., 2007). Los resultados de los estudios en receptoras (Gómez *et al*., 2020b; Gómez *et al*., 2020c; Gimeno *et al*., 2023a) y en el MC de los embriones (Gimeno *et al*., 2021) sugieren que los embriones sometidos a la criopreservación tienen un metabolismo y unos requerimientos nutricionales diferentes. Por tanto, cada tipo de embrión, fresco o criopreservado, podría necesitar un ambiente uterino específico para implantar y continuar el desarrollo. En esta línea, entre las rutas metabólicas diferencialmente expresadas entre receptoras gestantes transferidas con embriones frescos y congelados, figuran la biosíntesis de pterina y la biosíntesis de folatos (Gimeno *et al*., 2023a). La pterina es un precursor de la síntesis de folatos, los cuales son necesarios para la síntesis y metilación del DNA, y, por tanto, para mantener la rápida proliferación celular de los embriones (Laanpere *et al*., 2010). La deficiencia de folatos puede alterar o interrumpir el desarrollo embrionario y posterior crecimiento fetal. Otra de las rutas identificadas fue el metabolismo del triptófano. La concentración de triptófano en el fluido uterino aumenta en vacas gestantes durante el periodo de implantación (Groebner *et al*., 2011). En la gestación, el triptófano se precisa en cantidades crecientes para sostener la síntesis de proteínas en la receptora y en el embrión, y para sintetizar otros metabolitos importantes como la serotonina, niacina y quinurenina (Badawy, 2015). También identificamos cambios en la ruta de biosíntesis de

esteroides, metabolismo de andrógenos y estrógenos, metabolismo de androstenediona. Las vacas preñadas presentan una mayor expresión de genes propios de la biosíntesis de esteroides en el útero (Binelli *et al*., 2015). Además, la mayor actividad de estas rutas puede deberse a un aumento de la secreción de estradiol (Gómez *et al*., 2020d). Los animales con mayores niveles de estradiol durante el celo presentan mayor actividad antioxidante en el fluido uterino (Ramos *et al*., 2015). Por lo tanto, es posible que las receptoras aptas para gestar un determinado tipo de embrión presenten características metabólicas distintas, y, en particular, que las receptoras de embriones criopreservados deban tener un ambiente uterino adecuado para contrarrestar los efectos de la congelación o vitrificación en el embrión. En cambio, los embriones transferidos frescos no presentarían tales daños producidos por la criopreservación, lo que explicaría las diferencias observadas en los biomarcadores entre receptoras de embriones frescos y congelados.

Valoración de la aptitud física de los terneros procedentes de embriones frescos y criopreservados

Las tecnologías reproductivas producen cambios medibles en los embriones y en la descendencia. Según un reciente metaanálisis, la MIV y FIV en bovino alargan la gestación y aumentan el peso al nacimiento en la descendencia, y producen alteraciones análogas en otras cuatro especies (Beilby *et al*., 2023). En ratón, las técnicas de reproducción asistida modifican el metabolismo lipídico y el de la glucosa en la descendencia masculina. Estas alteraciones se dan en individuos sanos y fértiles. Sin embargo, aunque la congelación y la vitrificación alteran la fisiología de los embriones (de Oliveira Leme *et al*., 2016; Hayashi *et al*., 2019; Estudillo *et al*., 2021; Gutierrez-Castillo *et al*., 2021; Fryc *et al*., 2023), el impacto de la criopreservación de embriones bovinos no se ha evaluado en la descendencia. En el Capítulo IV se examinó la influencia de la congelación lenta y la vitrificación del embrión en la salud perinatal de los terneros nacidos. Para ello, se usaron técnicas de criopreservación asociadas a modificaciones en cultivos desarrollados en nuestro laboratorio que no aumentan la duración de la gestación ni el peso al nacimiento (Gómez *et al*., 2020a). De esta manera, se evitó que las complicaciones causadas por partos dificultosos e incluso distócicos -debidas en gran medida al sobrepeso de los terneros- influyesen en los parámetros estudiados,

contribuyendo así a definir con mayor nitidez los efectos de la vitrificación y la congelación de embriones en los terneros.

Los terneros nacidos de embriones IVP cultivados con un 20% de suero son de mayor tamaño que los obtenidos por IA (Jacobsen *et al*., 2000). Este incremento del tamaño del ternero es causa de dificultades durante el parto, afectando a los niveles de pH, lactato y presión parcial de CO_2 (PCO_2) en sangre. El mayor peso al nacimiento también se ha relacionado con menores valores de pH, HCO_3^- y BE, y mayor concentración de CO_2 total (TCO_2) y lactato (Kovács *et al*., 2017). En nuestro estudio, como era esperado, no observamos diferencias de peso entre los tres grupos de terneros, pero sí un efecto del FCS en cultivo sobre el peso al nacimiento. El FCS en cultivo afectó también al perímetro torácico, a la temperatura corporal, y a parámetros bioquímicos como el BE, HCO_3^-, TCO_2, urea, creatinina y anión gap. Sin embargo, estos efectos desaparecieron en día 15 y día 30, a excepción de la temperatura, donde el efecto del suero en cultivo se mantuvo durante el primer mes de vida. Esto también fue observado por Jacobsen *et al*. (2000), donde los terneros nacidos de embriones IVP con suero mostraron concentraciones más altas de lactato al nacer, pero tales diferencias desaparecieron a las 6 h tras el parto. El sexo del ternero también afectó al peso al nacimiento, el tamaño, Na^+, Cl^-, creatinina, hematocrito y hemoglobina. El efecto del sexo del ternero en los parámetros bioquímicos hemáticos es sin embargo constatable (Dillane *et al*., 2018) o no (Sayers *et al*., 2016) según las circunstancias de cada estudio.

La influencia de la criopreservación se reflejó en signos clínicos, como el CRT y la frecuencia cardíaca, y en parámetros bioquímicos, incluyendo el PCO_2, Na^+, creatinina, hematocrito y hemoglobina, analizados en día 0 (antes y después de la ingesta de calostro). Las diferencias debidas a la criopreservación en algunos parámetros fueron las mismas que se observaron usando otras tecnologías reproductivas. Por ejemplo, en terneros aparentemente sanos nacidos por transferencia de embriones SCNT, los valores de hematocrito, hemoglobina, urea y creatinina difieren de los medidos en terneros nacidos por IA (Chavatte-Palmer *et al*., 2002).

Los parámetros relacionados con el equilibrio ácido-base en día 0 mostraron pequeñas pero significativas diferencias entre grupos de terneros. Así, en terneros nacidos de embriones congelados la PCO_2 es más baja que en terneros procedentes de embriones frescos. Los valores de PCO_2 patológicos son propios de alcalosis respiratoria debida a

hipoxia (Constable, 2014), probablemente causada por la hiperventilación durante el parto. Tras la ingesta de calostro, el PCO_2 disminuyó en nuestros terneros procedentes de embriones frescos y congelados, al igual que en otros estudios (Jacobsen *et al*., 2000; Kovács *et al*., 2017). La reducción del PCO_2 refleja la adaptación del ternero a la vida extrauterina a través de la mejora de la función respiratoria (Varga *et al*., 2001). Sin embargo, la PCO_2 no se redujo por igual en los terneros de embriones vitrificados. Estas diferencias en el PCO_2 desaparecieron en día 15 y día 30, reduciéndose al tiempo que aumentaron los valores de PO_2. El equilibrio ácido-base puede verse alterado por la presencia de diarrea en terneros, la cual además causa deshidratación y un desequilibrio en los electrolitos (Sayers *et al*., 2016). Así, durante el estudio longitudinal, los valores de la temperatura, PCO_2, HCO_3^-, BE, TCO_2, además del Na^+, se vieron afectados por la presencia de diarrea, de acuerdo con Sayers *et al*. (2016). La diarrea disminuyó los valores de TCO_2, HCO_3^- y BE en día 30, con un efecto más pronunciado en el grupo de terneros procedentes de embriones vitrificados. Los valores del anión gap, indicador de la acidosis metabólica (Ewaschuk *et al*., 2003), aumentaron con la diarrea en los terneros nacidos de embriones vitrificados sobre los frescos. Si las posibles consecuencias de la vitrificación de embriones implican una mayor sensibilidad de los terneros a la diarrea -o un mayor compromiso inmunológico- merece ser investigado.

La vitrificación de embriones dio lugar a valores más altos de Na^+ en día 0 que los observados en terneros de embriones frescos y congelados. Por el contrario, los niveles de K^+ tendieron a aumentar en terneros de embriones congelados respecto a los frescos. Otros estudios han reportado diferencias en los electrolitos en sangre entre terneros nacidos de embriones IVP y de IA (Sangild *et al*., 2000; Rérat *et al*., 2005), aunque con resultados contradictorios. De este modo, la concentración hemática de K^+ tras el parto en terneros nacidos de embriones IVP se ha descrito tanto aumentada (Sangild *et al*., 2000) como reducida (Rérat *et al*., 2005). Este temprano aumento del nivel de K^+ se acompañó de una disminución de Na^+ y Cl^- (Sangild *et al*., 2000), aunque, siete días después, los niveles de K^+ y Na^+ se elevaron en el grupo de embriones IVP (Sangild *et al*., 2000).

La glucosa presentó una concentración inesperadamente alta en día 30 en el grupo de terneros procedentes de embriones vitrificados. No podemos establecer las causas de este aumento, aunque se han visto incrementos transitorios de glucosa hemática en terneros obtenidos de embriones IVP cultivados con suplementos de fluidos de oviducto y de

útero; no se observaron tales cambios en los terneros procedentes de cultivo con BSA y los nacidos por IA (Lopes *et al*., 2022). Sin embargo, en otros casos, los niveles de glucosa de terneros procedentes de embriones SCNT no difieren de los terneros de IA (Chavatte-Palmer *et al*., 2002), ni de los terneros procedentes de embriones IVP respecto a los de IA (Jacobsen *et al*., 2000; Sangild *et al*., 2000).

Las concentraciones de creatinina más elevadas tuvieron su origen tanto en la vitrificación como en la congelación de embriones, lo que indica una alteración propia de la criopreservación, independientemente de la técnica que se haya usado. Los terneros procedentes de embriones IVP también presentan valores más altos de creatinina que los terneros de IA (Hiendleder *et al*., 2006; Lopes *et al*., 2022). La creatinina en día 0 estuvo afectada no sólo por la criopreservación, sino también por el suero y el sexo del ternero, mientras que, con el paso del tiempo, únicamente se mantuvo el efecto del sexo. Se sabe que los machos presentan una concentración de creatinina mayor que las hembras, posiblemente debido a su mayor masa muscular (Rérat *et al*., 2005). Tras la ingesta de calostro, los valores de creatinina disminuyeron en los tres grupos de terneros, al igual que en otros estudios (Mohri *et al*., 2007; Kalaeva *et al*., 2019), e indicando un efecto reversible de la criopreservación sobre este parámetro.

El CRT fue mayor en los terneros nacidos de embriones criopreservados. Además, los valores de hematocrito se elevaron en el grupo de terneros de embriones vitrificados sobre los congelados. El CRT y el hematocrito indican el estado de hidratación del ternero, siendo más altos cuanto mayor es el grado de deshidratación (Kells *et al*., 2020; Roadknight *et al*., 2021). Nuestros resultados, por tanto, sugieren un cierto mayor grado de deshidratación en terneros procedentes de embriones criopreservados, especialmente en el grupo de vitrificados, aunque sin comprometer el estado de salud, pues en casos de deshidratación severa no es posible medir el CRT (Kells *et al*., 2020).

El calostro tuvo efectos comparables en los parámetros fisiológicos entre los tres grupos de terneros, indicando una capacidad adaptativa a la vida extrauterina similar entre ellos. Por ejemplo, todos los terneros presentaron valores de pH superiores a 7.2, que es el límite inferior indicativo de acidosis metabólica (Bleul & Götz, 2013). Así, los terneros de los tres grupos respondieron a la ingesta de calostro aumentando el pH a valores comprendidos entre 7.30 y 7.35. La saturación de oxígeno (sO_2) analizada en nuestro trabajo representa la cantidad de oxihemoglobina respecto a la hemoglobina total

(incluyendo oxihemoglobina y desoxihemoglobina). Al final de la gestación, el ternero responde a la hipoxia con una concentración transitoria más alta de hemoglobina y hematocrito, como observamos en nuestro estudio. A partir de este momento, la hemoglobina fetal es paulatinamente reemplazada por la hemoglobina adulta, proceso que finaliza a las 13 semanas de vida (Golbeck *et al*., 2019). En día 0, los valores del hematocrito y la hemoglobina fueron menores en los terneros nacidos de embriones congelados que en los nacidos de embriones vitrificados. Durante las primeras 24 h de vida, los terneros nacidos de embriones IVP también presentan niveles de hemoglobina más altos que los terneros obtenidos por IA (Sangild *et al*., 2000). En nuestro estudio, las diferencias en el hematocrito y la hemoglobina desaparecieron en día 15 y día 30. La sO_2 aumentó de día 0 a día 15 en los tres grupos de terneros, manteniendo unos niveles similares en día 30. Asimismo, el valor del hematocrito disminuyó gradualmente, reflejando el reemplazo de los eritrocitos fetales por las células adultas de menor volumen (Mohri *et al*., 2007; Golbeck *et al*., 2019). Tras la ingesta de calostro, y durante las primeras semanas de vida de los terneros, la disminución de la concentración de hemoglobina, de eritrocitos y del valor hematocrito, son cambios fisiológicos que cabe esperar (Muri *et al*., 2005; Mohri *et al*., 2007; Heidarpour *et al*., 2008).

En general, los valores de los parámetros hemáticos que obtuvimos se encontraron comprendidos en los rangos descritos como saludables en varios estudios, aunque no todos los valores que hemos encontrado se ajustan a los rangos definidos en un mismo estudio. Sin embargo, constatamos que nuestros parámetros se ajustaron al menos a uno de los valores de referencia. Cabe indicar dos excepciones, la urea, para la que medimos valores inferiores a otros estudios, y el TCO_2 que no fue medido en los estudios de referencia. De cualquier modo, es importante señalar que las condiciones de nuestra investigación fueron netamente diferentes a esos otros trabajos, también muy distintos entre sí, lo cual impide generalizaciones en el uso de valores de referencia, y entre los propios estudios. Las mayores influencias pueden ser debidas a factores genéticos (raza), nutricionales (dieta) y ambientales, como son el tipo de explotación y el manejo de los animales. También la edad influye en los valores de los parámetros bioquímicos analizados en sangre, y, por tanto, en los rangos definidos como normales (Mohri *et al*., 2007; Sayers *et al*., 2016; Dillane *et al*., 2018; Wenker *et al*., 2022).

Estudios futuros

Las bases moleculares de las alteraciones producidas por la criopreservación en la descendencia requieren ser investigadas. En bovino, los embriones vitrificados y congelados difieren en la metilación y expresión de genes implicados en el desarrollo respecto a los frescos (Stinshoff *et al*., 2011; Zhao *et al*., 2012; Gutierrez-Castillo *et al*., 2021). Las alteraciones en los embriones debidas a las técnicas de reproducción asistida pueden tener efectos a largo plazo, como el LOS/AOS, caracterizado por defectos genéticos y epigenéticos (Urrego *et al*., 2014; Rivera *et al*., 2021), aunque estos cambios también pueden darse en terneros aparentemente sanos (Rabaglino *et al*., 2021). Recientemente encontramos asociaciones significativas entre el metabolismo del embrión (medido en el MC) y el de la receptora (en plasma) con el peso y la talla de los terneros al nacer, así como con otros parámetros clínicos y bioquímicos (Gimeno *et al*., 2021b). Estas covariaciones, no verificadas a través de cambios en el epigenoma y en la expresión génica (Sinclair *et al*., 2016), reflejan que la aptitud física del ternero es ya perceptible en el embrión, y a niveles no genómicos. Más tarde, pudimos observar altos niveles postnatales de metilación de *IGF2* en linfocitos de terneros nacidos de embriones congelados, a la par que expresión génica de *IGF2* tendente a ser más baja que la que se encontró en terneros de embriones frescos (Gimeno *et al*., 2023b). Por tanto, sería interesante analizar el perfil de expresión y metilación génica y el metaboloma de los embriones antes y después de la congelación o vitrificación para entender las bases moleculares que determinan la supervivencia de un embrión a la criopreservación, y para investigar el origen de los cambios observados en la descendencia.

8| CONCLUSIONES

CONCLUSIONES

Las conclusiones de esta tesis son:

- La UHPLC-MS/MS es una técnica metabolómica válida y eficiente para identificar metabolitos biomarcadores en el medio de cultivo del embrión.

- La complejidad de los factores que afectan al metaboloma del embrión hace que no exista un procedimiento sencillo en la búsqueda de biomarcadores de rasgos de interés, como el sexo y la predicción de la gestación. De forma más específica se concluye que:

    - En una población heterogénea, la variedad de factores que afectan al metabolismo embrionario puede enmascarar la identificación de biomarcadores.

    - La identificación de biomarcadores es factible en grupos muestrales más homogéneos (bloques), que permitan discriminar uno o varios factores fijos como el medio de cultivo *in vitro*, la raza del toro productor de los espermatozoides, los estadios embrionarios entre los cuales transita el embrión durante el período de cultivo individual que sirve para analizar el metaboloma, y el estado en el que se transfiere el embrión, fresco o criopreservado.

    - El potente efecto de los factores aleatorios individuales, toro, y presumiblemente ovocito, se puede contrarrestar a base de integrar múltiples individuos y repeticiones.

    - No existe un biomarcador único para diagnosticar el sexo del embrión ni predecir la gestación en todas las condiciones analizadas en este estudio. No obstante, algunos metabolitos actúan como marcadores únicos altamente eficientes en unas condiciones concretas (es decir, raza, medio de cultivo, estadio, y estado del embrión transferido, o sus combinaciones).

- Los metabolitos biomarcadores de gestación identificados en el plasma de las receptoras pueden actuar en función del estado en el que se transfiere el embrión (fresco o congelado).

- Las receptoras preñadas y vacías, así como las receptoras preñadas de embriones frescos y congelados, presentan entre sí distintos niveles de actividad metabólica; por tanto, la optimización de la selección de receptoras debe tener en cuenta si el embrión que se transfiere es fresco o criopreservado.

- Hay menos embriones viables que receptoras con competencia para gestar. Así, la combinación de biomarcadores de la receptora y del embrión mejora la predicción de la gestación, especialmente en transferencias con embriones congelados.

- La criopreservación de embriones afecta a los terneros sanos, con cambios más pronunciados al nacer que en los siguientes días de vida. Sin embargo, la capacidad de los terneros para adaptarse a la vida extrauterina es similar entre los procedentes de embriones frescos y los que fueron criopreservados.

CONCLUSION

This thesis concludes that:

- UHPLC-MS/MS metabolomics is a valuable and efficient technique for identifying biomarker metabolites in the spent embryo culture medium.
- The complexity of factors that influence the embryo metabolome makes it difficult to obtain a simple procedure leading to the identification of biomarkers for traits of interest, such as sex and pregnancy prediction. More specifically, the following applies:
    - In a complex, multifactorial environment, the variety of factors affecting embryo metabolism obscures the identification of biomarkers.
    - The identification of biomarkers is feasible in homogeneous sample groups (blocks), which allow discriminating one or more fixed factors such as in vitro culture medium, bull breed, developmental stages during the individual embryo culture period that serves to analyze the metabolome, and the status of the embryo transferred, fresh or cryopreserved.
    - The significant effect of individual random factors, i.e., bull and presumably oocyte, can only be counteracted by integrating multiple individuals and replicates.
    - There is not a single biomarker to diagnose embryo sex or to predict pregnancy in all conditions analyzed in this study. However, some metabolites are highly efficient as single biomarkers in specific conditions (i.e., breed, culture medium, stage, and status of the embryo transferred, or their combinations).
- The pregnancy biomarker metabolites identified in recipient plasma perform according to the status of the embryo transferred (i.e., fresh or frozen).
- Pregnant and non-pregnant recipients, as well as recipients made pregnant with fresh and frozen embryos, show different levels of metabolic activity; thus,

optimization of recipient selection must consider the status of the embryo transferred, fresh or cryopreserved.

- There are fewer viable embryos than pregnancy competent recipients. Therefore, combining biomarkers from the recipient and the embryo improves pregnancy prediction, especially in the transfer of frozen embryos.

- Embryo cryopreservation impacts healthy calves with alterations more pronounced at birth than on the subsequent days of life. However, the ability of calves to adapt to extrauterine life is similar between those born from fresh embryos and those that were cryopreserved.

9| REFERENCIAS

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10| ANEXOS

PhD thesis: Isabel María Gimeno Miquel

The thesis, NON-INVASIVE IDENTIFICATION OF BIOMARKERS OF SEX AND PREGNANCY VIABILITY IN FRESH AND CRYOPRESERVED BOVINE EMBRYOS PRODUCED IN VITRO, has set out to examine the metabolic profiles of early bovine embryos and then relate these to a series of phenotypic markers, including embryonic sex and viability, as well as assessing the impact of cryopreservation.

The paucity of non-invasive biomarkers of embryonic phenotype is widely accepted as a limitation and knowledge gap on our understanding of embryo viability. In addition, the absence of markers of embryonic sex remains a challenge in agricultural practice. The work presented in this thesis has made a novel contribution to our understanding in this field, as evidenced by the fact that all of the data have reached the threshold for publication in peer reviewed journals.

The work has shown a number of fascinating observations; most notably the power of measuring combinations of metabolites to determine embryonic sex and viability. These observations form the major component of two published articles and thus meet the threshold of having made a novel contribution to knowledge.

The topic of the thesis is wide and has provided the candidate with an opportunity to demonstrate advanced skills in critical analysis of published works, as well as the scope to design and undertake technically challenging methodologies to perform high quality data synthesis. The data have been analysed appropriately and presented well. Finally, the candidate has shown an excellent ability to reflect on the work, demonstrated by interesting and thoughtful, yet considered discussions of the data.

This is a very impressive piece of work, of international quality and I am pleased to endorse award of an international PhD and offer my congratulations to Ms Gimeno and the supervisory team.

Professor Roger Sturmey
Hull York Medical School

April 25, 2023

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April 25th, 2023

To Whom This May Concern:

Ref: assessment of the PhD thesis of Ms. Isabel M. Gimeno Miquel

The thesis presented by Ms. **Isabel María Gimeno Miquel** to obtain the Ph.D. degree, entitled "Non-invasive identification of biomarkers for embryonic sex and pregnancy viability of bovine embryos produced in vitro fresh and cryopreserved", aimed to (1) investigate whether metabolites identified in the spent culture medium by the embryo could serve as biomarkers to diagnose embryonic sex and predict pregnancy outcome and live-births, and (2) to study the perinatal health of calves born from fresh and cryopreserved embryos.

The contribution of her thesis to the field of the identification of biomarkers is original and important in the international context. Particularly, the thesis made the following significant contributions:

- From the metabolomic analysis of the embryo culture medium, experiments reported provide new functional support to differences in metabolic activity between male and female embryos. Likewise, the metabolism of embryos that were able to remain viable differed from that of embryos that failed to survive and implant.

- Due to the complexity of the embryonic metabolome, it remains challenging to identify biomarkers of desirable traits, such as sex or pregnancy viability, in a heterogeneous population. This is because the variety of factors affecting embryonic metabolism hinders the identification of biomarkers. Nevertheless, the identification of biomarkers was feasible in groups that were more homogeneous.

- There was not a single biomarker to diagnose embryonic sex in all conditions analyzed, nor to predict the pregnancy outcome. However, some metabolites presented a highly favorable predictive efficiency under specific conditions (i.e., breed, culture medium composition, stage of development of the embryo transferred, or combinations thereof).

- Combining the metabolic information of recipients and embryos improved pregnancy prediction, especially in transfers with frozen embryos.

- Embryo cryopreservation affected calf fitness, with more pronounced changes at calving than in the following days of life. However, the ability of calves to adapt to extrauterine life was similar between those that originated from fresh embryos and those that originated from cryopreserved embryos.

Besides my own assessment, the high quality of the research performed during the course of Ms. Gimeno's Ph.D program has been certified by international experts, that evaluated and approved her work for publication on high impact-factor peer-reviewed scientific journals on the field of reproductive sciences.

In conclusion, based on the quality of the research performed by Ms. **Isabel María Gimeno Miquel** and the evidence presented, I highly recommend her for the mention of an International Ph.D. degree.

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Mario Binelli, PhD
Assistant Professor of Physiology

FACULTY OF VETERINARY MEDICINE
accredited by EAEVE

DEPARTMENT OF INTERNAL MEDICINE, REPRODUCTION AND POPULATION MEDICINE
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Report on the Ph.D. thesis

The thesis carried out by Ms. **Isabel María Gimeno Miquel** to obtain the Ph.D. degree, entitled "Non-invasive identification of biomarkers for embryonic sex and pregnancy viability of bovine embryos produced in vitro fresh and cryopreserved" aimed to investigate whether metabolites identified in the spent culture medium by the embryo could act as biomarkers to diagnose embryonic sex and predict pregnancy and birth, and to study the perinatal health of calves born from fresh and cryopreserved embryos.

The contribution of her thesis to the field of the identification of biomarkers is original and important. Particularly, the thesis made significant contributions, as follows:

- From the metabolomic analysis of the embryo culture medium it provided novel support in identifying metabolic differences between male and female embryos. Similarly, the metabolism of embryos that were capable of inducing a pregnancy differed from that of embryos that failed to implant.

- Due to the complexity of the embryo metabolome, it is not easy to search for biomarkers of specific interesting traits, such as sex or pregnancy prediction in a heterogeneous population. This is because the variety of factors affecting embryonic

metabolism hides the identification of biomarkers. Nevertheless, the identification of biomarkers is feasible in more homogeneous groups (blocks).

- There is not a single biomarker to diagnose embryonic sex in all conditions analyzed, nor to predict pregnancy. However, some metabolites are highly efficient under specific conditions (i.e., breed, culture medium, stage, and stage of the transferred embryo, or combinations thereof) that facilitate the use of one metabolite per embryo.

- There are fewer embryos with pregnancy competence than recipients. Thus, combining the metabolic information of recipients and embryos improves pregnancy prediction, especially in transfers with frozen embryos.

- Embryo cryopreservation affects calf fitness, with more pronounced changes at birth than in the following days of life. However, the ability of calves to adapt to extrauterine life is similar between those from fresh embryos and those that were cryopreserved.

The quality of the research performed by the Ph.D. student has been already certified by different international experts, as it has been already published in high impact-factor peer-reviewed scientific journals of reproduction science.

In conclusion, based on the quality of the research performed by Ms. **Isabel María Gimeno Miquel** and the evidence presented, I highly recommend her for the mention of an International Ph.D. degree.

Sincerely,

Prof. dr. Ann Van Soom

Nouzilly, le 26/04/2023

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Isabel Maria Gimeno Miquel

International thesis evaluation

Since the development of genomic selection in cattle few years ago, the use of embryo transfer in selection schemes and for the diffusion of genetic gain increased rapidly. In addition, the statistics gathered by international societies (AETE, IETS) shows that the ratio of in vitro produced (IVP) embryos is growing fast, compared to in vivo derived ones. It is well known that IVP embryos are less viable and more sensitive to conventional cryopreservation techniques than in vivo derived ones, resulting in lower success rate after embryo transfer, especially after cryopreservation or cell biopsy for genetic diagnosis. High research efforts have been devoted to culture conditions for IVP in relation with embryo gene expression profile (omics approaches) to establish a molecular signature of embryo quality and trying to identify markers to predict it. However, most studies missed to address the important aspects of embryo- recipient interaction which also affect the final success, i.e. establish a viable pregnancy producing viable and healthy offspring.

In this context, Isabel Miquel developed a very innovative and original work with very powerful experimental designs allowing to study the interactions between culture conditions, embryo gender and metabolism, and recipient characteristics, for a comprehensive evaluation of the embryo transfer success conditions. This huge work leaded to the writing of four papers (three published and one accepted), bringing up a considerable quantity of highly relevant, valuable, and interesting results.

A first set of experiments (published in Metabolomics) used UHPLC coupled to tandem mass spectrometry to analyze the metabolites present in the medium used for 24h culture of single IVP embryos of different stages, from different bulls (7 bulls from 2 breeds) and developed in different media (+/- FCS). It is concluded that the variability of metabolites related to embryo sex and viability post TE is hidden by the variations induced by genetic origin and IVP conditions. However, when given panels of metabolites (not single ones) are used as markers for subsets of embryos with homogeneous IVP origin, they can efficiently predict embryo sex and TE success.

A second paper published in Metabolites (a MDPI Journal) used a similar approach to compare embryos produced in the same way with culture conditions and genetic (bulls) differences reports similar results with markers of ET success were shared between fresh and frozen/thawed transferred embryos while many others were specific.

A third paper, published in Frontiers in Veterinary Science (in which the candidate is last author) compared the health status between calves born after transfer of fresh or cryopreserved (vitrification or slow freezing) IVP embryos. While observing some metabolic and physiologic differences between the three calves groups at birth, they all adapted normally after colostrum uptake while IVP with serum induced some temperature and metabolic disorders. Groups became similar at Day 15, except a higher diarrhea susceptibility it the vitrified group.

A fourth paper (accepted in J Dairy Science) reports the use of H+ nuclear magnetic resonance (NMR) to analyze the blood of recipient cows and the spent culture medium of single IVP embryos transferred fresh or frozen/thawed. This study allowed to identify sets of markers reflecting recipient aptitude, amongst which creatine was well represented. It is important to combine embryo and recipient metabolic markers to reach an accurate prediction of pregnancy success.

To conclude, Isabel Gimeno Miquel did a huge, original and well conducted work. She published her results in top rated international journals in the field of reproduction, bringing up highly valuable results for the scientific and practitioner communities worldwide. Therefore, I warmly recommend delivering to her work the truly justified label of International PhD degree.