Journal of Animal Science, 2024, **102**, skae081
https://doi.org/10.1093/jas/skae081
Advance access publication 3 April 2024
Reproduction

The recipient metabolome explains the asymmetric ovarian impact on fetal sex development after embryo transfer in cattle

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Abstract

In cattle, lateral asymmetry affects ovarian function and embryonic sex, but the underlying molecular mechanisms remain unknown. The plasma metabolome of recipients serves to predict pregnancy after embryo transfer (**ET**). Thus, the aim of this study was to investigate whether the plasma metabolome exhibits distinct lateral patterns according to the sex of the fetus carried by the recipient and the active ovary side (**AOS**), i.e., the right ovary (**RO**) or the left ovary (**LO**). We analyzed the plasma of synchronized recipients by $^{1}H^{+}$NMR on day 0 (estrus, $n = 366$) and day 7 (hours prior to ET; $n = 367$). Thereafter, a subset of samples from recipients that calved female ($n = 50$) or male ($n = 69$) was used to test the effects of embryonic sex and laterality on pregnancy establishment. Within the RO, the sex ratio of pregnancies carried was biased toward males. Significant differences ($P < 0.05$) in metabolite levels were evaluated based on the day of blood sample collection (days 0, 7 and day 7/day 0 ratio) using mixed generalized models for metabolite concentration. The most striking differences in metabolite concentrations were associated with the RO, both obtained by multivariate (OPLS-DA) and univariate (mixed generalized) analyses, mainly with metabolites measured on day 0. The metabolites consistently identified through the OPLS-DA with a higher variable importance in projection score, which allowed for discrimination between male fetus- and female fetus-carrying recipients, were hippuric acid, L-phenylalanine, and propionic acid. The concentrations of hydroxyisobutyric acid, propionic acid, L-lysine, methylhistidine, and hippuric acid were lowest when male fetuses were carried, in particular when the RO acted as AOS. No pathways were significantly regulated according to the AOS. In contrast, six pathways were found enriched for calf sex in the day 0 dataset, three for day 7, and nine for day 7/day 0 ratio. However, when the AOS was the right, 20 pathways were regulated on day 0, 8 on day 7, and 13 within the day 7/day 0 ratio, most of which were related to amino acid metabolism, with phenylalanine, tyrosine, and tryptophan biosynthesis and phenylalanine metabolism pathways being identified throughout. Our study shows that certain metabolites in the recipient plasma are influenced by the AOS and can predict the likelihood of carrying male or female embryos to term, suggesting that maternal metabolism prior to or at the time of ET could favor the implantation and/or development of either male or female embryos.

Lay Summary

This study explored how the active ovary side (**AOS**, i.e., left or right) and the sex of the calf carried by the recipient relate to the plasma metabolome in blood. For this purpose, we analyzed blood samples from heifers at two specific times: the day of the estrus and the day of the embryo transfer. We found significant differences in the sex ratio of pregnancies carried in the right ovary, and in the levels of certain metabolites depending on whether the active ovary was on the right or left and whether the calf was male or female. As examples, the concentrations of hydroxyisobutyric acid, propionic acid, L-lysine, methylhistidine, and hippuric acid were lowest when male calves were carried, in particular when the right ovary was active. Interestingly, the calf sex also influenced certain metabolic pathways, especially in the right AOS, several of them related to amino acid metabolism. However, no significant metabolic pathway changes were observed based solely on which ovary was active. Overall, the study suggests that the metabolism of the recipient, influenced by the AOS, might play a role in the successful implantation and development of embryos of a certain sex. This insight could potentially help to predict and improve pregnancy outcomes in cattle through embryo transfer techniques.

Key words: embryonic sex, metabolism, pregnancy, recipient

Abbreviations: AM, Asturiana de la Montaña; AOS, active ovary side; AV, Asturiana de los Valles; CL, corpus luteum; COC, cumulus–oocyte complexes; ET, embryo transfer; FCh, fold change; FCS, fetal calf serum; GnRH, gonadotropin-releasing hormone; IVP, in vitro-produced; LH, luteinizing hormone; LO, left ovary; mSOF, modified synthetic oviduct fluid; NMR, Nuclear Magnetic Resonance; OPLS-DA, Orthogonal Partial Least Square-Discriminant Analysis; PVA, polyvinyl-alcohol; P4, progesterone; RO, right ovary; UF, uterine fluid; VIP, variable importance in projection.

Introduction

Lateral asymmetry in follicular activity between the left ovary (LO) and the right ovary (RO) has been described in various species (Ginther, 2019). This phenomenon has been extensively studied in the cattle genital tract at several levels in natural cycles, including the ovary (Karamishabankareh et al.,

Received December 7, 2023 Accepted April 2, 2024.

2015; Ginther, 2019) and uterus (Geres et al., 2011; Trigal et al., 2014). However, in general, these valuable studies were observational, often focusing only on the side of the occurrence of ovulation and pregnancy. Ovulation occurs more frequently in the RO than in the LO (Karamishabankareh et al., 2015; Ginther, 2020a). Similarly, the frequency of gestation on the right side is higher than on the left side (60% on the right vs. 40% on the left) (Hylan et al., 2009; Gharagozlou et al., 2013). Oocytes collected from the RO are more competent in developing into blastocysts in vitro compared to those isolated from the LO (Karamishabankareh et al., 2015). Moreover, more embryos can be recovered from the RO than from the LO after multiple embryo transfer (**ET**) to synchronized recipients and flushing (Trigal et al., 2014).

The term intraovarianism was coined by Ginther (2020b) to describe mechanisms that locally affect follicle and luteal activity within an ovary. The increased frequency of ovulation in the RO over the LO is a faithful representation of such processes, as well as the greater number of antral follicles found in the RO in calves (Ginther, 2020a; Ginther et al., 2021). However, as cited in Ginther (2020b), the mechanisms underlying such differences, reported very early in the last century, remain unknown. It has been proposed that the mechanisms that govern the enhanced propensity for ovulation of the RO reside in the LO (Ginther and Gomez-León, 2020), and they can be explained by physical more than by biochemical or hormonal facts (Ginther, 2020a). Thus, the propensity for greater diameter deviation—the process that determines the selection of the dominant follicle—in the RO can be attributed to the differences between the RO and LO (Ginther, 2020a; Ginther, 2020b; Ginther and Gomez-León, 2020). Other traits that underlie lateral ovarian differences include higher weight and a higher number of 4 to 6 mm diameter follicles in the RO in newborn calves (Casida et al., 1935; Dangudubiyyam and Ginther, 2019), and higher DNA and protein content of the RO reported in humans (Dangudubiyyam and Ginther, 2019; Ginther, 2020a). The lower follicle development reported in the LO in newborn calves continues during adulthood (Dangudubiyyam and Ginther, 2019; Ginther et al., 2021). Therefore, the greater likelihood for the RO to ovulate may be congenital and a consequence of a negative tendency for ovulation on the left side (Ginther, 2020a).

Asymmetry in the reproductive tract can also affect embryonic sex, resulting in fetuses distributed between uterine horns following unbalanced, sex-based patterns. Such a peculiar distribution consists of more males seen in the horn adjacent to the active RO than in the left horn (Vazquez et al., 1993; Hylan et al., 2009; Giraldo et al., 2010; Karamishabankareh et al., 2015). Similarly, more male than female embryos were obtained in vitro with oocytes collected from the RO (Hylan et al., 2009). However, the sex ratio of calves born from ET does not differ between the left and right horns (Hylan et al., 2009), suggesting that the ovary of origin is more responsible for the skewed sex ratio than the uterine horn of gestation. The rationale for such skewed sex distribution has not been explained yet. Sperm transport after artificial insemination has been investigated for lateral asymmetry, although with controversial conclusions (Larsson and Larsson, 1985; Larsson, 1986; López-Gatius, 1997). Testosterone levels in follicular fluid are higher in RO follicles (Grant and Irwin, 2005), and oocytes exposed to higher testosterone levels are more likely to be fertilized with Y-bearing spermatozoa (Grant et al., 2008). Alternatively, ovaries may secrete different compounds into their ipsilateral uterine horn which would favor the implantation of male or female embryos (Clark et al., 1994). However, sex laterality could be breed-specific or influenced by unknown factors, since it was not observed in Bos Indicus cattle (Borges et al., 2017), nor in other Holstein studies (Gharagozlou et al., 2013).

Fertility in cattle is closely related to their nutritional status (Butler, 2000; Ferraretto et al., 2014). Thus, the levels of certain metabolites found in recipient plasma collected prior to the ET time (i.e., days 0 and 7) are associated with success in pregnancy (Gómez et al., 2020a, 2020b; Gomez et al., 2021; Gimeno et al., 2023). Some of these metabolites have their concentrations reflected in the uterus (Hugentobler et al., 2007). Several authors have found that the endometrium reacts differently to embryonic sex (Gross et al., 2017; Gómez et al., 2018; Mathew et al., 2019). In fact, male and female in vitro-produced (**IVP**) embryos release contrasting amounts of molecules with signaling effects (Gómez et al., 2013). Therefore, such local dialogue could influence the establishment of pregnancies based on a lateral asymmetry (i.e., when the active ovary side (**AOS**) is the left or the right) and the sex of the embryo. Otherwise, it is unknown whether embryos of each sex need—in whole or in part—an already adapted sex- and pregnancy-competent recipient to develop. If the former is true, recipients capable of carrying male or female pregnancies might differ in their blood metabolic profiles prior to ET. On the contrary, if all competent recipients adapt to the sex of the embryo transferred, or if the embryonic sex does not affect the recipient, no metabolically different, recipient populations would be observed before the ET time.

Considering that transuterine migration of embryos to a preferred uterine horn for gestation is infrequent (McMillan and Peterson, 1999), a means to narrow down sex and lateral asymmetry effects to specific sections in the genital tract is the transfer of a blastocyst. This is so because previous steps from intraovarian oocyte growth and maturation, ovulation, fertilization, and embryo cleavage stages occur outside the uterus and are overcome within ET. Therefore, although the AOS may be responsible for conducting the asymmetry from the beginning, a contribution to identifying the systemic effects of such regulation in different anatomical locations is needed.

Currently, laterality mechanisms have not been studied in depth in a way that such knowledge could lead to specific improvements, if any, in cattle reproductive technology. Laterality in the AOS does not show a genetic basis, since it changes through subsequent reproductive cycles, with the pairs of ovulation cycles right–right, left–right, and right–left exhibiting similar frequency of occurrence (28%), and the left–left pair bearing the lowest frequency (16%) (Ginther and Gomez-León, 2020; Ginther et al., 2021). Thus, we hypothesized that if corpus luteum (**CL**) side and embryo or calf sex are mechanistically related, such phenotypes should be better concerned by environmental, nutritional and management issues, which would be reflected in the recipient blood metabolome. Therefore, the objective of this study was to investigate whether the early collected, plasma metabolome of cattle is already committed to follow lateral patterns in AOS and sex of the fetus carried by each recipient of embryos. This investigation aims to provide a deeper understanding of the underlying mechanisms that may influence fetal sex selection and ovarian side preference.

Materials and Methods

The present study analyzed blood plasma samples collected in two experimental herds in Spain and France.

Experimental procedures in Spain 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 the guidelines of European Community Directive 86/609/EC. Experimental procedures in France followed the European Directive 2010/63/EU approved by the French Ministry of National Education, Higher Education, Research and Innovation after review by the local ethics committee "Comité d'Ethique en Expérimentation Animale Val de Loire" (APAFIS number 32529-2021072010588v3).

Reagents were purchased from SIGMA unless otherwise stated.

In vitro embryo production

Embryos were produced from oocytes within ovaries collected at slaughterhouses.

General procedures for in vitro embryo production in Spain have been described in previous studies (Gómez et al., 2020c; Gimeno et al., 2021). The following are descriptions in brief. Cumulus–oocyte complexes (**COC**) were matured in vitro in TCM199 supplemented with $NaHCO_3$ (2.2 mg/mL), 10% (v/v) fetal calf serum (**FCS**) (F4135), 1.5 µg/mL of porcine FSH-LH (Stimufol; ULg FMV, Liège, Belgium) and 1 µg/mL 17β-estradiol, for 22 to 24 h at 38.7 °C, 5% CO_2 and high humidity. Oocytes were in vitro fertilized (day 0) with commercial frozen/thawed semen from Asturiana de los Valles (**AV**) bulls ($n = 5$) and Holstein ($n = 12$) bulls with proven fertility. Motile sperm were selected by a swim-up protocol. COC and sperm cells (2×10^6 cells/mL) were co-incubated in 4-well dishes containing 500 µL of pre-equilibrated fertilization medium (Fert-TALP) with heparin (10 µg/mL; Calbiochem, La Jolla, CA, USA) for 18 to 20 h at 38.7 °C in a 5% CO_2 atmosphere with saturated humidity. Subsequently, cumulus cells were removed using a vortex, and fertilized oocytes were cultured in commercial media BO-IVC (71005, IVF Bioscience, UK), or in modified synthetic oviduct fluid (**mSOF**) containing 45 µL/mL basal medium eagle (BME) amino acids solution (B6766), 5 µL/mL minimum essential medium (MEM) nonessential amino acids solution (M7145), citrate (0.1 µg/mL), myo-inositol (0.5 µg/mL), and 6 mg/mL Bovine serum albumin (BSA) (A3311) with or without 0.1% (v/v) FCS (F4135), under mineral oil. In vitro culture was carried out in groups ($n = 35$ to 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 postinsemination) good quality morulae, early blastocysts and blastocysts were cultured individually in 12 µL mSOF with 0.5 mg/mL polyvinyl-alcohol (**PVA**, P8136) with or without recombinant hepatoma-derived growth factor (**HDGF**), under mineral oil for 24 h. On day 7 (168 h postinsemination), expanding and fully expanded blastocysts were transferred fresh or were frozen or vitrified. Embryo cryopreservation procedures were described in detail in Gómez et al. (2020c). Exceptionally, day 7 early blastocysts and blastocysts derived from day 6 morulae were individually re-cultured again for 24 h with fresh medium and those that reached the fully expanded blastocyst stage on day 8 were frozen or vitrified. The remaining embryos were discarded.

In France, two sets of $n = 29$ and $n = 30$ embryos were produced in vitro. Out of them, a set of $n = 29$ embryos was produced as published (Janati Idrissi et al., 2021). Briefly, COCs were washed three times in embryo flushing media (Euroflush, IMV Technologies, France) and matured in TCM199 supplemented with 10% FCS (v/v), 10 µg/mL pFSH/pLH, 1 µg/mL 17β-estradiol, 5 ng/mL epidermal growth factor and 5 µg/mL gentamicin for 22 h at 38.5 °C in a 5% CO_2 humidified atmosphere. Oocytes were fertilized with commercial semen from a Holstein bull with proven fertility (GIAGI, FR1532181070, Evolution Cooperative, France). Motile spermatozoa were separated by a Percoll gradient. Oocytes and spermatozoa (10^6 cells/mL) were co-incubated in 500 µL of Fert-TALP containing 10 µg/mL heparin, 6 g/L BSA, 20 µM penicillamine, 10 µM hypotaurine, 1 µM epinephrine, and 20 µM sodium pyruvate for 18 h at 38.5 °C in a 5% CO_2 high humidified atmosphere. Cumulus cells were detached by pipetting. Zygotes were washed twice and cultured in group in 30 µL micro-drops of SOF (Minitüb, Gmbh, Germany) supplemented with 1% estrus cow serum, 2% MEM 100×, 1% BME 50×, 0.33 g/L Na-Pyruvate and 6 g/L fatty-acid-free BSA covered with mineral oil (Liquid Paraffin, Origio, Måløv, Denmark) at 38.5 °C in a humidified atmosphere containing 5% O_2, 5% CO_2, and 90% N_2. On day 7, fully expanded blastocysts were frozen as described (Janati Idrissi et al., 2021). A second group of $n = 30$ embryos was produced in vitro following a not yet disclosed experimental procedure. This group of embryos was considered an independent replicate for ET dates and embryo production conditions.

Animal management and embryo transfer to recipients

In Spain, animals were housed in an experimental herd (Centro de Biotecnología Animal, SERIDA, Deva), and were selected, managed, and fed in a uniform manner throughout a 9-yr period. Animal management involves a combination of indoor and outdoor pasture feeding systematically implemented to maintain a body condition score between 2.5 and 3.5 (scale ranging from 0 to 5). Comprehensive details regarding animal diets and management practices were described in previous studies (Gómez et al., 2020b, 2020d). Briefly, recipient heifers from Holstein, AV, Asturiana de la Montaña (AM), and their crossbreeds were synchronized in estrus using an intravaginal progestogen device (PRID Alpha; CEVA, Barcelona, Spain) for 8 to 11 d, followed by an injection of a prostaglandin F2α analogue (Dynolitic, Pfizer, Madrid, Spain) 48 h prior to progestogen removal. Recipients for ET were selected based on observation of standing estrus on day (–1) and on day 0—expected estrus—by experienced carekeepers 2 to 3 times per day, and heat monitoring with an automated sensor system (Heatphone, Medria, Humeco, Huesca, Spain). When estrus signs were not evident, progesterone (P4) levels were used to select recipients, with P4 fold change day 7/day 0 > 8, along with day 7 P4 values > 3.5 ng/mL. Prior to ET, all recipients underwent clinical examination for the detection of a healthy CL in one of the ovaries by ultrasonography. Embryo transfer to recipients was performed nonsurgically under epidural anesthesia into the uterine horn tip ipsilateral to the CL detected, at a fixed time 9 d + 4 to 6 h after progestogen removal. The animals were transferred with fresh, frozen and vitrified embryos up to 5 times if nonpregnant in previous ETs. Pregnancy was diagnosed by ultrasonography on days 40, 62, and monitored until birth, and ETs were repeated after 30 to 60 d from the former nonpregnant diagnosis.

In France, three rounds of ET (December 2021 to March 2022) were performed on recipients housed in an experimental

farm in a free stall barn. Animals were fed with straw ad libitum and supplemented with 5 kg concentrate per day (Floria, Sanders, Bruz, France) distributed twice over the day. Heifers were subjected to estrus synchronization using an intravaginal progesterone-releasing device (Prid Delta, 1.55 g, Ceva, Libourne, France) followed by 2 mL intramuscular injection of a prostaglandin F2α analogue (Estrumate, MSD Santé Animale—Intervet, France; equivalent to 0.5 mg cloprostenol) 8 to 10 d later. The device was removed 24 h after cloprostenol injection. Estrus was detected approximately 40 h after intravaginal device removal using activity and rumination monitoring systems (Heatime, Evolution XY, France) and by observation of standing estrus. Ovarian ultrasound exams were performed 5 and 7 d after the induced estrus to assess the CL side.

Blood plasma collection and processing

Blood was collected from the coccygeal vein of recipients in EDTA-vacuum tubes at a fixed time (10:00 a.m.) at estrus (day 0) and 4 to 6 h prior to ET (day 7). The blood tubes were then refrigerated at 4 °C and centrifuged at 2,000 × *g*. Supernatant plasma layer was separated and stored at −150 °C until P4 and Nuclear Magnetic Resonance ($1H^+$NMR) analysis.

For measurement of plasma P4, an ELISA test was used (EIA-1561, DRG Diagnostics, USA), which operates on a 0 to 40-ng/mL scale. The sensitivity of the test started at 0.045 ng/mL, demonstrating less than 1% cross-reactivity with steroids other than P4. The variation coefficients of inter- and intra-assay were 7% and 6%, respectively.

All plasma samples were collected from nulliparous heifers, but eight samples were taken from recipients that had calved once; these cows were dry (i.e., they were not in lactation). In a previous study, we did not observe differences in metabolite concentration between primiparous and nonlactating cows and heifers on day 0 and day 7 (Gómez et al., 2020d). Two plasma samples were collected from all ETs, but one plasma sample at day 0 and another at day 7 from a fresh ET were not obtained.

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

Plasma samples were thawed at room temperature for 5 to 10 min, and a volume of 300 µL was mixed with 300 µL of chloroform–chilled methanol and 300 µL of chilled methanol. After vortexing, the solution was kept at −20 °C for 30 min. The polar phase was separated by centrifugation at 15,000 × *g* and 4 °C for 10 min. Then, the sample was evaporated in a Speedvac (ThermoScientific) at 35 °C and stored at −20 °C.

For $1H^+$NMR, 200 µL of 0.2 M potassium phosphate buffer in deuterium oxide (D_2O) serving as a field frequency lock (pH 7.4 ± 0.5) and 10 µL of 3.2 mM trimethylsilylpropanoic acid (**TSP**), used as a chemical shift reference, were added to the dried plasma samples. The resulting mixture was then transferred into conventional 3-mm $1H^+$NMR tubes.

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

Spectra were assigned using ChenomX 7.1 software (ChenomX, Edmonton, Canada), an in-house database, and the Livestock Metabolome Database (LMDB http://lmdb.ca/) (Goldansaz et al., 2017). Metabolite quantification was performed using the TSP signal as a reference with a known concentration ([TSP] = 152 µM).

Experimental design and statistical analysis

Randomization was achieved by incorporating different batches of recipients from different breeds across two countries over a 9-yr period, which were transferred with IVP embryos derived from different oocyte sources, and fertilized with different bulls and bull breeds. Moreover, embryos were cultured in five different culture systems and were transferred as either fresh or cryopreserved, using two cryopreservation methods, freezing and vitrification. Statistical analyses were conducted using Metaboanalyst 5.0 (Pang et al., 2021) and GraphPad Prism 9.0 (GraphPad Software, Boston, Massachusetts).

Metabolomics for AOS in the whole herd: determination of metabolic plasma baseline

We first tested the metabolic asymmetry in the recipient's plasma collected on days 0 and 7 in accordance with the activity of the ovarian side involved. Thus, a metabolite baseline of the entire herd of recipients selected for ET was obtained disregarding embryonic and pregnancy factors.

The AOS (LO or RO) was analyzed in $n = 478$ estrus cycles. For metabolomic analysis, we had available a subset of plasma samples collected on day 0 ($n = 366$) and day 7 ($n = 367$) from animals that served as embryo recipients in the experimental herd. Metabolite measurements were performed by NMR in three analytical replicates (sample batches). For multivariate analysis, when data were not normally distributed, metabolite concentrations were normalized by auto-scaling, which consisted of mean-centering and dividing by the standard deviation of each variable. Differences in metabolite levels were evaluated in accordance with the day of blood sample collection (days 0 and 7) and in terms of changes in concentration from days 0 to 7 (expressed as day 7/day 0 ratio). Differences were considered to be significant ($P < 0.05$) after mixed generalized models (analysis for metabolite concentration as LSM ± SEM); stringent statistical requirements were applied with parametric and nonparametric analysis in both models. Parameters (covariates) analyzed included: AOS, country (Spain, France), year, season, number of ETs repeated in the same recipients (1 to 5), NMR replicate, parity (heifer or dry cow), recipient breed (Holstein, AV, AM, crossbred), and recipient age (linear and quadratic differences with the age herd average).

Concentrations of P4 were also compared between recipients pregnant to term and nonpregnant according to the AOS (LO or RO).

Metabolomics in the pregnant-to-term recipient subset: calf sex and ovary side

In this experiment, we hypothesized that embryonic sex and laterality could exert a role in pregnancy establishment in cattle. For this purpose, we selected a subset of $n = 119$ day 7 and $n = 119$ day 0 plasma metabolomes ($n = 91$ from Spain and $n = 28$ from France) from recipients that were transferred

with one embryo (either fresh, frozen, or vitrified). The AOS was determined by CL ultrasonography prior to ET; the pregnant horn side was confirmed at the time of the first pregnancy diagnoses (day 40) but only pregnancies to term were recorded since offspring sex was determined at birth. The chi-square test was used to evaluate birth frequency based on embryonic sex and ovarian laterality. Multivariate and univariate analyses were subsequently performed.

Multivariate study

Predicted estimates were drawn from metabolite concentrations and covariates: calf sex, AOS, year, season, country (Spain, France), NMR analysis batch ($n = 3$), recipient breed, embryo cryopreservation (fresh, frozen, vitrified), individual bull, and recipient age, measured as linear (Ldays) and quadratic (Qdays) differences with the mean herd average at the time of each ET. Subsequently, Orthogonal Partial Least Square-Discriminant Analysis (OPLS-DA) was used as a supervised method that uses class information to maximize the separation between groups. To test the likelihood of overfitting, the discrimination accuracy was parametrized with R2 and Q2 indexes. Within significant OPLS models, features that contributed to classification accuracy were ranked by the variable importance in projection (**VIP**) scores, which estimates the importance of each variable to discriminate within the projection underlying the OPLS-DA analysis. Scores ≥ 1 were considered important.

Univariate study

Metabolite differences in concentration and fold change were considered to be significant either for calf sex, for AOS (presence of CL) or for calf sex*AOS interaction ($P < 0.05$) after mixed generalized models (analysis for metabolite concentration). Covariates whose influence was determined consisted of parameters from the recipient (i.e., calf sex, AOS, year, season, number of ETs performed [1 to 5], NMR replicate, parity [heifer or dry cow], recipient breed [Holstein, AV, AM, crossbred], and recipient age [linear and quadratic differences with the age herd average]) and parameters from the embryo (fertilizing bull, bull breed, embryo culture until day 6 [with or without FCS], embryo culture from days 6 to 7, embryonic stage at the beginning and end of the individual culture, embryonic age [day 7 or day 8] and embryo cryopreservation).

Metabolic pathway study

Two pathway studies were conducted using the pathway analysis tool in Metaboanalyst 5.0 against the Bos taurus pathway library:

Pathway study according to the calf sex or AOS

We analyzed the independent effects of sex and AOS on metabolic enrichment pathways using concentration value estimates from the entire pregnant-to-term recipient dataset. Pathways with values of $P < 0.05$ and FDR < 0.10 were considered to be significantly enriched.

Pathway study for calf sex and AOS interaction

We examined regulated pathways in recipient plasma on a calf sex*AOS interaction basis, with four combinations being analyzed on days 0, 7 and day 7/day 0 (left AOS, male vs. female; right AOS, male vs. female; left AOS male vs. right AOS male; and left AOS female vs. right AOS female). We considered the remaining two combinations (i.e., males in the LO vs. females in the RO, and vice-versa) as noninterpretable and they were excluded.

Results

Metabolomics for AOS in the whole herd: determination of metabolic plasma baseline

The AOS was determined in all recipients that had a detectable CL after estrus synchronization ($n = 478$), resulting in $n = 217$ LO (45.4%) and $n = 261$ RO (54.6%); such a difference was significant ($P < 0.01$).

By mixed generalized models, no metabolite differed in concentration between RO and LO (AOS) on day 0 plasma ($P > 0.10$). In contrast, significant differences ($P < 0.05$) were observed in day 7 plasma for L-methionine, L-phenylalanine, and propionic acid with nonparametric analysis, and dimethylsulfone with parametric analysis (Table 1). Furthermore, seven metabolites (L-lysine, butyric acid, mannose, 1-methylhistidine, 2-hydroxyisobutyric acid, isobutyric acid, and 3-Methyl-2-oxovaleric acid) tended to differ in concentration ($P < 0.10$) with nonparametric and/or parametric analysis (Table 1 and Supplementary Table 1). When the day 7/day 0 ratio was examined, L-methionine, mannose,

Table 1. Metabolites that differed in their plasma concentrations measured on day 7 and as day 7/day 0 ratio when the CL was present in the left or in the right ovary[1]

	Day 7 concentration (mg/L)[1]				Day 7/day 0 ratio[2]			
Metabolite	Right	Left	P[3]	NP[4]	Right	Left	P[3]	NP[4]
L-Methionine	5.285 ± 0.153	5.517 ± 0.155	**0.0759**	0.0083	1.004 ± 0.016	1.047 ± 0.017	**0.0684**	0.0214
L-Phenylalanine				0.0336				
Propionic acid	21.42 ± 0.44	22.28 ± 0.43	**0.0745**	0.0135				
Dimethylsulfone	6.668 ± 0.219	6.419 ± 0.214	0.0417					
Mannose				**0.0846**				0.0289
Sarcosine					1.000 ± 0.024	1.041 ± 0.024	**0.0508**	0.0294
Dimethylamine								0.0491

[1]Data, expressed as LSM ± SEM, obtained from measurements in the whole herd ($n = 192$ from the RO, $n = 175$ from the LO).
[2]Data, expressed as LSM ± SEM, obtained from measurements in the whole herd ($n = 191$ from the RO, $n = 175$ from the LO).
[3]Parametric analysis: Mixed generalized models. Tendencies ($0.05 < P < 0.10$) are given in bold.
[4]Non-parametric analysis: Kruskal–Wallis test. Tendencies ($0.05 < P < 0.10$) are given in bold.

sarcosine, and dimethylamine differed ($P < 0.05$) in concentration between both AOS with nonparametric analysis (Table 1), and 1-methylhistidine tended to differ ($P < 0.10$) with parametric analysis (Supplementary Table 1). Covariates used in each statistical model for day 7 and day 7/day 0 concentration ratio analyses are shown in Supplementary Table 2. No significant effects were observed between the eight cows and heifers used in this study.

Metabolites and P4 concentrations did not show significant correlations on day 0 and day 7 or in the day 7/day 0 ratio. Furthermore, no differences were observed in P4 between the RO and LO. Concentrations of P4, as far as the pregnancy rates to term were considered upon detection of AOS, did not differ ($P = 0.1540$ [experimentwise] Supplementary Table 3). This is in spite of independent predicted differences identified between pregnant-to-term and nonpregnant recipients after subsequent AOS detection and ET in the left horn ($P = 0.0486$), and between recipients pregnant in the left vs. the right horn ($P = 0.0421$).

Metabolomics in the pregnant-to-term recipient subset: calf sex and ovary side

In this experiment, we collected plasma samples from recipients that later carried female calves ($n = 50$; $n = 24$ in LO and $n = 26$ in RO) and male calves ($n = 69$; $n = 26$ in LO and $n = 43$ in RO). The distribution of males and females across the LO and RO significantly differed from the theoretically expected frequencies (50% male and 50% female for each ovary; $P = 0.047$), with the RO showing a higher proportion of male pregnancies.

Multivariate study

Sample classification within the entire pregnant-to-term recipient dataset

The OPLS-DA model was first used to classify the sample value estimates from the entire, pregnant-to-term, recipient dataset. Separation between male and female samples was validated by permutation on days 0, 7 and day 7/day 0 ratio (Fig. 1A, C, and E, respectively). However, although the separation was significant at the three timepoints (empirical P values, Q2: $P < 0.001$; R2Y: $P < 0.001$), partial sample overlapping was observed on day 7, with obvious sample separation on day 0 and separation and tighter clustering with the day 7/day 0 ratios. Indeed, the multivariate model for day 7 was scarcely predictive and overfitted (Q2: 0.113; R2Y: 0.288; Fig. 1C), with divergent R2 and Q2 values. In contrast, day 0 was more predictive and adjusted (Q2: 0.558; R2Y: 0.843; Fig. 1A), while day 7/day 0 had the best fitting (Q2:0.784; R2Y:0.903; Fig. 1E). Importantly, whereas the values of days 0 and 7 were auto-scaled to get close to normal distribution, day 7/day 0 ratios appeared normally distributed and were not transformed prior to OPLS-DA analysis. The top VIP values identified were hippuric acid, mannose, L-phenylalanine and propionic acid on day 0 (Fig. 1B); tryptophane, L-phenylalanine, mannose and dimethylamine on day 7 (Fig. 1D); and tryptophane, oxoglutaric, hippuric acid, and sarcosine on day 7/day 0 (Fig. 1F). Metabolites consistently represented throughout the analyses were hippuric acid and L-phenylalanine, and propionic acid with lower VIP scores.

The OPLS-DA model was next used to separate the AOS. The model was nonsignificant for any day or condition (day 7/day 0) analyzed (not shown as figures).

Sample classification according to the ovary side and calf sex

The interactions between the ovary side and calf sex were analyzed. The OPLS-DA study identified significant metabolite concentration changes on day 0 between recipients carrying male calves in the left vs. the right AOS (Fig. 2A). Differences were also observed between males and females within the same AOS when analyzing the day 7/day 0 concentration ratio (Fig. 2C). Interestingly, sex differences showed the highest predictive value and fitting (i.e., higher and closer Q2 and R2Y values) in the right AOS with day 7/day 0 ratio (Fig. 2C). The values with extreme comparisons (i.e., females in the left AOS vs. males in the right AOS) were significant on day 7 (Fig. 2B) and with day 7/day 0 ratio (Fig. 2C).

VIP scores of metabolites involved in the interactions between AOS and calf sex were identified (Fig. 3). The highest VIP score was obtained within extreme comparisons (i.e., females in the left AOS vs. males in the right AOS) on day 7 (Fig. 3B) and day 7/day 0 (Fig. 3D). On day 0 (Fig. 3A), L-lysine, creatine, and creatinine showed the highest VIP score when comparing males in the LO vs. the RO. On day 7 (Fig. 3B), L-phenylalanine, L-tryptophane, and dimethylamine held the highest VIP values when comparing females in the left AOS vs. males in the right AOS. For the day 7/day 0 ratio, oxoglutaric acid and L-tryptophane were the most represented, both showing the highest VIP score within the three comparisons (males and females in the LO, Fig. 3C; females in the LO vs. males in the RO, Fig. 3D; males and females in the RO, Fig. 3E). The metabolite most represented through the entire analysis was L-tryptophane. Other metabolites widely represented through the analyses, but with lower VIP scores, were L-phenylalanine, oxoglutaric acid, and hippuric acid.

Univariate study

Independent effects of calf sex and AOS

The abundance of metabolites that differed in concentration and FCh between recipients carrying male and female fetuses was higher on day 0 plasma compared to day 7 and the day 7/day 0 ratio (13, 4, and 6 metabolites affected, respectively). As seen in Table 2, on day 0, butyric acid, 2-hydroxyisobutyric acid, mannose, 1-methylhistidine, isobutyric acid, isocaproic acid, and propionic acid fulfilled all statistical requirements ($P < 0.05$) for calf sex regarding concentrations and FCh. L-Lysine also showed significant differences in concentration on day 0, although these did not extend to the FCh. Furthermore, hippuric acid, L-phenylalanine, and L-Isoleucine differed for calf sex in FCh, with tendencies in concentration ($P < 0.10$); this is contrary to 2-oxoisocaproic acid, which differed in concentrations but tended to differ in FCh ($P < 0.10$). L-Methionine showed close to significant differences between male and female calves in concentration ($P = 0.0503$) and FCh values ($P < 0.10$; Supplementary Table 4). Isocaproic acid and hippuric acid were the only metabolites with FCh differences for sex that statistically differed both as adjusted and nonadjusted. Notably, all metabolites that differed on day 0 for calf sex had lower abundance in recipients that carried male fetuses. Besides the above changes dealing with the sex of the calf carried, no metabolite varied significantly between AOS. However, concentration and FCh values of some metabolites that did not differ for fetus sex (L-threonine, L-glutamine, oxoglutaric acid, and sarcosine) tended to differ ($P < 0.10$) based on AOS (Supplementary Table 4).

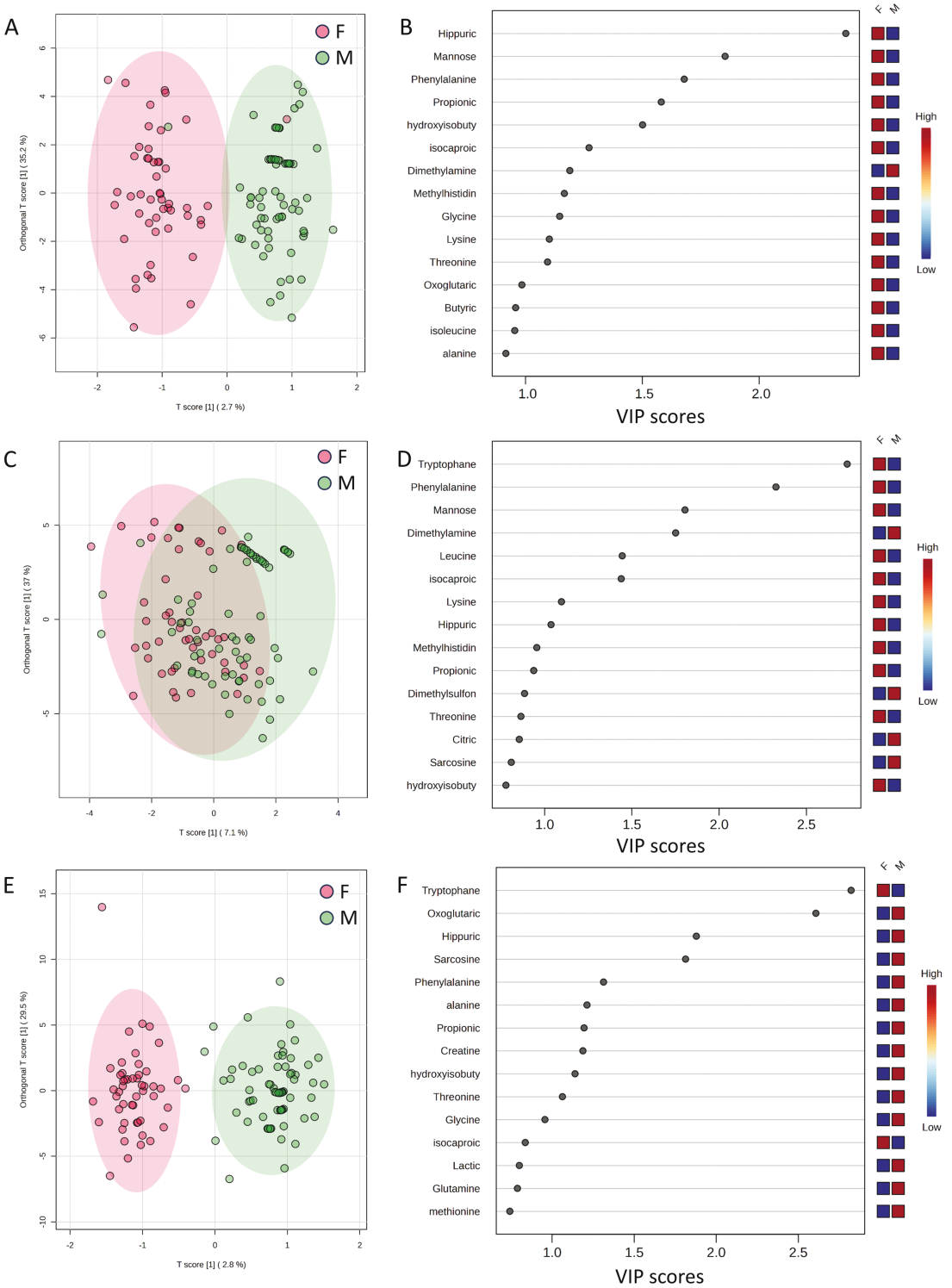

Figure 1. Orthogonal partial least square-discriminant analysis (OPLS-DA) performed with plasma samples collected on day 0 (A), day 7 (C) and day 7/day 0 ratio (E) from recipients delivering male (M) and female (F) calves within the entire pregnant-to-term dataset. Key metabolites identified by OPLS-DA using VIP score for day 0 (B), day 7 (D), and day 7/day 0 ratio (F) are also shown. Colored circles represent 95% confidence intervals, and colored boxes represent the relative metabolite concentrations in the plasma from recipients carrying M and F fetuses.

On day 7 plasma (Table 3), metabolite changes for fetus sex affected sarcosine and citric acid concentration and FCh, although citric acid FCh exhibited a tendency ($P < 0.06$). Creatine concentrations varied significantly between recipients carrying male and female fetuses, and L-phenylalanine tended to differ (not shown in tables: P value = 0.0932; female: 11.94 ± 0.35 mg/L vs. male: 11.15 ± 0.30 mg/L). Differences between AOS were recorded for citric acid, affecting both concentrations and FCh and for 2-hydroxyisobutyric acid, which impacted only FCh and was not affected by calf sex. Interestingly, and in contrast to day 0 findings, the concentration of metabolites that differed on day 7 for calf sex had

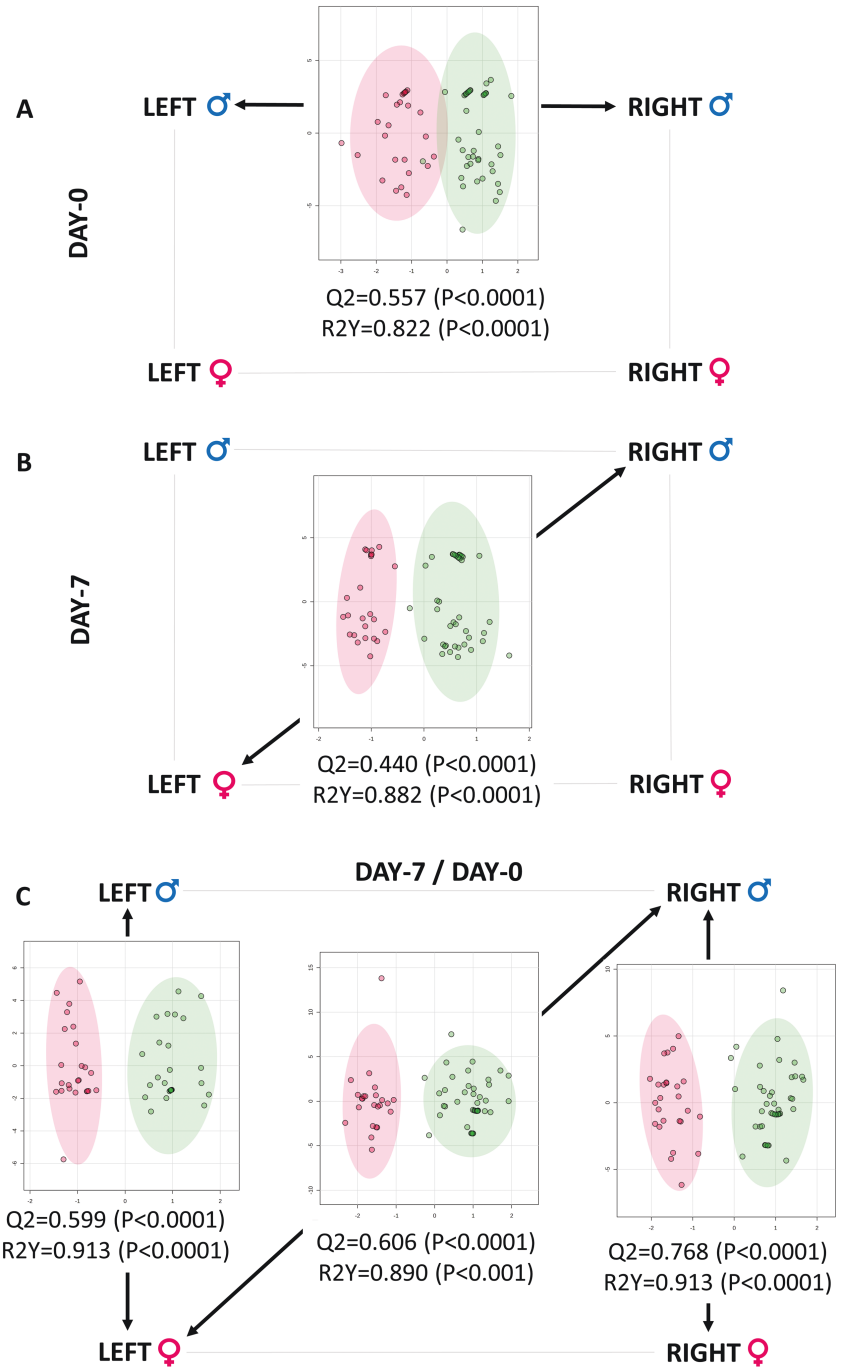

Figure 2. Orthogonal partial least square-discriminant analysis (OPLS-DA) performed with plasma samples collected on day 0 (A), day 7 (B), and day 7/day 0 ratio (C) from recipients in accordance with the AOS (right or left) and the calf sex. Colored circles represent 95% confidence intervals.

a higher abundance in the plasma from recipients carrying male fetuses.

On day 7/day 0 concentration ratio, the analysis can be seen as a snapshot parameter of metabolite concentration evolution through the estrual and early luteal periods in line with the sex carried. Such a dataset showed normal distribution, which allowed the exclusion of data transformation and the use of GLM statistics. As shown in Table 4, metabolites whose day 7/day 0 ratio showed significant ($P < 0.05$) concentration differences between male- and female-fetus carriers were sarcosine, isocaproic acid, isobutyric acid, butyric acid, and propionic acid, while 2-hydroxyisobutyric acid tended to differ (not shown in tables: P value = 0.0592; female: 0.980 ± 0.039 vs. male: 1.070 ± 0.039). No significant differences were found for AOS. In terms of interpretation, FCh was not calculated as it already incorporates a ratio between two values (day 7 and day 0), making an additional fold change calculation redundant.

Covariates used in the statistical models for days 0, 7 and day 7/day 0 ratio analysis are shown in Supplementary Table 5.

Interactions between calf sex and ovary side

day 0 concentrations of metabolites were described in Table 5 in a 2 × 2 factorial design (ovary side × calf sex). Significant

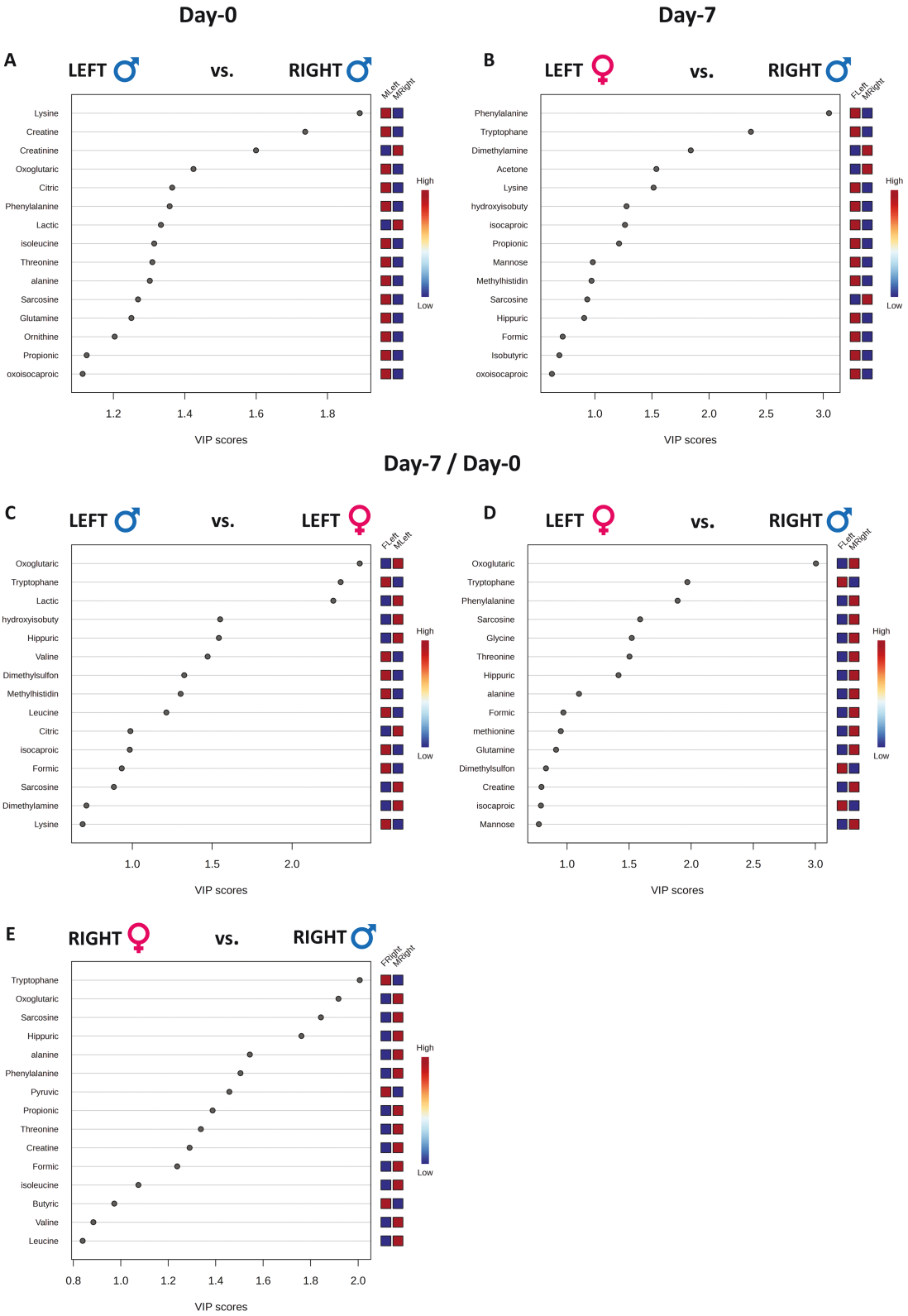

Figure 3. Key metabolites identified by OPLS-DA using VIP score for day 0 (A), day 7 (B), and day 7/day 0 ratio (C, D, and E) plasma samples according to the calf sex and ovary side. Colored boxes represent the relative metabolite concentrations in the plasma from recipients carrying M and F fetuses.

interactions ($P < 0.05$) concerned hydroxyisobutyric acid and propionic acid, with a close to significant trend ($P < 0.06$) for L-lysine, methylhistidine, and hippuric acid. Interestingly, the lowest concentrations of these metabolites were recorded in the RO when male fetuses were carried, except for methylhistidine, which showed the lowest level in the LO when males were carried. Such a pattern was also observed for other metabolites that tended to differ ($P < 0.10$), with butyric and isobutyric acid showing their lowest concentrations in the RO when carrying male fetuses (Supplementary Table 6).

Table 2. Absolute concentrations and logarithm fold change (LogFCh) of metabolites measured by 1H⁺NMR analysis in recipient plasma on day 0 in accordance with the sex of the fetus carried to term

	Concentration (mg/L)[1]			LogFCh	
Metabolite	Female	Male	*P* value	Sex	*P* value
Butyric acid	3.40 ± 0.17	3.01 ± 0.14	0.0185	−0.210	0.0158
Hippuric acid	9.91 ± 0.29	9.17 ± 0.25	**0.0581**	−0.521	0.0115*
2-Hydroxyisobutyric acid	0.52 ± 0.02	0.47 ± 0.02	0.0141	−0.431	0.0022
Mannose	6.16 ± 0.21	5.63 ± 0.19	0.0354	−0.424	0.0472
1-Methylhistidine	9.66 ± 0.42	8.71 ± 0.39	0.0326	−0.256	0.0107
L-Phenylalanine	12.35 ± 0.39	11.32 ± 0.38	**0.0569**	−0.554	0.0153
Isobutyric acid	1.47 ± 0.11	1.36 ± 0.11	0.0050	−0.539	0.0041
Isocaproic acid	4.59 ± 0.22	4.22 ± 0.24	0.0423	−0.144	0.0231*
Isoleucine	14.32 ± 0.39	13.59 ± 0.37	**0.0764**	−0.460	0.0232
L-Lysine	14.96 ± 0.86	13.53 ± 0.86	0.0223		
2-Oxoisocaproic acid	2.40 ± 0.16	2.23 ± 0.15	0.0459	−0.271	**0.0759**
Propionic acid	25.90 ± 1.94	23.27 ± 1.80	0.0011	−0.556	0.0028

[1]Data are expressed as LSM ± SEM. *P* values of tendencies (0.05 < *P* < 0.10) are given in bold.
*Significant when adjusted and non-adjusted.

Table 3. Absolute concentrations and logarithm fold change (LogFCh) of metabolites measured by 1H⁺NMR analysis in day 7 recipient plasma in accordance with the sex of the fetus carried to term, and the effect of the ovary (left or right) bearing the CL

	Concentration (mg/L)[1]				LogFCh		
	Sex		*P* value			*P* value	
Metabolite	Female	Male	Sex	Ovary	Sex	Sex	Ovary
Sarcosine	0.72 ± 0.03	0.79 ± 0.03	0.0088		0.505	0.0069	
Citric acid	42.90 ± 1.10	45.86 ± 0.95	0.0440	0.0171	0.337	**0.0573**	0.0385
Creatine	21.20 ± 1.54	22.82 ± 1.51	0.0425				
2-Hydroxyisobutyric acid							0.0381

[1]Data are expressed as LSM ± SEM. *P* values of tendencies (0.05 < *P* < 0.10) are given in bold.

Table 4. Day 7/day 0 concentration ratio of metabolites that differed between recipients carrying male and female calves to term

	Day 7/day 0 ratio[1]		
Metabolite	Female	Male	*P* value
Sarcosine	0.969 ± 0.027	1.042 ± 0.023	0.0412
Isocaproic acid	1.016 ± 0.040	1.140 ± 0.042	0.0241
Isobutyric acid	0.944 ± 0.045	1.011 ± 0.043	0.0474
Butyric acid	0.889 ± 0.057	1.002 ± 0.044	0.0441
Propionic acid	1.020 ± 0.036	1.105 ± 0.036	0.0484

[1]Data are expressed as LSM ± SEM.

The concentrations of these metabolites were lower in the RO when comparing recipients carrying male and female fetuses (as shown by predicted difference values), disregarding lysine and methylhistidine. Methylhistidine showed particular differences, affecting concentrations between females and males in the LO.

No significant interactions were detected in metabolite concentrations on day 7 between calf sex and AOS. On day 7/day 0 ratio, interactions that were detected as tendencies ($P < 0.10$) included methyloxovaleric acid, pyruvic acid, and L-threonine (Supplementary Table 6). Covariates incorporated in the statistical models analyzing day 0 and day 7/day 0 ratio are shown in Supplementary Table 7.

Metabolic pathway study

Pathway study according to the calf sex or AOS

No pathways were significantly regulated in accordance with the AOS. In contrast, there were six top enriched pathways for calf sex in the whole day 0 recipient plasma (Fig. 4A), three pathways in day 7 plasma (Fig. 4B), and nine enriched pathways in the day 7/day 0 concentration ratios (Fig. 4C). Interestingly, phenylalanine metabolism and phenylalanine, tyrosine, and tryptophan biosynthesis pathways were found enriched at the three timepoints (Table 6).

Pathway study for calf sex and AOS interaction

Pathway analysis based on a calf sex*AOS interaction showed that the combination "right AOS, male vs. female" concentrated the highest variability identified (Table 7). This is explained by the fact that the combination "male LO vs. male RO" only showed two regulated pathways on day

Table 5. Variation of concentrations of blood plasma metabolites on day 0 in accordance with the interaction between the sex of the fetus carried (F: female; M: male) and the ovary (left or right) bearing the CL[1]

		Female		Male		
Metabolite	*P* value[2]	Left	Right	Left	Right	Predicted differences
Hydroxyisobutyric acid	0.0404	0.493 ± 0.023	0.546 ± 0.023	0.491 ± 0.024	0.467 ± 0.020	F-Right vs. M-Right: $P = 0.0045$ F-Right vs. M-Left: $\mathbf{P = 0.0637}$ F-Left vs. F-Right: $\mathbf{P = 0.0905}$
Propionic acid	0.0104	25.97 ± 2.110	25.95 ± 1.994	23.93 ± 1.956	23.01 ± 1.847	F-Right vs. M-Right: $P = 0.005$ F-Left vs. M-Right: $P = 0.0074$ F-Right vs. M-Left: $\mathbf{P = 0.0725}$
L-Lysine	**0.0557**	14.86 ± 0.853	13.86 ± 0.802	13.98 ± 0.867	12.65 ± 0.707	F-Left vs. M-Right: $P = 0.0089$
Methylhistidine	**0.0533**	10.85 ± 1.119	10.27 ± 1.034	9.042 ± 0.997	9.683 ± 1.034	F-Left vs. M-Left: $P = 0.0099$ F-Left vs. M-Right: $P = 0.0484$ F-Right vs. M-Left: $\mathbf{P = 0.0621}$
Hippuric acid	**0.0538**	9.450 ± 0.431	10.42 ± 0.418	9.407 ± 0.415	8.930 ± 0.323	F-Right vs. M-Right: $P = 0.006$ F-Right vs. M-Left: $\mathbf{P = 0.091}$

[1]All data are expressed as LSM ± SEM (mg/L).
[2]*P* value: Generalized Mixed Procedures. *P* values of tendencies ($0.05 < P < 0.10$) are given in bold.

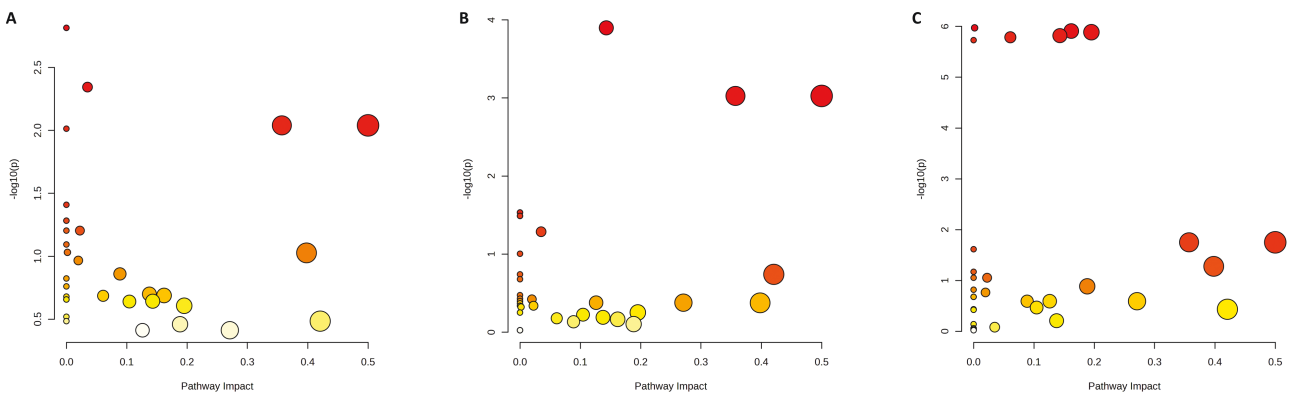

Figure 4. Metabolic pathways differentially affected by the sex of the fetus carried to term in plasma samples collected on day 0 (A), day 7 (B), and day 7/day 0 ratio (C) from all recipients transferred with an embryo. The metabolome view shows all matched pathways in accordance with the *P* values from the pathway enrichment analysis and pathway impact values (dot size) from the pathway topology analysis.

0 (Lysine degradation and Biotin metabolism; both with *P* value = 0.005 and FDR = 0.0854; not shown in tables), and the combinations "left AOS, male vs. female" and "female LO vs. female RO" showed scarce significantly regulated pathways, all of them with nonsignificant FDRs (>0.10), or no pathways were identified. The strong variability concentration in RO male vs. female can be explained as the top pathways regulated between males and females across the entire dataset on days 0, 7 and day 7/day 0 (Table 6) were also recorded between males and females when the AOS was the right (Table 7). Similar to the pathways differentially regulated for calf sex, phenylalanine metabolism and phenylalanine, tyrosine, and tryptophan biosynthesis were consistently identified across the three timepoints between males and females in the right AOS.

Importantly, as expected from the above univariate and multivariate analysis, day 0 plasma was actually supportive of pathway regulation within "right AOS male vs. female" (Table 7). Indeed, day 0 plasma showed 20 pathways regulated, with 9 of them recorded in its homolog calculation on day 7/day 0 ratio. In contrast, day 7 plasma showed 8 significantly activated pathways, out of which five were common with pathways identified on day 0, but only 3 were shared with those obtained in the day 7/day 0 ratio analysis.

Discussion

The present study provides the first evidence of metabolic differences on day 0 (estrus) and day 7 (prior to ET) in cattle bearing left and right AOS and carrying male or female fetuses. Specific metabolites present in plasma before ET are predictive of pregnancy and birth (Gómez et al., 2020a, 2020b; Gomez et al., 2021; Gimeno et al., 2023). Understanding the ovary-side influences on the sex ratio can improve the efficiency of ET when embryos fertilized with sex-sorted spermatozoa are used. Thus, concentrations of a pregnancy biomarker could be refined in accordance with the ovary side and the presumed sex of the embryo to select the most favorable recipients.

In the entire herd of this study, L-phenylalanine, L-methionine, propionic acid, and dimethylsulfone on day 7 plasma differed in their concentrations between ovary sides, with no changes recorded on day 0 and no pathways regulated in accordance with the AOS at any timepoint. When the

Table 6. Pathways differentially affected in plasma on days 0, 7 and day 7/day 0 ratio between male and female calves carried

Metabolic pathway	Raw *P*	FDR	Impact
Day 0			
Fructose and mannose metabolism	0.0015	0.026	0
Amino sugar and nucleotide sugar metabolism	0.0015	0.026	0
Galactose metabolism	0.0045	0.051	0.035
Phenylalanine, tyrosine, and tryptophan biosynthesis	0.0091	0.055	0.5
Phenylalanine metabolism	0.0091	0.055	0.357
Propanoate metabolism	0.0097	0.055	0
Day 7			
Tryptophan metabolism	0.0001	0.004	0.143
Phenylalanine, tyrosine, and tryptophan biosynthesis	0.0009	0.011	0.5
Phenylalanine metabolism	0.0009	0.011	0.357
Day 7/day 0			
Lipoic acid metabolism	1.07E–06	1.06E–05	0.002
Alanine, aspartate and glutamate metabolism	1.23E–06	1.06E–05	0.162
Citrate cycle (TCA cycle)	1.30E–06	1.06E–05	0.195
Tryptophan metabolism	1.52E–06	1.06E–05	0.143
Arginine biosynthesis	1.64E–06	1.06E–05	0.061
Butanoate metabolism	1.86E–06	1.06E–05	0
Phenylalanine, tyrosine, and tryptophan biosynthesis	0.0177	0.075	0.5
Phenylalanine metabolism	0.0177	0.075	0.357
Selenocompound metabolism	0.0243	0.092	0

metabolite abundances were measured as dynamic changes (i.e., day 7/day 0 concentration ratio), L-methionine, sarcosine, mannose, and dimethylamine were identified, suggesting that the differences between the LO and RO may also reflect the involvement of asymmetry regulation in the transition from estrual to luteal phases. Despite these observations, there were no differences in P4 concentration between the left and right AOS in the present study, and no significant correlation was found between metabolite concentrations and P4 levels, as seen in Gómez et al. (2020d). In a previous study, Trigal et al. (2014) observed higher P4 concentrations in recipients diagnosed as pregnant on days 38 to 53 after ET when the LO was the AOS. However, despite the predicted differences observed between pregnant-to-term and open recipients within the LO in this study, the lack of experimentwise significance in our study precluded the drawing of conclusions regarding P4 levels.

In the framework of the pregnant-to-term recipients, the sex distribution within the RO was biased towards males, in agreement with previous studies with artificially inseminated cows (Giraldo et al., 2010; Karamishabankareh et al., 2015) and natural mount, although not after transfer of in vivo collected embryos (Hylan et al., 2009). Moreover, metabolic differences in recipients based on the sex of the fetus were more pronounced on day 0 than on day 7. Thus, more significantly regulated metabolic pathways for calf sex were identified on day 0 than on day 7. Notably, all metabolites that differed on day 0 for calf sex were higher in female-carrying recipients and were not affected by AOS, consistent with the absence of differences reported on day 0 with AOS as a sole criterion. The amino acids L-lysine, L-phenylalanine, isoleucine, and 1-methylhistidine varied with calf sex. Male and female embryos exhibit distinct preferences towards certain amino acids during early development, notably phenylalanine (Sturmey et al., 2010; Rubessa et al., 2018; Gimeno et al., 2022). Since the concentration of several amino acids in plasma, including phenylalanine, isoleucine, and lysine, is positively related to their concentration in the oviduct and uterine fluids (**UF**) (Hugentobler et al., 2007), differences in metabolism due to embryonic sex might account for the disparities we observed in our research. Phenylalanine metabolism and phenylalanine, tyrosine, and tryptophan biosynthesis pathways were enriched for calf sex, especially within the right AOS. Interestingly, in the presence of a female embryo, cultured epithelial endometrial cells produce more phenylalanine than those challenged by a male embryo (Muñoz et al., 2020). Phenylalanine increases in the pregnant UF from days 12 to 18 (Groebner et al., 2011) and, by day 19, together with isoleucine and lysine, in the UF of recipients exposed to a male conceptus (Forde et al., 2016). Although we cannot extend our observations on day 0 to later stages, phenylalanine seems to be involved in embryonic sexual dimorphism in utero.

We also observed significantly higher levels of propionic, isobutyric, and butyric acid in day 0 plasma from female-carrying recipients, but a higher day 7/day 0 ratio in male-carrying recipients. These volatile, short-chain fatty acids are produced by fermentation in the rumen. Butyric acid is metabolized into ketone bodies, which can be used as an energy source, while propionic acid acts as a precursor for glucose production in the liver (Danfaer, 1994; Baldwin and Connor, 2017). Glucose concentration in plasma is related to its levels in oviduct fluid (Hugentobler et al., 2008). In vitro, elevated glucose levels shift the sex ratio toward males (Kimura et al., 2005; Rubessa et al., 2011), although female embryos seem to survive better in a highly glucose-rich environment (Jiménez et al., 2003). Similarly, cows with greater body condition between calving and conception are more likely to

Table 7. Pathways differentially affected in plasma on days 0, 7 and day 7/day 0 between recipients carrying male and female fetuses within the right AOS

Metabolic pathways	Raw *P*	FDR	Impact
Day 0			
Propanoate metabolism	0.0009	0.01	0
Lysine degradation	0.0014	0.01	0
Biotin metabolism	0.0014	0.01	0
Fructose and mannose metabolism	0.0016	0.01	0
Amino sugar and nucleotide sugar metabolism	0.0016	0.01	0
Galactose metabolism	0.0017	0.01	0.035
Valine, leucine, and isoleucine biosynthesis	0.0055	0.024	0
Glycine, serine, and threonine metabolism	0.0055	0.024	0.398
Phenylalanine, tyrosine, and tryptophan biosynthesis	0.0101	0.033	0.5
Phenylalanine metabolism	0.0101	0.033	0.357
Valine, leucine, and isoleucine degradation	0.0108	0.033	0.02
Histidine metabolism	0.0125	0.035	0
Glutathione metabolism	0.0146	0.037	0.089
Arginine biosynthesis	0.0153	0.037	0.061
Butanoate metabolism	0.0172	0.039	0
Arginine and proline metabolism	0.019	0.04	0.188
Lipoic acid metabolism	0.0223	0.045	0.002
Primary bile acid biosynthesis	0.028	0.049	0.022
Porphyrin metabolism	0.028	0.049	0
Selenocompound metabolism	0.0288	0.049	0
Day 7			
Phenylalanine, tyrosine, and tryptophan biosynthesis	0.0021	0.035	0.5
Phenylalanine metabolism	0.0021	0.035	0.357
Tryptophan metabolism	0.0081	0.066	0.143
Lysine degradation	0.0097	0.066	0
Biotin metabolism	0.0097	0.066	0
Fructose and mannose metabolism	0.017	0.077	0
Amino sugar and nucleotide sugar metabolism	0.017	0.077	0
Galactose metabolism	0.0181	0.077	0.035
Day 7/day 0			
Lipoic acid metabolism	0.0002	0.003	0.002
Tryptophan metabolism	0.0002	0.003	0.143
Glycine, serine, and threonine metabolism	0.0006	0.007	0.398
Alanine, aspartate, and glutamate metabolism	0.0012	0.01	0.162
Citrate cycle (TCA cycle)	0.0016	0.011	0.195
Butanoate metabolism	0.0044	0.025	0
Selenocompound metabolism	0.012	0.058	0
Arginine biosynthesis	0.0166	0.071	0.061
Phenylalanine, tyrosine, and tryptophan biosynthesis	0.0247	0.079	0.5
Phenylalanine metabolism	0.0247	0.079	0.357
Arginine and proline metabolism	0.0257	0.079	0.188
Tyrosine metabolism	0.0298	0.084	0
Propanoate metabolism	0.0359	0.094	0

produce male offspring (Roche et al., 2006). In the context of ovary side effects, glucose plays a crucial role in supporting the release of the gonadotropin-releasing hormone (GnRH), and thus the luteinizing hormone (**LH**) (Ohkura et al., 2004). Propionic acid, whose abundance in this study responded to interactions between ovary and calf sex, increases the secretion of LH and insulin, which influences follicular dynamics (Rutter et al., 1983; DiCostanzo et al., 1999). Therefore, it is plausible that the observed variations in propionic levels in response to calf sex and AOS may be attributed to their downstream effects on glucose metabolism. Interestingly, propionic acid is a potential estrus biomarker in cattle (Sankar and Archunan, 2008, 2011), whose concentrations in feces markedly increase from 24 to 12 h before ovulation (Mozūraitis et

al., 2017). Since artificial insemination carried out late in the estrus can increase the male/female ratio at birth (Martinez et al., 2004), an involvement of propionic acid in such effects may occur. Our study points out that propionic acid, which predicts pregnancy on day 62 and birth (Gómez et al., 2020a, 2020b), is also a potential biomarker to differentiate recipients more likely to carry either a male or female embryo.

Hippuric acid, a pregnancy biomarker (Gomez et al., 2021), also changed with the calf sex. In the rumen, phenolic compounds are transformed into phenyl acids, from which hippuric acid is produced in the liver (Cremin et al., 1995). In our study, hippuric acid was affected by the season, consistent with the April to late October grazing period on pasture in our animals. This seasonal dependence could limit the use of this metabolite as a biomarker.

On day 7, sarcosine, creatine, and citric acid concentrations were increased in recipients who carried male calves. Sarcosine and creatine are intermediate products in the glycine, serine, and threonine metabolism as well as arginine and proline metabolism pathways, which were regulated by calf sex in our study, particularly in the right AOS. The presence of an embryo lowers the sarcosine levels in the UF of cows by day 7 (Sponchiado et al, 2019). Since glycine levels in plasma correlate with those in UF (Hugentobler et al., 2007), and male embryos deplete more glycine than females from the culture medium (Sturmey et al., 2010), the sex-based changes observed with sarcosine can be linked to activity in the glycine pathway. In contrast, female embryos deplete citrate from the culture medium (Gimeno et al., 2022), and the sex ratio of embryos cultured in citrate-supplemented medium is skewed toward females (Rubessa et al., 2011). However, we observed higher citric acid and creatine in male-carrying recipients. This would suggest higher energy demand by male embryos, possibly due to their faster early development (Avery et al., 1992; Estrella et al., 2024), since creatine and citric acid can be used for energy production (Scantland et al., 2014; Gardner and Harvey, 2015).

Discrimination by calf sex was observed at each ovary side level, being more robust in the right AOS than in the left. Furthermore, such separation was supported by concentrations of some metabolites varying with the sex of the fetus and the ovary bearing the CL. On day 0, such metabolites were hydroxyisobutyric acid, propionic acid, L-lysine, methylhistidine, and hippuric acid; all showed the lowest concentrations when male fetuses were carried, especially when the AOS was the RO, which was consistently reflected in the corresponding regulated metabolic pathways. Thus, a different metabolic profile of recipients prior to or at the time of ET could operate in favor of embryos with specific sex, which in turn will develop and implant. Some studies have observed bilateral asymmetry in offspring sex in cattle (Vazquez et al., 1993; Hylan et al., 2009; Giraldo et al., 2010; Karamishabankareh et al., 2015), while others do not (Gharagozlou et al., 2013; Borges et al., 2017); however, the mechanism underlying sex laterality is currently unknown. It has been suggested that ovaries could secrete different compounds into their ipsilateral uterine horn that favor the implantation of male or female embryos (Clark et al., 1994), which may fit in with our findings. Thus, differences in the development or sensitivity to environmental conditions of male and female embryos within the female reproductive tract could lead to a selective loss of embryos of one sex before implantation (Rosenfeld and Roberts, 2004). The UF collected on day 8 by uterine lavage from the left and right uterine horns differs in the abundance of nine proteins and hexoses (Trigal et al., 2014), and the UF obtained from recipients pre-exposed to either male or female IVP embryos differs in 23 proteins and fructose levels (Gómez et al., 2013). Thus, while the ovary of origin appears to impact the sex ratio of embryos (Grant and Irwin, 2005; Grant et al., 2008; Hylan et al., 2009), it is still unclear whether and how the uterus contributes to embryonic sex selection. In accordance with this, some authors suggest that placentas from early female fetuses exhibit greater adaptability and therefore higher protection against adverse uterine environments, improving female survival (Aiken and Ozanne, 2013; Hufnagel and Aiken, 2023). These adaptations of females would require a higher energy investment by the mother (Aiken and Ozanne, 2013), which fits with the higher concentration of most plasma metabolites in recipients carrying female fetuses in this study.

We should assume a number of not-yet-confirmed postulates and limitations. One is that embryo selection by recipients on a sex and ovary side basis should involve particular conditions present in the uterine horn at the time of ET. Second, despite concentrations between blood and the genital tract fluids correlating in a number of metabolites (Hugentobler et al., 2007, 2008), others do not show such a correlation, and we do not have evidence of such correlations for some relevant metabolites in our study. Third, the progress of pregnancies in terms of specific losses of male or female fetuses was not monitored and perhaps an early diagnosis of sex may give another picture of our findings.

Conclusions

Our work provides evidence that some metabolites identified in the plasma of recipients are influenced by the AOS and inform on the propensity of such a recipient to carry a male or female fetus to term. Whether the observed changes respond to mechanisms or merely reflect differences based on anatomical facts (i.e., associated with differences in the ovary size and contents) continues to be an intriguing question. The most remarkable differences in metabolite concentrations were observed on day 0 plasma, particularly from the right AOS, which was also responsible for an altered sex ratio in the pregnancies carried. In the same way, enrichment in metabolic pathways associated with the calf sex was more pronounced when the AOS was the one on their right, suggesting inherent ovary-specific mechanisms. However, no pathways were significantly enriched according to the AOS. Our study provides new knowledge in cattle asymmetry and suggests that, in future biomarkers studies, the ability of certain biomarkers to predict pregnancy could be improved by considering laterality effects.

Supplementary Data

Supplementary data are available at *Journal of Animal Science* online.

Acknowledgments

This project was partially funded by the European Commission's Horizon 2020 Research and Innovation Program via the GLOMICAVE project under grant agreement

No. 952908. Fondo Europeo de Desarrollo Regional (FEDER). Ann Hannigan-Breen is thanked for the English review of the manuscript.

Conflict of interest statement.

The authors declare that they have no competing interests.

Authors' contributions

Isabel Gimeno (Conceptualization, Data curation, Formal Analysis, Investigation, Software, Visualization, Writing—original draft, Writing—review & editing), Pascal Salvetti (Conceptualization, Funding acquisition, Methodology, Supervision, Writing—review & editing), Susana Carrocera (Investigation, Resources, Visualization, Writing—review & editing), Julie Gatien (Data curation, Formal Analysis, Investigation, Resources, Software, Writing—review & editing), Daniel Le Bourhis (Investigation, Writing—review & editing), and Enrique Gómez (Conceptualization, Data curation, Formal Analysis, Funding acquisition, Methodology, Software, Supervision, Visualization, Writing—original draft, Writing—review & editing). All authors read and approved the final manuscript.

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