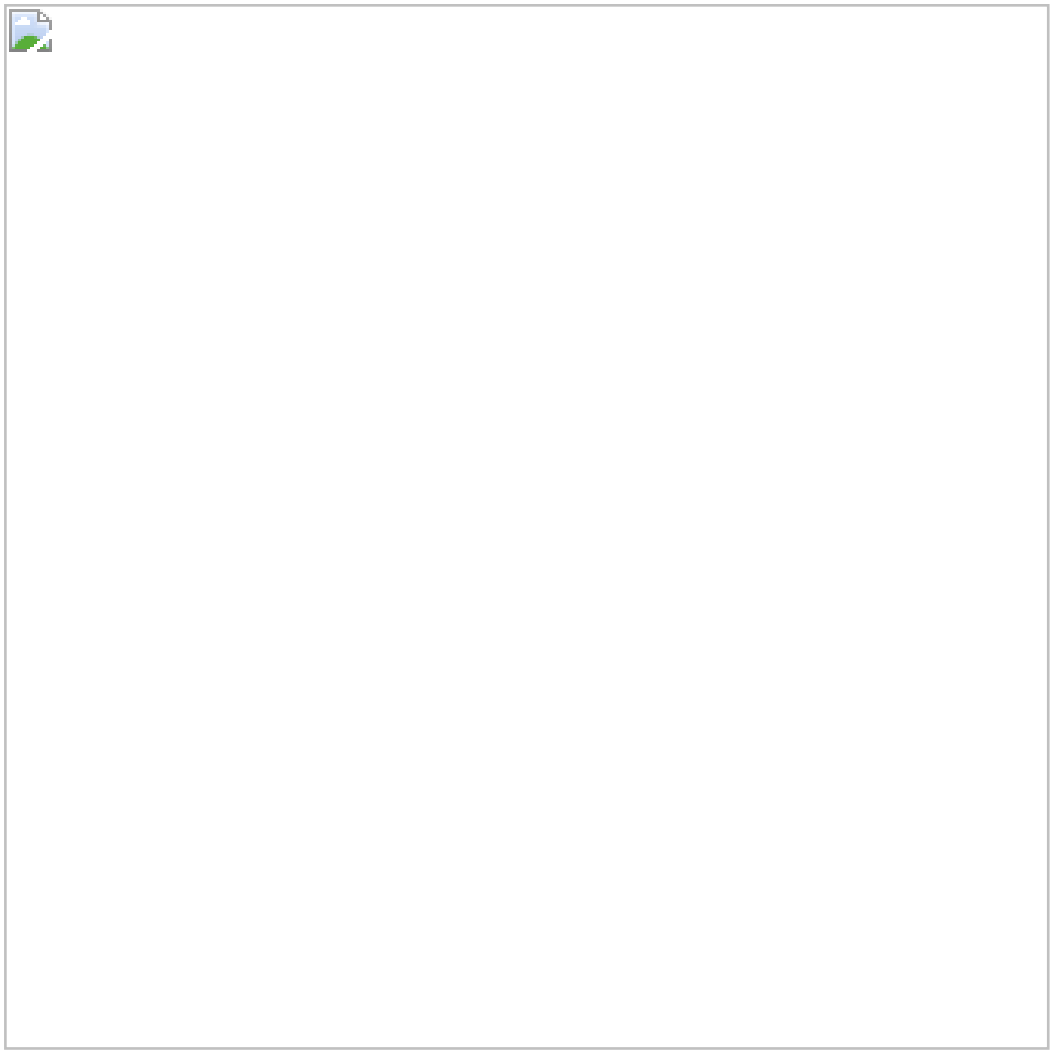

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Research Article

Effects of the donor factors and freezing protocols on the bovine embryonic lipid profile†

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Abstract

Embryo lipid profile is affected by in vitro culture conditions that lead to an increase in lipids. Efforts have been made to optimize embryo lipid composition as it is associated with their quality. The objective of this study was to evaluate whether the diet supplementation of donor cows (n-3 or n-6 polyunsaturated fatty acids), or the slow freezing protocols (ethylene glycol sucrose vs. glycerol-trehalose), or the physiological stage of the donor (nulliparous heifers vs. primiparous lactating cows) may impact the bovine embryo lipid profile. Lipid extracts of 97 embryos were individually analyzed by liquid chromatography-high resolution mass spectrometry, highlighting 246 lipids, including 85% being overabundant in cow embryos compared to heifer embryos. Among 105 differential lipids, 72 were overabundant after ethylene glycol sucrose protocol, including a single glycerophosphate PA(32:1) representing 27.3% of the significantly modulated lipids, suggesting that it is degraded when glycerol-trehalose protocol is used. No lipids were different according to the n-3 or n-6 supplementation of the donor cows. In conclusion, the embryonic lipid profile was mainly affected by the physiological stage of the donors and the slow freezing protocols. The overabundance of lipids in lactating cow embryos and the resulting lower quality of these embryos are consistent with the lower pregnancy rate observed in cows compared to heifers. Unlike glycerol-trehalose protocol, ethylene glycol sucrose freezing allowed to preserve glycerophospholipids, potentially improving the slow freezing of in vitro-produced embryos. Further studies are required to modulate embryo quality and freezability by modulating the lipidome and by integrating all stages of embryonic production.

Keywords: cryopreservation, embryo quality, lipidomic, N-3 PUFA, in vitro embryo production, bovine

Introduction

Lipid composition is an important factor in ensuring proper embryonic development and thus achieving high pregnancy rates [1, 2]. Moreover, it has been shown that the embryonic origin (vivo or vitro) as well as embryo freezing had a significant impact on the lipid profile of bovine embryos [3]. In fact, in vitro-produced embryos seemed to present an overabundance of oxidized glycerophospholipids and triglycerides, while the in vivo ones had an overabundance of phospholipids. An overabundance of phospholipids, particularly phosphatidyl choline, appears to be considered as a positive biomarker for successful cryopreservation [4]. These differences contribute to the lower quality of in vitro-produced embryos [5]. Indeed, an in vitro culture system induces lipid droplet accumulation in blastomeres. This phenomenon is responsible for a higher cryo-susceptibility [6, 7] and it also stimulates the oxidative metabolism and thereby the oxidation of fatty acids (FA) derived from the breakdown of triglycerides by the addition of growth factors hormones and serum during embryo culture [8, 9]. Preserving phospholipids therefore seems to be of interest to improve embryo quality.

Given the importance of the embryonic lipid profile, several authors have tried to modulate it by supplementing the donor cow diet that is modifying PUFA intake in cows. It has already been demonstrated that n-3 PUFA supplementation has beneficial effects on dairy cow reproduction [10]. In fact, addition of n-3 PUFA (alpha-linolenic acid ALA, eicosapentaenoic acid EPA, and docosahexaenoic acid DHA) in the cow diet led to 8% reduction in the embryo mortality rate [11] and tended to increase the conception rate by reducing prostaglandin $F_{2\ alpha}$ ($PGF_{2\ alpha}$) levels and therefore pregnancy losses [12, 13]. Recently, Freret et al. [14] demonstrated that a short period of supplementation with n-3 PUFA in cow diet was sufficient to induce variations in plasma and follicular fluid FA composition. Such n-3 PUFA supplementation led to changes in the oocyte phospholipid composition, particularly in phosphatidylcholine and sphingomyelin, which are major components of cell membranes and involved in membrane structure

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stabilization [14, 15]. Indeed, phospholipid composition, and especially the level of unsaturation in glycerophospholipid and the amount of cholesterol in the membrane, can affect the resistance of membrane during cryopreservation and its elasticity [16, 17]. The higher the membrane fluidity, the better the cryopreservation survival would be [17]. Indeed, during cooling, the molecular motion in the membrane lipid bilayer decreases, allowing interaction between lipid molecules and affecting fluidity and functioning of the membrane. The addition of DHA (1 μM) directly into bovine oocyte maturation medium increased the rates of cleaved embryos and day 7 blastocysts and tended to increase the total cell number per blastocyst [18]. This addition also induced a decrease in lysophosphatidylcholine (LPC), the precursor of lysophosphatidic acid, a change that could explain some of the beneficial effects reported regarding oocyte quality [19].

Cryopreservation of in vitro-produced embryos is a crucial step that is limiting the spread of valuable high-merit animals [20]. The freezing protocol can damage membrane integrity by causing membrane-chilling injuries [21, 22]. In fact, the lipid phase transition, followed by separation, is one of the major causes of cryo-damages in lipid-rich oocytes and embryos [21, 23]. It has also been shown that embryo and oocyte cryopreservation protocols (slow freezing and vitrification) lead to a decrease in the phospholipid and LPC content [3, 24]. However, LPCs are precursors of lysophosphatidic acid, which is a modulator of embryos quality markers, such as insulin-like growth factor 2 receptor and placental associated 8 as well as pluripotency factors like sex-determining region Y box 2 and octamer-binding transcription factor 4, indicating that a decrease in LPC content can affect bovine embryo quality and can lead to a dysregulation of pluripotency pathway [25, 26].

Most slow freezing media contain sugars, such as trehalose, sucrose, and glucose that help maintain the integrity of membrane proteins. Trehalose addition in media improves the cryopreservation of mammalian cells, especially oocytes [27, 28]. Membrane proteins protect the lipids from an increase in temperature during the lipid phase transition that occurs during chilling and decrease the size of ice crystals [29, 30]. Membrane permeability properties are affected by chilling process and by cryoprotectants. This increase in permeability would induce the absorption of membrane impermeable molecules like trehalose [31]. Disaccharide uptake during freezing protocols facilitates intracellular protection, stabilizing cells during cryopreservation, [32] and reduces chromatin degradation during storage [33].

Non-lactating Holstein heifers present higher rates of good-quality embryos and blastocysts compared to lactating Holstein cows [34, 35]. Indeed, only 13.1% of lactating Holstein cows' embryos were categorized as excellent compared with 62.5% of the non-lactating Holstein heifers' embryos. Moreover, non-lactating Holstein heifers exhibited paler embryos, meaning they had a lower lipid content [34]. On the contrary, the reduced reproductive performance of lactating cows observed over the last decades is a phenomenon associated with intense genetic selection aiming to increase milk production [36, 37], an approach that is closely related to energy balance [38]. High milk yield requires high dietary intake and altered peripheral concentrations of metabolic hormones like insulin-like growth factor 1 and insulin and metabolites that are associated with subfertility through an altered follicular function, resulting in delayed ovulation [39]. Indeed, high genetic merit cows presented a decrease in serum glucose, IGF-1, and insulin concentrations, from 15 days post-partum [39], suggesting that high merit cows had a lower energy balance than low merit cows [40, 41]. However, IGF-1 regulates the sensitivity/response of follicles to gonadotropin hormones and may influence follicle development and oocyte maturation [42]. Lactating cows present a reduction in peak circulating estradiol (E2) and an increased size of the ovulatory follicle compared to nulliparous heifers. Those parameters, could lead to shorter or less intense estrus behavior, poor fertilization, poor early embryonic development, and to a tendency to increase pregnancy loss [43].

We therefore hypothesized that the embryonic lipid profile is altered by lipid supplementation given to the donor cow, a change in the slow freezing protocol, or according to the physiological stage of the donor. Thus, the objectives of this study were to evaluate, using mass spectrometry, the lipid content of single bovine grade 1 expanded blastocysts from cows that had been supplemented with n-3 PUFA. The embryonic lipid profiles from heifers vs. lactating cows were also evaluated. Finally, the effect of two slow freezing protocols on the embryonic lipid profiles was assessed.

Methods

All chemicals were purchased from Sigma-Aldrich unless otherwise stated.

Ethics statement

All experimental protocols were conducted following the European Directive 2010/63/EU on the protection of animals used for scientific purposes and were approved by the French Ministry of National Education, Higher Education, Research and Innovation after ethical assessment by the local ethics committee "Comité d'Ethique en Expérimentation Animale Val de Loire" (protocols registered under APAFIS, numbers 20013-2019032818243107v2 and 2098-2015100115278976_v5). The present study is also in accordance with the ARRIVE guidelines.

Experimental design

Experiment 1

To assess the effects on the embryonic lipid profile of PUFA supplementation in the donor diet, embryos were produced by ovum pick-up followed by in vitro fertilization (OPU-IVF) from Holstein primiparous cows that were supplemented with either n-3 PUFA (fish oil) or n-6 PUFA (soy oil = control) in their diet [14]. The OPU-IVF was performed at an average of 114.3 ± 17.6- and 123.5 ± 15.6-days post-partum for, respectively, n-3 and n-6 cows. Three OPU-IVF sessions were carried out on 12 n-3 cows and 10 control n-6 cows corresponding to 2, 5, and 7 weeks of PUFA supplementation, respectively. This procedure allowed the production of 113 Q1 expanded blastocysts at the rate of about 20 embryos per week (Table 1). As described by Freret et al. [14], an impregnation of the follicular fluid and therefore a change in the lipid profile of the oocytes were observed after 5 weeks of supplementation of donor females. However, an increase in transferrable embryos (Q1-Q2 blastocyst) was noticeable after 2 weeks of supplementation. Considering this effect, a total of 27 n-3 and 29 n-6 embryos have been selected from 10 to 12 different cows submitted to OPU-IVF at 2, 5, and 7 weeks of PUFA

Table 1. Embryo production per OPU-IVF sessions, for n-3 and n-6 cows' group and heifers

	n-3 cows		n-6 cows		Heifers	
Embryo production data	**Mean ± SEM per session**	**Total**	**Mean ± SEM per session**	**Total**	**Mean ± SEM per session**	**Total**
Nb punctured follicles	32.9 ± 2.8^{a}	559	33.7 ± 3.3^{a}	505	15.6 ± 1.9^{b}	312
Nb recovered COC	11.8 ± 1.5	201	10.7 ± 1.3	161	8.9 ± 0.9	177
Nb oocytes in IVM	10.1 ± 1.4	172	8.8 ± 1.2	132	8.0 ± 0.8	159
Nb cleaved embryos	8.1 ± 1.3	138	7.5 ± 1.0	112	7.4 ± 0.8	147
Nb D6 M + BL	5.3 ± 0.9	90	3.6 ± 0.6	54	4.9 ± 0.7	98
Nb D7 blastocyst	4.9 ± 0.6	84	4.1 ± 0.4	62	4.9 ± 0.6	98
Nb D7 expanded blastocysts Q1	3.8 ± 0.4	64	3.3 ± 0.3	49	3.2 ± 0.4	64
Nb D7 expanded blastocysts Q2	$0.8 \pm 0.2^{a,b}$	13	0.3 ± 0.2^{b}	5	1.0 ± 0.2^{a}	20
Cleaved embryos (%)	80.2 ± 4.4^{b}		84.8 ± 5.4^{b}		92.5 ± 2.1^{a}	
D6 M + BL (%)	52.3 ± 5.3^{a}		40.9 ± 7.4^{b}		61.6 ± 0.9^{a}	
D7 BL (%)	48.8 ± 5.4^{b}		47.0 ± 5.1^{b}		61.6 ± 4.7^{a}	
D7 BL Q1 (%)	37.2 ± 3.2		37.1 ± 5.9		40.3 ± 0.05	
D7 BL Q2 (%)	$7.6 \pm 3.4^{a,b}$		3.8 ± 1.7^{b}		12.6 ± 0.02^{a}	

Wilcoxon tests were performed between the number of developed embryos of the different comparisons, and Khi^2 test were realized between development rates of the different comparisons, Superscripts a, b, c highlight the significant differences. Nb: number; M: morula; BL: blastocyst; D6 and D7: days 6 and 7 of development; Q1 and Q2: embryo quality based on International Embryo Technology Society recommendations.

supplementation. In this study, the lactating cows are the same that the cows used by Freret et al. [14] for the analysis of oocyte lipid profile and follicular fluid.

Experiment 2

Seven Holstein heifers were subjected to oocytes collection by OPU-IVF. Heifers were 43.6 ± 2.0 months old and weighed 527.7 ± 15.5 kg at the time of embryos production. To avoid any individual effects, the same 7 Holstein heifers underwent an average of 2.6 ± 0.4 OPU-IVF sessions to obtain about 20 expanded grade-1 slow-frozen blastocysts according to two different slow freezing protocols, glycerol-trehalose (GLY-TRE, $n = 19$) and ethylene glycol-sucrose (EG-S, $n = 22$) at the rate of about 11.2 embryos per week.

Experiment 3

To evaluate the impact of the donor metabolic status on the embryonic lipid profile, embryos produced by OPU-IVF from the heifers from experiment 2 were compared to embryos produced by OPU-IVF from the lactating cows supplemented with n-6 PUFA from experiment 1. OPU-IVF of lactating cows was performed in 2015–2016, while the OPU-IVF of heifers was performed in 2019. All embryos were preserved in liquid nitrogen and all the lipid extractions were performed in 2019.

Animal feeding management

Experiment 1

Each Holstein lactating cows received a diet supplemented with n-6 or n-3 PUFA for 9 weeks, starting approximately 11 weeks after calving [14]. Briefly, cows were fed with a basic diet described in Supplementary Table S1.

The PUFA supplements were added to the above diets. The supplements were OMG750®, a n-3 PUFA microencapsulated fish oil (Kemin, Nantes, France), and OMGSOY®, a microencapsulated n-6 PUFA soy oil (Kemin, Nantes, France). They were distributed at 1% of the total dry matter. Cows were fed ad libitum.

Experiment 2

Each Holstein heifer were fed with straw ad libitum that was complemented with 5 kg of concentrate (Floria, Sanders, Bruz, France) distributed twice over the day (2.5 kg portion), as described in Supplementary Table S1.

Estrus synchronization

Experimental heifers were subjected to estrus synchronization, as described by Janati Idrissi et al. [3]. Briefly, synchronization treatments were performed by insertion of an intravaginal progesterone releasing device (Prid® Delta, 1.55 g, Ceva, Libourne, France) and this was followed, 6 days later, by 2 mL intramuscular injection of a prostaglandin F2 alpha analog (Estrumate®, MSD Santé Animale—Intervet, Beaucouzé, France; equivalent to 0.5 mg cloprostenol). The removal of the intravaginal device was performed 24 h after cloprostenol injection. Reference heat was detected by the monitoring of activity and rumination (Heatime®, Evolution XY, Noyal-Sur-Vilaine, France) of heifers around 48 h after intravaginal device removal. Dominant follicles (follicles with a diameter > 8 mm) were ablated between 8 and 12 days later.

Experimental cows were also subjected to synchronization and ovarian stimulation, as described by Freret et al. [14]. Briefly, each cow received a synchronization treatment consisting of a 3.3 mg norgestomet subcutaneous implant (Crestar SO®, MSD Santé Animale, Beaucouzé, France) and intramuscular injection of 0.001 mg buserelin (Crestar Pack®, MSD Santé Animale, Beaucouzé, France). Seven days later, each cow received an intramuscular injection of 15 mg of PGF2a (Prosolvin®, Virbac, Carros, France) and, 2 days later, the subcutaneous implant was removed. Reference heat was detected by the monitoring of activity and rumination (Heatime®) of cows around 48 h after subcutaneous implant removal. The estrus corresponded to the first day of supplementation, and a new subcutaneous implant of 3.3 mg norgestomet was inserted on that day. In this way, every 10 days, the subcutaneous implant was replaced, and this continued until the end of the protocol to prevent the occurrence of estrus between OPU sessions.

In vitro embryo production

For Holstein heifers, ovarian stimulation was performed as previously described [3] 36 h after the removal of the dominant follicles. Briefly, a new intravaginal progesterone device (Prid® Delta, 1.55 g, Ceva, Libourne, France) was inserted, and the ovarian stimulation was performed by five intramuscular injections of decreasing porcine follicle stimulating hormone/porcine luteinizing hormone (pFSH/pLH) doses (Stimulfol®, Reprobiol, Ouffet, Belgium), every 12 h, over 2.5 days.

- Day 0.5: 7 p.m–75 μg FSH/15 μg LH
- Day 1: 7 a.m–62.5 μg FSH/12.5 μg LH
- Day 1: 7 p.m–50 μg FSH/10 μg LH
- Day 2: 7 a.m–37.5 μg FSH/7.5 μg LH
- Day 2: 7 p.m–25 μg FSH/5 μg LH

For Holstein lactating cows, ovarian stimulation was performed as described by Freret et al. [14] by five intramuscular injections of decreasing pFSH/pLH doses (Stimulfol®, Reprobiol, Ouffet, Belgium), every 12 h, over 2.5 days.

- Day 0.5: 7 p.m–112 μg FSH/22.4 μg LH
- Day 1: 7 a.m–100 μg FSH/20 μg LH
- Day 1: 7 p.m–75 μg FSH/15 μg LH
- Day 2: 7 a.m–63 μg FSH/12.6 μg LH
- Day 2: 7 p.m–50 μg FSH/10 μg LH

Cumulus oocyte complexes (COCs) were collected by OPU. After locoregional anesthesia, follicles from both ovaries, from 6 to 12 mm in diameter, were aspirated by transvaginal way using ultrasonography and COCs were recovered in a tube containing 1 mL of flushing solution (Euroflush®; IMV Technologies, L'Aigle, France) added with heparin (1:50), maintained at 37°C.

In vitro development

In vitro development was performed as previously described [3]. Whatever the groups, 82–90% of the recovered COCs were selected. The recovered COCs were selected under a stereomicroscope, and only COCs with homogenous, non-granular cytoplasm and having at least three layers of granulosa cells were used [44]. The COCs were matured in in vitro maturation medium, which consisted of TCM-199 that was supplemented with 10 μg/mL pFSH/pLH, 1 μg/mL 17-beta-œstradiol, 5 ng/mL epidermal growth factor 5 μg/mL gentamicin, and 10% fetal calf serum (FCS) (v/v). All COCs were incubated at 38.5°C for 22 h under a maximum humidity atmosphere of 5% CO_2 in the air. Matured oocytes were fertilized in 500 μL of a modified Tyrode's bicarbonate buffered solution containing 10 μg/mL heparin, 20 μM penicillamine, 10 μM hypotaurine, 1 μM epinephrine, 6 g/L bovine serum albumin (BSA), and 20 μM sodium pyruvate [45]. A single ejaculate from one bull (FR1532181070, Evolution cooperative, Noyal-Sur-Vilaine, France) of proven fertility was used for all the IVF experiments for fertilization of COCs from both lactating cows and heifers. Spermatozoa were coincubated with COCs at 1×10^6 spermatozoa/mL at 38.5°C for 18 h in a maximal humidified atmosphere of 5% CO_2 in the air. After fertilization, cumulus cells and spermatozoa were removed from presumptive zygotes. Zygotes were cultured in a 30 μL micro drop of synthetic oviductal fluid (Minitüb, Gmbh, Germany) supplemented with 1% of estrus cow serum, 2% Minimum Essential Medium 100× containing non-essential amino acids, 1% basal medium 50× containing essential nutrients, 0.33 g/L Na-Pyruvate, and 6 g/L FA free BSA at 38.5°C in a maximal humidified atmosphere of 5% O_2, 5% CO_2, and 90% N_2 and covered with mineral oil (Liquid Paraffin, Origio, Måløv, Denmark). Cleavage rates were assessed under stereoscopic microscopy 48 h post-fertilization (day 2). Blastocyst development rates and embryo quality were recorded at day 6 and day 7 according to the International Embryo Technology Society morphological criteria (Chapter 9 and Annex D, IETS Manual, third edition). Only grade-1 expanded blastocysts were used for the experiment, that is, expanded blastocysts with a compact inner cell mass, uniformly colored blastomeres, few irregularities or excluded cells, and an intact and smooth zona pellucida. Wilcoxon tests were carried out to compare the numbers of embryos at the different stage of development, and Chi^2 tests were carried out on the percentages of embryos development at different stage (Table 1).

Embryo freezing

Ethylene glycol sucrose protocol

The embryos were frozen in EG-S solution following a protocol previously described [3]. Briefly, embryos were washed in Embryo Holding Medium (EHM, IMV Technologies, L'Aigle, France) and were placed in 1.5 M ethylene glycol embryo freezing medium containing BSA (ET freezing media, IMV, Technologies, L'Aigle, France) with 0.1 M sucrose added. The embryos were individually mounted in 250-μL straws. Straws were placed in the cryochamber of the freezer (Freeze Control®, Cryologic, Melbourne, Australia), which was previously equilibrated at −6°C. After 2 min, the seeding was manually induced. The temperature was stabilized at −6°C for 8 min post-seeding and was then dropped to −32°C at a rate of −0.3°C/min. The entire protocol, from the moment the straws were placed in the cryochamber, until the freezer has stabilized at −32°C, lasted for 96 min.

GLY-TRE protocol

Embryos were washed in EHM and were placed in dehydration solution composed of EHM added with 20% FCS, 5% glycerol during 10 min (solution 1). Embryos were then placed in freezing solution composed of EHM that was added with 20% FCS, 9% glycerol, and 0.2 M trehalose during 10 min (solution 2). During this time, the embryos were mounted in 250-μL straws respecting the following proportion 4:2:4. Specifically, the first column was composed of solution 1, the second contain embryos in the solution 2, and the third column was composed of solution 1. The straws were placed in the cryochamber of the freezer (Freeze Control®, Cryologic, Melbourne, Australia) at room temperature. The temperature was dropped to −7°C at a rate of 1.5°C/min and was stabilized at −7°C during 3 min. The seeding was manually induced, and the temperature remained at −7°C for an additional 3 min. Then, temperature dropped to −32°C at a rate of −0.3°C/min. The entire protocol, from the moment the straws were placed in the cryochamber, until the freezer has stabilized at −32°C, lasted for 110 min.

At the end of both programs, the straws were directly plunged in liquid nitrogen and were stored before thawing and lipid extractions. For thawing, whatever the cryopreservation

protocol used, straws were kept 5 s in ambient air and were then immersed in a water bath at 35°C for 30 s. Embryos were washed three times in PBS and were transferred in 1.5-mL vials in minimum volumes for lipid extractions.

Lipid analysis by mass spectrometry, sample preparation, and data acquisition

Liposoluble extraction and lipid analysis by mass spectrometry were performed as previously described [3]. Briefly, liposoluble fraction of each embryo was extracted by methanol/chloroform protocol. The lower phase (350 μL) corresponding to the nonpolar fraction was recovered and was put in glass tubes for solvent evaporation using a SpeedVac (Thermo Fisher Scientific, Waltham, MA). The residue was then reconstituted with 100 μL of a 6:3:1 mix of acetonitrile/water/isopropanol which was followed by centrifugation for homogenization before mass spectrometry analysis.

Liquid chromatography-high resolution mass spectrometry (LC-HRMS) was performed in positive ionization mode (ESI+) using an UPLC Ultimate WPS-3000 system (Dionex, Germany), which was coupled to a QExactive mass spectrometer (Thermo Fisher Scientific, Bremen, Germany). The autosampler temperature was set at 4°C, and the injection volume for each sample was 5 μL. During the full-scan acquisition, the instrument operated at 70 000 resolution, which ranged from 250 to 1600 m/z, with an automatic gain control target of 1×10^6 charges and a maximum injection time of 250 ms. The instrumental stability was evaluated by multiple injections ($n = 9$) of a quality control (QC) sample obtained from a pool of 10 μL of all samples analyzed. This QC sample was injected once at the beginning of the analysis, then after every 10 injections, and at the end of the run.

LC-HRMS data processing and statistical analysis

Data processing and statistical analysis were performed as previously described [3]. Briefly, Galaxy Workflow4metabolomics was used to process the raw data for the detection of features and for retention time correction. Considering all the samples of an analytical campaign, features with QC variance greater than sample variance and variance intensities >30% in QC samples were removed as well as those identified as background noise or poorly integrated after visual inspection [46]. For all the remaining features, lipid annotation was performed on SimLipid®. The peak intensities were normalized by the sum of the areas of the features considered, transformed into a logarithm using the automatic transformation tool of SIMCA® software (Umetrics, Umeå, Sweden). Lipids were annotated (<0.01 ppm) according to either the homemade SimLipid® (highlighted in yellow in Supplementary Table S2) database or according to the LIPID MAPS® Lipid Classification System [47, 48] (not highlighted in Supplementary Table S2). (https://www.lipidmaps.org/resources/tools/bulk_structure_searches.php?database=LMSD).

Principal component analysis (PCA) was performed on the data as an exploratory unsupervised analysis representing the distribution of the embryos for the different comparisons according to their entire lipid profile. PCA allowed to observe the distribution of embryos according to their lipid profile without supervision and allowed a visual description of the results. Multivariate analysis was performed with orthogonal partial least square discriminant analysis (O-PLS-DA), which was performed on the data set in the form of supervised analysis to predict groups by maximizing the explained variance between groups using the SIMCA® Software [49]. Unlike PCA, O-PLS-DA was supervised and did only concern a part of the lipid profile. O-PLS-DA made it possible to create a prediction model associating several features. Ions with low variable importance in the projection (VIP) were repeatedly excluded until there was maximum improvement of the quality of the models. All VIPs have been presented in Supplementary Table S3. Model quality was evaluated with cross-validated residuals ANOVA (CV-ANOVA) and cross-validation by Q^2 (goodness of prediction). The univariate analysis of lipid levels between groups was based on nonparametric Wilcoxon tests with false discovery rate (FDR) correction and a fold-change (FC) >1.5 or <0.66 using Metaboanalyst, version 4.0. (https://www.metaboanalyst.ca/). All significantly different lipids are presented in Supplementary Table S2. For each comparison, the embryos frozen with EG-S or GLY-TRE (green column) and embryos from Holstein heifers or Holstein lactating cows (blue column), the *P*-value, the average peak intensity, and the FC are presented. Univariate analysis is represented by a volcano plot combining the *P*-value and the FC of the *t*-tests. The *y*-axis represents the *P*-value that corresponds to the *t*-test comparing the differences between the variables (negative base 10 logarithmic scale), and the *x*-axis represents the FC between the subject groups (base 2 logarithmic scale).

Results

Embryo production data

Forty sessions of OPU-IVF were performed to produce the embryos needed for the different comparisons. These procedures allowed the production of 64, 49, and 64 grade-1 expanded blastocysts from, respectively, 201, 161, and 177 COCs from n-3 cows, n-6 cows, and heifers (Table 1). The average number of in vitro-produced Q1 expanded blastocysts was, respectively, 3.8 ± 0.4, 3.3 ± 0.3, and 3.2 ± 0.4 per OPU session (Table 1).

Lipid composition of bovine embryos

Ninety-seven individual Q1 expanded blastocysts were produced by OPU-IVF, and among them, 56 were produced from lactating cows supplemented with either n-6 ($n = 27$) or n-3 PUFA ($n = 29$) and 41 were produced from heifers, including 19 expanded blastocysts frozen according to a GLY-TRE protocol and 22 that were frozen according to an EG-S protocol (Figure 1). The LC-HRMS spectra were obtained for these 97 individual Q1 expanded blastocysts using ultra-high pressure liquid chromatography and 1686 features were identified. After visual inspection of the data, elimination of ions with coefficient of variation > 30% in QC and isotope elimination, 515 features were conserved. Among those 515 features, 127 were annotated using the SimLipid Database and LIPID MAP. Among the 127 annotated lipids, 105 have only one annotation proposal. Among them, a majority of glycerophospholipids (glycerophosphocholine, ethanolamine, glycerol, serine, and inositol) have been annotated 23/105, sterols 15/105, FA 11/105, and ceramides 11/105 (Supplementary Table S2).

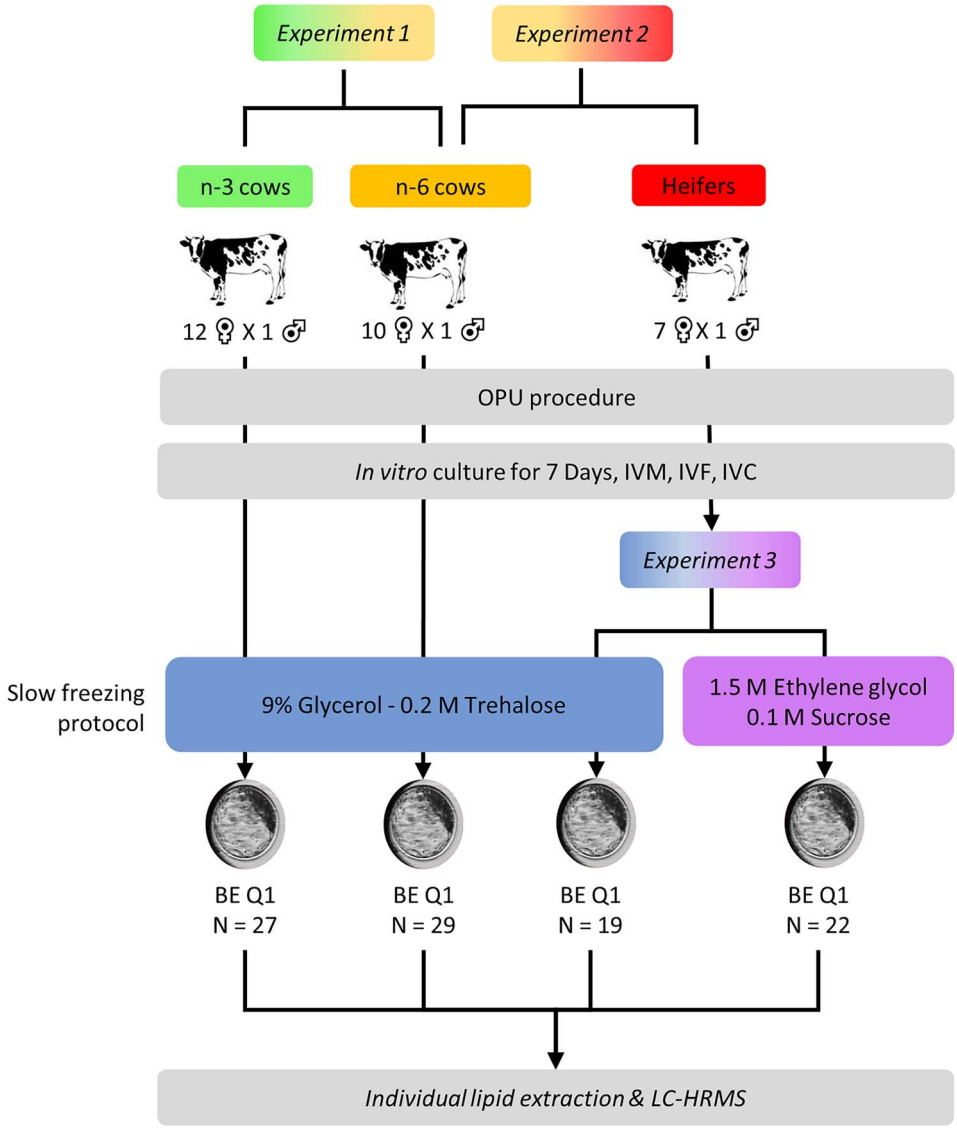

Figure 1. Experimental design. In vitro grade 1 expanded blastocysts from Holstein lactating cows supplemented with n-6 or n-3 PUFA, in their diet, were produced by OPU-IVF and were frozen by a GLY-TRE protocol after 7 days of development. Seven Holstein heifers enabled the production of in vitro embryos by OPU-IVF. After 7 days of development, all grade 1 expanded blastocysts were frozen: 19 were frozen by a GLY-TRE protocol and 22 were frozen by an EG-S protocol. All embryos underwent lipid extraction. Lipid extracts were analyzed by LC-HRMS, which allowed the detection of 515 features.

Mass spectrometry lipid signature of in vitro-produced embryos from donor cows supplemented with either n-3 or n-6 PUFA

To evaluate the effects of n-3 or n-6 PUFA dietary supplementation on embryo lipid profiles, 56 individual expanded blastocysts were used, including 27 embryos produced from oocytes collected from cows supplemented with n-6 PUFA in their diet and 29 n-3 embryos produced from cows supplemented with n-3 PUFA in their diet (Figure 1). The PCA showed no discrimination between n-6 and n-3 embryos (Supplementary Figure S3A). No significant difference in the average of peak intensities' sums was highlighted between n-3 and n-6 embryos, respectively, $2.0E{+}09 \pm 1.2 + 08$ vs. $1.41E{+}09 \pm 1.00 + 08$, $P = 0.28$. The O-PLS-DA showed a discrimination between the two distinct profiles of n-6 (yellow dots) and n-3 (green dots) embryos, with a cross-validated predictive ability (Q^2) of 0.64 and with good reliability of this model evaluated by CV-ANOVA ($P < 0.0001$) (Supplementary Figure S3B). The fitted model included 69/515 features, meaning that the association of these 69 features participated in explaining the differences between the n-6 and n-3 embryonic lipid profile (Supplementary Table S2).

Among these 69 features, 22 could be annotated. There were 12 overabundant lipids in n-6 embryos: two ceramides, Cer(39:0;O4) and Cer(41:1;O4); a diacylglycerol, DG(38:3); an eicosanoid, FA(22:3;O3); a fatty amide, NAT(18:2); three fatty esters, FAHFA(25:1;O), FAHFA(26:1;O), and CAR(22:0); a glycerophosphoethanolamine, LPE(O-19:0;O); and three triacylglycerols, TG(52:1), TG(52:3), and TG(54:6). There were 10 overabundant lipids in n-3 embryos: an eicosanoid, FA(20:2;O3); two FA, FA(22:0;O) and FA(18:8;O2); a fatty alcohol, FOH(22:12); two fatty amides, NA(22:2) and NA(22:2;O2); a glycerophosphate, PA(32:1);

a glycerophosphocholine, LPC(O-14:0); a glycerophosphoethanolamine, LPE(22:4); and an oxidized glycerophospholipid, PS(26:3;O2). However, the univariate analysis did not show any difference between n-6 and n-3 embryonic lipid profiles (Supplementary Figure S3C).

Mass spectrometry lipid signature of in vitro-produced embryos from lactating cows or heifers

To evaluate the impact of physiological stage on embryo lipid profiles, 46 individual expanded blastocysts were used, including 27 from Holstein lactating cows and 19 from Holstein heifers (Figure 1). The PCA showed a discrimination between in vitro-produced embryos from Holstein lactating cows (turquoise dots) or Holstein heifers (red dots) (Figure 2A). The semi-quantitative analysis highlighted that the average of peak intensities' sums of embryos from cows was higher than that of heifers, $1.8E{+}09 \pm 1.0 + 08$ vs. $1.4E{+}09 \pm 7.5 + 07$, respectively, $P = 0.004$. The O-PLS-DA showed a clear discrimination of the two distinct between the two distinct profiles of embryos from Holstein lactating cows (turquoise dots) and Holstein heifers (red dots) with a cross-validated predictive ability (Q^2) of 0.93 and with good reliability of this model evaluated by CV-ANOVA ($P < 0.0001$) (Figure 2B). The fitted model included 449 features, meaning that the majority of the detected features (449/515) participated in explaining the lipid profile of embryos from Holstein lactating cows or Holstein heifers (Supplementary Table S2). The univariate analysis highlighted 246 significantly different features between embryos from Holstein lactating cows and Holstein heifers ($P < 0.05$ and FC <0.66 or >1.5), including 120 annotated lipids (Supplementary Table S2). Among the 246 significant features, 210 had a FC >1.5, including 68 with a FC >10, indicating an increased abundance in embryos from Holstein lactating cows. Furthermore, 41 features exhibited a FC <0.66, indicating a decreased abundance in embryos from Holstein lactating cows (Table 2). The volcano plot, based on both the FC and the *P* value, highlighted a lipid enrichment of embryos from Holstein lactating cows, particularly in FA, sterols, fatty amides, oxidized glycerophospholipids, and glycerophospholipids like LPC(O-16:1), LPC(18:2), LPC(O-18:1), PC(O-36:4), and others, while embryos from Holstein heifers seemed to be enriched in ceramides (9/41) (Figures 2C, 3 and Supplementary Figure S2).

Mass spectrometry lipid signature of in vitro-produced embryos frozen either with GLY-TRE or EG-S protocol

To evaluate the effects of slow freezing protocols on embryo lipid profiles, 41 individual expanded blastocysts were used, including 19 frozen with GLY-TRE and 22 with EG-S protocol (Figure 1). The PCA mostly discriminated embryos frozen with EG-S protocol (blue dots) and GLY-TRE (purple dots) embryos despite the overlap of five embryos (Figure 4A). No significant difference in the average of peak intensities' sums was highlighted between EG-S and GLY-TRE embryos, $2.3E{+}09 \pm 7.8 + 08$ vs. $1.4E{+}09 \pm 8.1 + 07$, respectively, $P = 0.37$. The O-PLS-DA showed a clearer discrimination of the two distinct groups of embryos, either frozen with the EG-S protocol (blue dots) or with the GLY-TRE (purple dots) protocol, with a cross-validated predictive ability (Q^2) of 0.86 and with good reliability of this model evaluated by CV-ANOVA ($P < 0.0001$) (Figure 4B). The fitted model included 89/515 features, meaning that the association of these 89 features participated in explaining the lipid profile differences

Table 2. The 30 most differentially expressed lipids between in vitro-produced embryos from Holstein lactating cows or Holstein heifers

Observed m/z	Lipid annotation	FDR *P*.ajusted	Mean cow	Mean heifer	Fc cow/heifer	Observed m/z	Lipid annotation	FDR *P*.ajusted	Mean cow	Mean heifer	Fc cow/heifer
453.1693	Cissoic acid	1.21E−06	2.12E−04	0	Absence	614.5740	Cer(37:0;O4)	4.67E−08	9.67E−04	4.56E−04	9760.85
456.2654	N-arachidonoyl glutamic acid	3.32E−08	8.05E−05	0	Absence	550.0878	ND	4.46E−10	5.97E−05	8.99E−06	20143.79
473.1976	3,12-diketo-4,6-petromyzonene-24-sulfate	3.32E−08	1.08E−04	0	Absence	663.4549	ND	3.19E−07	1.53E−02	7.44E−03	23337.99
484.2966	PS(O-16:0/0:0)	3.32E−08	6.84E−05	0	Absence	343.1745	FA(22:10)	1.74E−09	9.20E−05	4.16E−05	23911.35
503.3276	ST(28:1;O6)	3.32E−08	9.38E−05	0	Absence	663.5346	PA(O-34:0)	6.28E−09	6.22E−04	2.06E−04	32875.75
520.3176	ND	3.32E−08	5.85E−05	0	Absence	663.5346	SM(d18:0/13:0)	6.28E−09	6.22E−04	2.06E−04	32875.75
524.2362	PC(8:2(2E,4E)/ 8:2(2E,4E))	3.32E−08	6.35E−05	0	Absence	619.5292	DG(36:3)	6.28E−09	1.27E−03	5.76E−04	91724.00
527.2330	20-acetoxy-clavulone	3.32E−08	6.74E−05	0	Absence	512.4175	ND	2.12E−10	1.73E−04	2.82E−05	133266.29
527.2330	PG(18:4(6Z,9Z, 12Z,15Z)/0:0)	3.32E−08	6.74E−05	0	Absence	653.2921	ND	1.27E−11	6.68E−05	3.43E−06	268974.66
573.2287	ND	3.32E−08	5.92E−05	0	Absence	465.1098	ND	1.27E−11	8.29E−05	5.50E−06	431076.12
575.1658	ND	3.32E−08	5.21E−05	0	Absence	618.0758	ND	1.27E−11	4.62E−05	6.79E−06	532413.27
596.2369	ND	3.32E−08	1.45E−04	0	Absence	324.2032	ND	1.27E−11	1.92E−04	7.03E−06	551746.06
602.1848	ND	3.32E−08	6.03E−05	0	Absence	523.2477	ND	1.27E−11	3.07E−04	9.00E−06	705690.02
630.2169	ND	3.32E−08	6.87E−05	0	Absence	551.2147	ND	1.27E−11	1.80E−04	2.42E−05	1899426.58
312.2392	ND	3.45E−02	6.99E−05	4.19E−05	0.077	647.5607	DG(38:3)	1.27E−11	1.27E−03	3.84E−04	30088832.56

In this table, only the 30 most significantly different lipids among the 246 differentially expressed lipids between lactating cow and heifer embryos with a FC > 1.5 or <0.66 are presented. The *P* values with the FDR are presented here. Supplementary Table S2 provides a complete list of all the significant features and lipid annotations.

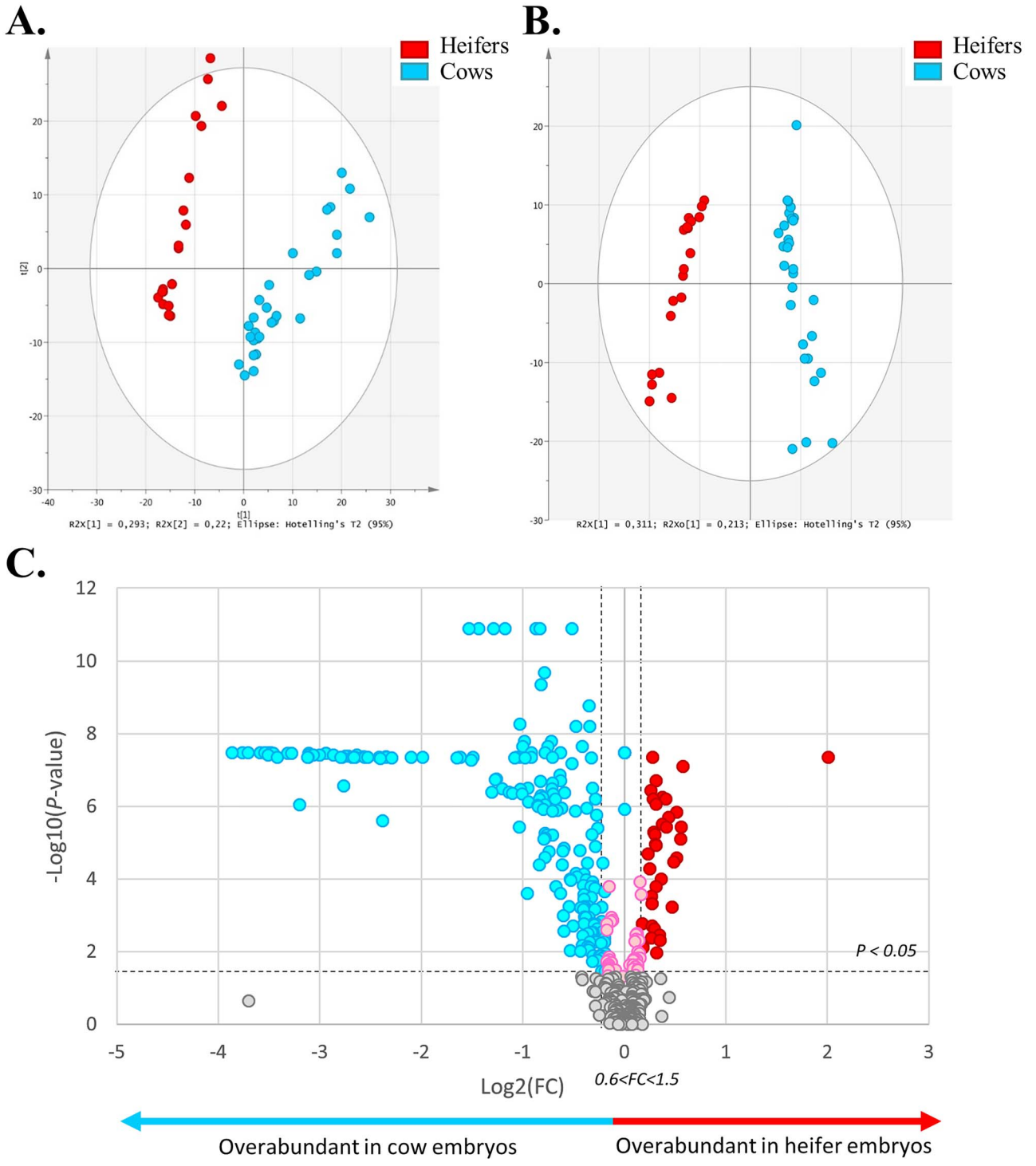

Figure 2. (A) PCA plot representing variance among in vitro-produced embryos from Holstein lactating cows (turquoise dots) or from Holstein heifers (red dots). (B) Multivariate analysis by O-PLS-DA, discriminating the embryo origin according to the embryonic lipid profile. (C) The univariate analysis via volcano plot, based on FC and *P*-value, highlighted several lipids. Pink dots correspond to lipids with significant *P* values but a FC between 0.66 and 1.5. Turquoise dots correspond to significantly overabundant lipids in embryos from Holstein lactating cows, while red dots corresponding to significantly overabundant lipids in embryos from Holstein heifers. Statistical significance was determined at $P < 0.05$ with a FC >1.5 or <0.66.

between embryos frozen with the EG-S and GLY-TRE (purple dots) protocols (Supplementary Table S2). The univariate analysis, represented by the volcano plot and based on both the FC and the *P* value, highlighted 105 significantly different features between the embryos frozen with the EG-S or GLY-TRE protocol ($P < 0.05$ and FC <0.66 or >1.5), including 53 annotated lipids (Supplementary Table S2). Among the 105 significant features, 47 were highlighted by the O-PLS-DA and 72 had a FC >1.5, indicating an increased abundance in embryos frozen with the EG-S protocol, and 33 features exhibited a FC <0.66, indicating a decreased abundance of embryos frozen with the EG-S protocol (Table 3).

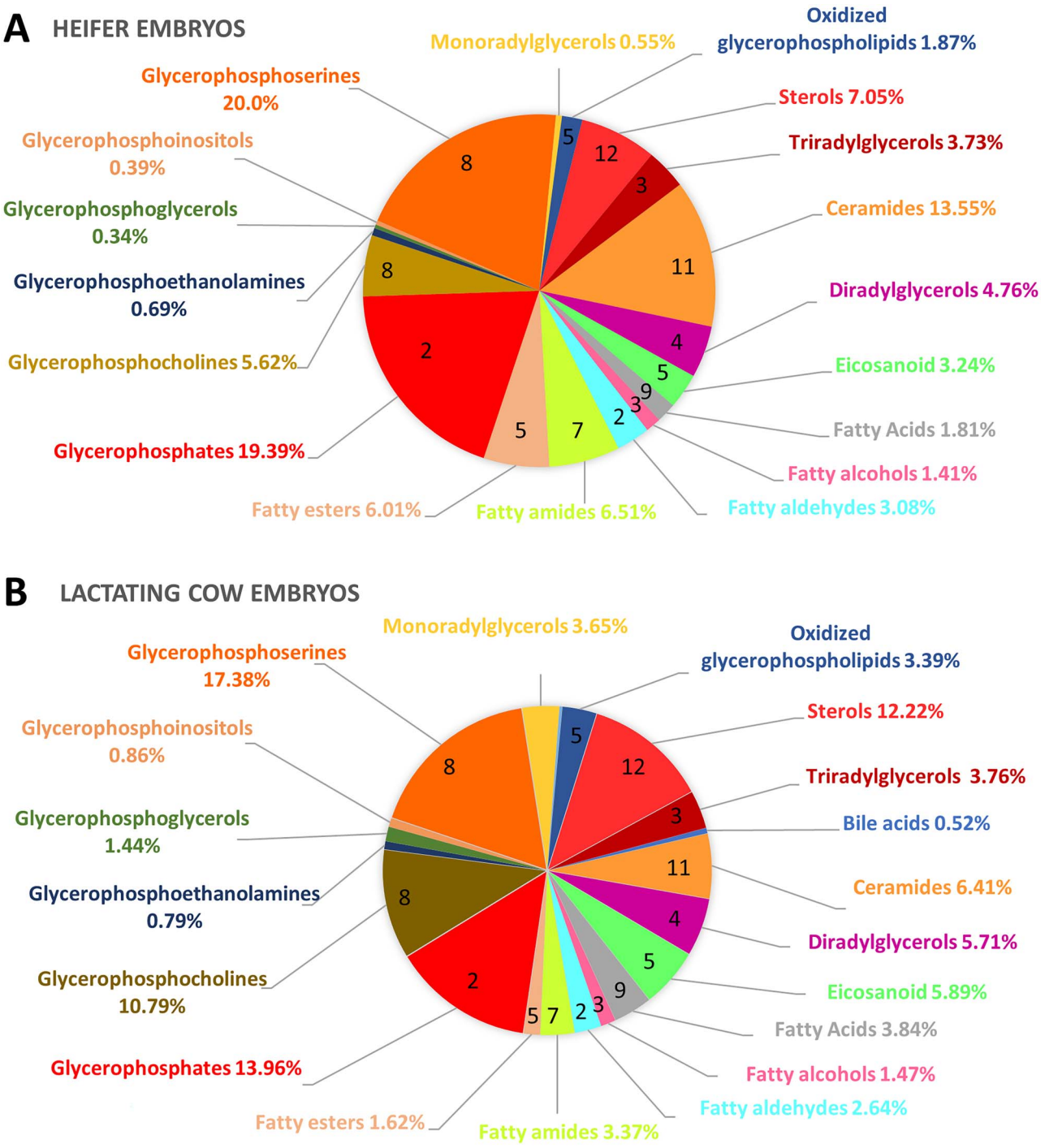

Figure 3. Graphic representation of lipid profile of in vitro-produced embryos from (A) Holstein heifers or (B) Holstein lactating cows. The mean intensity of differential peaks ($n = 246$) belonging to each lipid class was represented (%). Not determined and not attributable (ND and NA, respectively) features accounted for 79% and 76% of the lipid profile of, lactating cows and heifers, respectively, and therefore have been removed to streamline the data presentation. The number of features participating in the composition of each class is noted on the pie chart; it varied between 2 and 12.

The volcano plot highlighted a lipid enrichment of embryos frozen with the EG-S protocol, particularly in free FA, FA(21:4;O6), FA(22:0;O), FA(18:1;O), FA(18:3), and tricosanedioic acid; in glycerophospholipids, PI(40:3), PI(15:1), PC(O-36:4), PS(O-30:0), PG(16:0/0:0), and PC(16:4); in oxidized glycerophospholipids like PS-PA and OHOOA-PS; and in sterols, 19-oxodesacetylcinobufagin, asparacemosone C, and 5-beta-hydroxybufotalin. On the contrary, embryos frozen with the GLY-TRE protocol were enriched in PE(30:3) and in sterols ST(27:0;O7), theonellasterol and norcholestanol (Figure 4C and Supplementary Figure S1). As highlighted in Figure 5A and B, a quarter of the lipids could not be annotated because of either a lack of correspondence (ND) or because of too many possibilities corresponding to their mass (NA). The embryos frozen with the EG-S protocol exhibited an enrichment in glycerophosphates (27.4% of the differentially expressed lipids) of the embryonic lipid profile compared with the embryos frozen

with the GLY-TRE protocol (Figure 5 and Supplementary Figure S1).

Discussion

The present work is the first attempt, to our knowledge, to modulate the lipid profile through a modification in the slow freezing protocol, supplementation of the donor's diet, or the donor's physiological stage. To evaluate the effects of these changes on the lipid composition of in vitro-produced embryos, several comparisons were made: two slow freezing protocols, two donor diets, supplemented with either n-6 or n-3 PUFA, and in vitro-produced embryos from heifers and lactating cows. Our result suggested that the embryonic lipid profile is mainly impacted by the physiological stage of the donor, followed by the slow freezing protocol and is only mildly affected by the diet of the donors.

The lipid profile of in vitro-produced embryos was greatly affected when we compared in vitro-produced embryos from Holstein lactating cows and Holstein heifers. Our result demonstrated that most of the lipids constituting the profile contributed to explain the differences between the two groups (449/515 feature constituting the O-PLS-DA models), and more than half of them (246 features) were differentially abundant between lactating cows and heifers, including 85% of overabundant features in the embryonic lipid profiles from Holstein lactating cows. This huge difference can be partly attributable to the physiological stage of the animals. It is known that lactation induces distinct changes in the metabolic status of post-partum lactating cows, especially in amino acid and lipid composition of the follicular fluid [50]. Of note, non-lactating cows and heifers had similar follicular fluid FA and amino acid composition, reflecting the similarity in their metabolic profile [50]. It is known that embryo quality is also affected by the physiological stage of the animal. Indeed, Leroy et al. [34] demonstrated that only 13.1% of lactating Holstein cows embryos were categorized as excellent compared with 62.5% of the non-lactating Holstein heifers' embryos. The biggest difference was that 22% of the lactating cow embryos were darker compared with the others, while none of those from heifers had a dark inclusion. It is known that dark inclusion were associated with lipid accumulation in embryonic morphology evaluation [51, 52], and the dark embryos contain significantly more lipids in lipid droplets compared to pale embryos [53]. In vivo-produced embryos are also more likely to develop to the blastocyst stage when they are from heifers (23.2%) than from lactating cows (4.0%) [34]. Therefore, the lipid profile of embryos from heifers, allowing the production of embryos more capable to develop and thus favorable for gestation, could be considered optimum. Nowadays, embryos are indeed in majority produced from heifers in a genetic improvement consideration and more anecdotally on cows. Of note, embryos produced from Holstein lactating cows were produced 4 years before the heifer embryos and did not follow the exact same diet due to their different needs. We cannot rule out that confounding factors, such as feeding management or environmental factors, could induce biases. The Holstein lactating cow embryos, which were produced before the Holstein heifer embryos, showed an overabundance of lipids that were therefore not degraded because of a longer storage duration, allowing the comparison. In addition, experimental

Table 3. The 30 most differentially expressed lipids between in vitro-produced embryos that were frozen either with EG-S or with GLY-TRE protocol

Observed m/z	Lipid annotation	FDR *P*.ajusted	Mean EG-S	Mean GLY-TRE	FD EG-S/GLY-TRE	Observed m/z	Lipid annotation	FDR *P*.ajusted	Mean EG-S	Mean GLY-TRE	FD EG-S/GLY-TRE
456.2654	N-arachidonoyl glutamic acid	1.23E−02	2.72E−07	0.00E+00	Absence	331.2856	MG(16:0)	5.43E−04	1.09E−04	8.90E−07	123.04
503.3276	ST(28:1;O6)	1.23E−02	5.39E−07	0.00E+00	Absence	568.2056		5.54E−04	2.82E−06	2.09E−08	135.11
520.3176		2.07E−02	4.65E−07	0.00E+00	Absence	397.2732	LPA(O-16:0/0:0)	8.56E−03	6.76E−05	4.76E−07	142.23
524.2362	PC(16:4)	4.31E−03	6.72E−07	0.00E+00	Absence	500.2167		1.35E−03	3.74E−05	2.19E−07	170.27
527.2330	20-acetoxy-clavulone	2.74E−03	1.15E−06	0.00E+00	Absence	415.2132	19-oxodesacetylcin-obufagin	3.86E−03	7.20E−05	3.78E−07	190.42
527.2330	PG(18:4(6Z,9Z,12Z,15Z)/0:0)	2.74E−03	1.15E−06	0.00E+00	Absence	315.3270	FOH(20:0;O)	1.69E−02	5.50E−05	2.36E−07	233.29
573.2287		7.37E−03	3.19E−07	0.00E+00	Absence	768.5816	PC(O-36:4)	8.13E−04	5.08E−05	2.04E−07	249.33
596.2369		3.82E−04	1.25E−05	0.00E+00	Absence	511.2575	PS-PA	2.21E−03	9.90E−05	3.96E−07	250.15
602.1848		9.76E−04	1.71E−06	0.00E+00	Absence	421.2552		3.82E−04	7.75E−06	2.89E−08	268.06
630.2169		3.82E−04	5.62E−06	0.00E+00	Absence	359.3170	MG(18:0)	9.76E−04	1.70E−04	6.11E−07	278.95
368.3903	15 ou 17-tetracosenal	1.93E−04	4.47E−05	3.64E−04	0.12	432.2287	19-oxodesacetylcin-obufagin	4.92E−04	4.02E−05	1.22E−07	330.12
516.4264		1.80E−03	7.23E−05	3.79E−04	0.19	310.2389	FA(18:4;O)	1.23E−02	5.33E−05	1.32E−07	403.66
261.1316		1.27E−03	2.90E−04	1.08E−03	0.27	310.2389	Panaquinquecol 1	1.23E−02	5.33E−05	1.32E−07	403.66
506.4218	O-behenoylcarnitine	3.41E−04	2.97E−04	8.57E−04	0.35	483.2262	5beta-hydroxybufotalin	7.69E−04	5.65E−05	1.22E−07	462.72
256.1761		5.00E−03	1.96E−03	4.75E−03	0.41	485.3603		5.18E−03	1.52E−04	4.47E−08	3412.50

In this table, only the 30 most significantly different lipids among the 105 differentially expressed lipids with a FC > 1.5 or <0.66 are presented. The *P* values with the FDR are presented here. Supplementary Table S2 provides a complete list with all the significant features and lipid annotations.

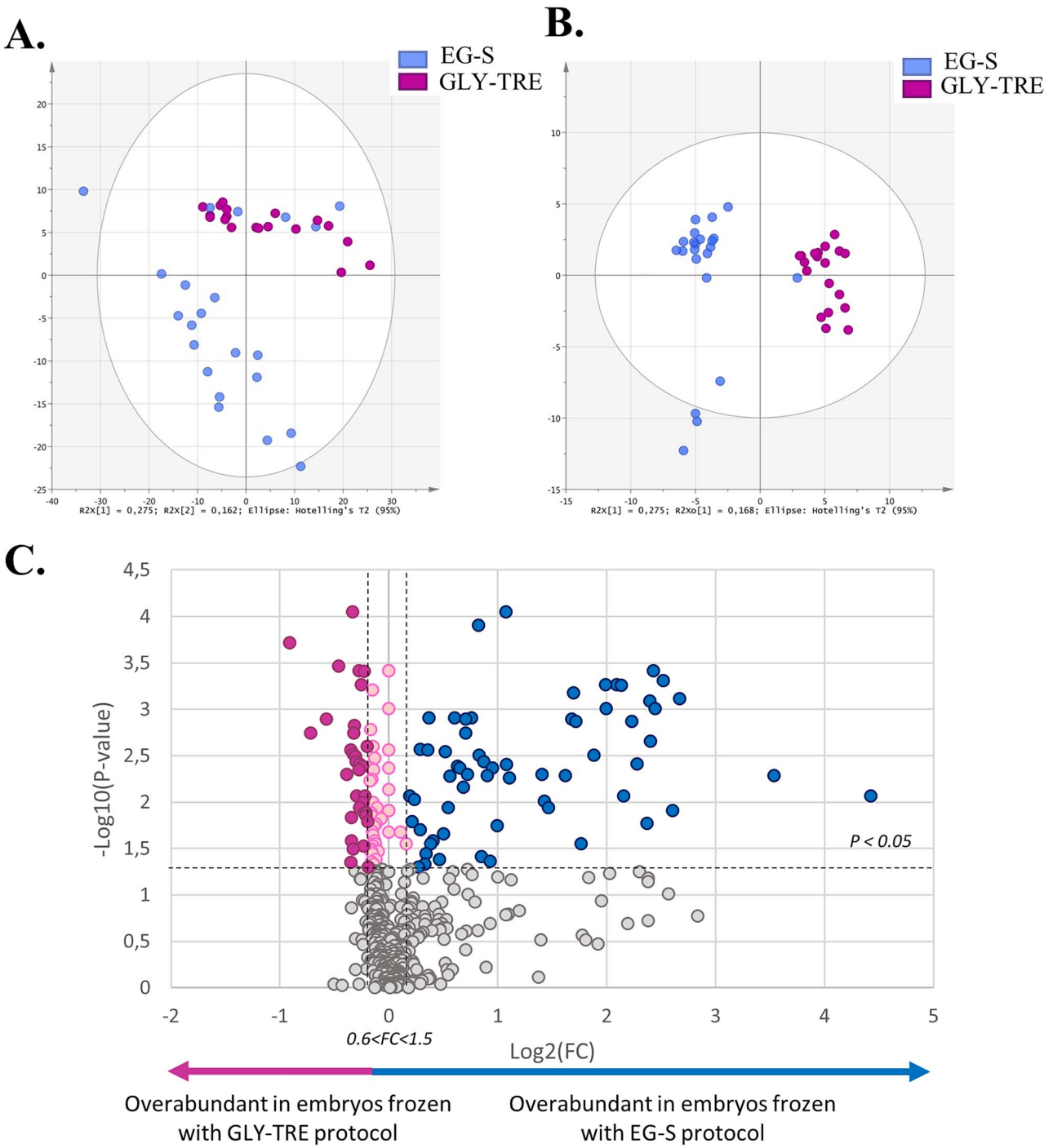

Figure 4. (A) PCA plot representing variance among in vitro-produced embryos frozen either with the GLY-TRE or the EG-S protocol. The blue dots show data for embryos frozen with the EG-S protocol, and the purple dots show data for embryos frozen with the GLY-TRE protocol. (B) Multivariate analysis by O-PLS-DA, discriminating these two protocols according to the embryonic lipid profile. (C) Univariate analysis via volcano plot based on the FC and the *P* value. Pink dots correspond to lipids with significant *P* values but a FC between 0.66 and 1.5. Blue dots correspond to significantly overabundant lipids in embryos frozen with the EG-S protocol, while purple dots correspond to significantly overabundant lipids in embryos frozen with the GLY-TRE protocol. Statistical significance was determined at $P < 0.05$ and FC >1.5 or <0.66.

conditions (protocols, laboratory, and incubators) remained unchanged between the two-production periods. Moreover, as discussed below, it would seem that the impact of the diet is smoothed out by the in vitro culture; hence, we can hypothesize that the impact of the lipid profile was mainly due to the physiological stage of the animals and to a lesser extent to the environment production of the embryos. In fact, if this smoothing effect could not be evaluated in that study, we suggest that the differences are high enough between lactating cow and nulliparous heifer oocytes to remain at the end of embryonic culture. As we cannot demonstrate that the observed effect is only due to the physiological status, this effect should rather be referred to a physiological/year of production effect.

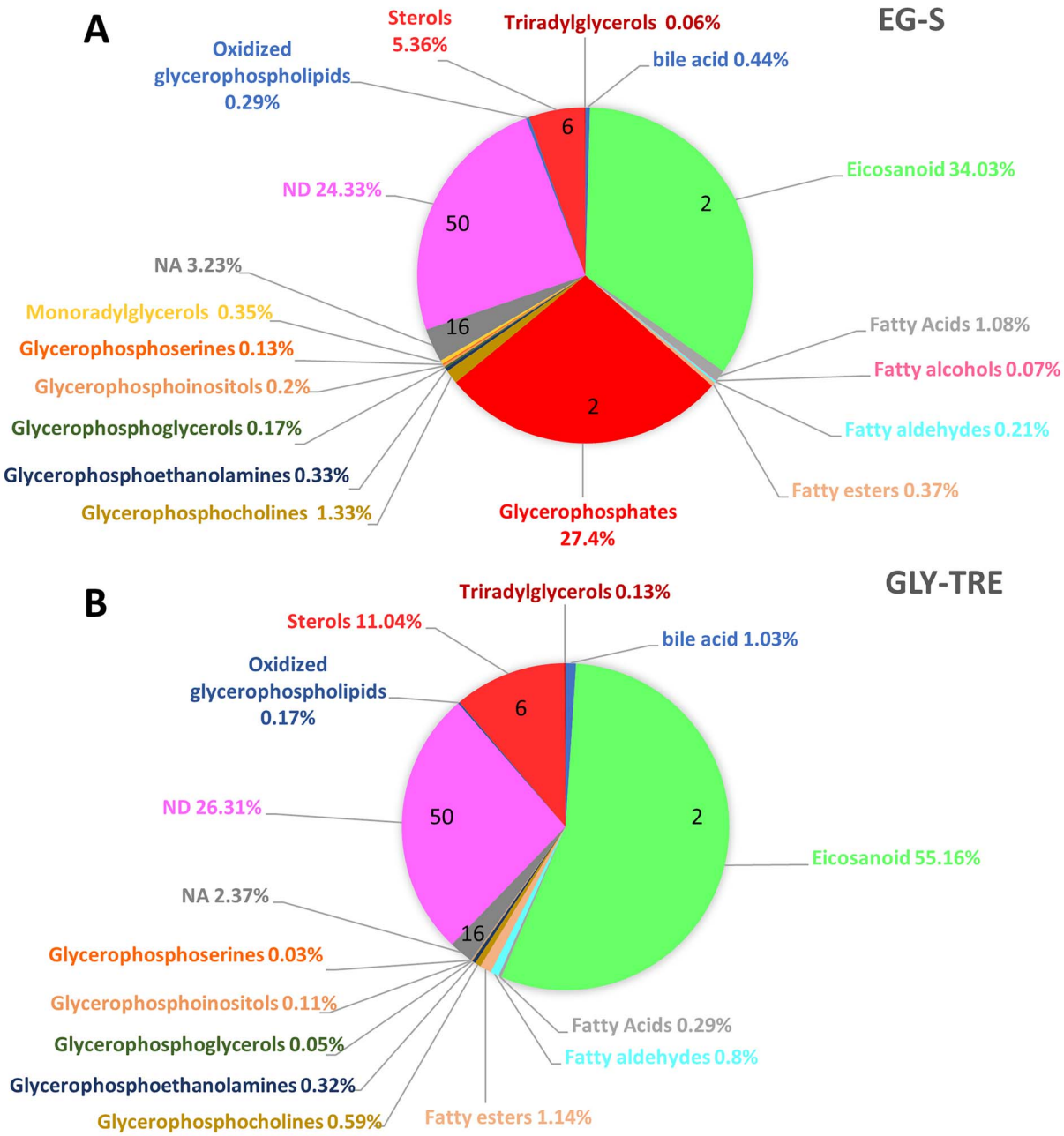

Figure 5. Graphic representation of the lipid profile of in vitro-produced embryos slow-frozen with either (A) the EG-S protocol or (B) the GLY-TRE protocol. The mean intensity of the differential peaks ($n = 105$) belonging to each lipid class is represented (%). ND correspond to "not determined" when there was no proposition for the corresponding mass, and NA corresponds to "not attributable" when there were too many lipid propositions for the corresponding mass. The number of features participating in the composition of each class is noted on the pie chart; it varied between 2 and 50.

The embryonic lipid profile is also greatly modulated by the slow freezing protocol, meaning that the choice of the slow freezing media and/or kinetics strongly modifies the embryonic lipid composition (Figure 5). As demonstrated here, a large part of significantly different features had an EG-S/GLY-TRE FC >1.5 (70/105), indicating an enrichment of lipids in the in vitro-produced embryos frozen with EG-S protocol. They were especially enriched in glycerophospholipids, oxidized glycerophospholipids, and FA. On the other hand, in vitro-produced embryos frozen with GLY-TRE protocol contain fewer lipids that were mainly not identified, but they contained glycerophospholipids and sterols. As the embryos were produced similarly from the same seven Holstein heifers, we can therefore suppose that this variation comes from the freezing protocols, either by preserving the lipids from degradation or by providing them via the composition of the medium. The lipid profile of EG-S embryos was notably enriched in a single glycerophosphate, the diacylglycerophosphate PA(32:1), which was present in large quantities in EG-S embryos (2.16e−2, i.e., 27.3% of the significant lipids). Due to its abundance, one can speculate that it comes from a huge degradation occurring in GLY-TRE protocol, or it

could come from the freezing medium that could provide it or its precursors. Diacylglycerophosphate is a derivative of 1-acylglycerophosphate, which itself is a derivative of phosphatidic acid, known to be a common intermediate for the synthesis of triacylglycerol and glycerophospholipids [54]. We previously showed that the quantity of PA(32:1) was 6-fold higher in fresh in vivo-produced embryos compared with frozen ones using EG-S protocol [3]. It would therefore seem that, despite not being sufficient to prevent the degradation of PA(32:1) compared to fresh embryos, the EG-S protocol could be more suitable for the freezing of expanded bovine expanded blastocysts.

In the eukaryotic membrane, phospholipids are the most abundant lipids. Indeed, PI, PC, PE, and PG are structural units of the membrane [55], and their concentration determines most of the physicochemical cell membrane properties, like fluidity, permeability, and thermal phase behavior [56]. Therefore, we can speculate that the EG-S protocol is less harmful for glycerophospholipids than the GLY-TRE protocol (Supplementary Figure S1). Interestingly, whether the freezing media contained ethylene glycol or glycerol does not affect pregnancy rates. In fact, after direct transfer of frozen–thawed bovine embryos, pregnancy rates were not different between control group (69%) consisting of embryos frozen in 1.8 M ethylene glycol and embryos frozen with either 1.8 M ethylene glycol +0.25 M sucrose (52%), or 1.4 M glycerol +0.25 M sucrose (60%) after being equilibrated for 10–20 min in freezing solution [57].

Most of the recovery, holding and cryopreservation media of bovine embryos are commercially available and commonly contains animal derived products, such as FCS or BSA [58–61]. Although the mechanisms through which serum affects embryos have not yet been fully described, serum is known to cause lipid accumulation in embryos [6, 62] thus compromising their ability to metabolize lipids properly. This dysfunction is strongly correlated with apoptosis and cryosusceptibility [7, 52, 63, 64]. In our experiments, the GLY-TRE freezing media contained BSA and 20% of FCS while the EG-S protocol contained only BSA. FCS or BSA have surfactant properties, which reduce the surface tension in the medium, preventing embryos from sticking to plastic surfaces or floating at the surface thus facilitating their handling [59, 65, 66]. BSA can also be used for its anti-oxidant and chelator effects in embryo media, especially those made with water or containing salts that could possibly be contaminated by heavy metals [59]. The addition of serum or BSA seems to protect embryos from possible toxic effect of cryoprotectants during the cryopreservation protocol. Despite the advantages of using BSA and FCS in embryo media, their composition is not stable and varies between batches. They contain vitamins, growth factors, amino acids, energy substrate, and minerals, and they are known to exhibit a variable ability to support blastocyst development in culture [63, 67]. The literature does not allow us to conclude whether EG or GLY protocol is more effective since there are no/few pregnancy data available [57] and the studies comparing the EG and GLY protocols do not show any significant difference [68, 69] in survival and hatching rates. Because no other study compares these same two protocols and variations in the use of sugars, in the cryoprotectant concentration and in the temperature curve , it this therefore difficult to make proper comparisons. Therefore, future studies, in particular, embryo transfer, are needed to explore the functional consequences of different lipid profiles.

However, in view of our results, EG-S protocol allowed the preservation of glycerophospholipids compared to the GLY-TRE protocol. It might be possible that supplementing GLY-TRE with PA(32:1) leads to a reduction in the differences observed. Only transfer results comparing these two protocols would allow us to know whether this difference in lipid profile has a functional impact beyond the blastocyst stage. Moreover, we cannot discuss our results further regarding the freezing protocol effects compared with the literature because the present paper seems to be one of the first to describe the effects of these two freezing protocols on the in vitro-produced bovine embryonic lipid profile. However, we hypothesized that the EG-S protocol is more suitable for bovine blastocyst cryopreservation. Further studies should confirm the differences observed in this paper and should proceed to embryo transfer so that it could link embryo lipid differences to implantation or pregnancy success and therefore to embryo quality.

The lipid profile of in vitro-produced embryos was, in a lesser extent, affected by the donor's diet, particularly the addition of n-6 or n-3 PUFA. As reported in the present paper, despite the absence of differential lipids, the association of 69/515 lipids was able to predict to which group the embryos belonged with 64.4% probability. Researchers have shown that there was a significantly higher COC recovery rate for n-3 PUFA-supplemented cows compared with n-6 PUFA-supplemented cows (38.0% vs. 32.8%, respectively; diet effect $P = 0.0035$). These authors also highlighted an increase in the good-quality blastocyst rate in n-3 PUFA-supplemented cows compared with n-6 PUFA-supplemented cows (42.2% vs. 32.7%, respectively; diet effect $P = 0.0217$) [14]. The analysis of the lipid profile of their oocytes indicated that oocytes collected from n-3 PUFA-supplemented donors would be more suitable for obtaining good quality embryos. Oocytes from cows supplemented with n-3 or n-6 PUFA exhibited 110 differentially abundant features, including 42 that were identified: 12 PC, 3 PE, 2 SM, and 1 LPC were more abundant in n-3 PUFA oocytes, whereas 15 PC, PE(30:0), SM(34:1), 2 LPC, and 2 TG were more abundant in n-6 oocytes [14]. These results were partly confirmed by our experiments. Indeed, three TG were more abundant in n-6 embryos, while LPC, 1 LPE, and 2 FA were more abundant in n-3 embryos, which is a finding that suggests a greater level of lipolysis in n-3 embryos [19, 70]. However, the differences observed in embryonic lipid profiles were milder compared with the differences reported in oocytes, therefore, suggesting that the 7-days in vitro culture system could smooth out the lipid profile differences. Our previous work also showed that when embryos are produced in vitro, there was a decrease in the difference between fresh and frozen embryos (4/496), while greater differences were observed for embryos produced in vivo (35/496) [3], a phenomenon that would seem to be in favor of a smoothing the differences by the in vitro culture. We can therefore hypothesize that, as the diet did not impact the blastocyst lipid profile, there will be no difference in the transfer rate. To confirm this result, it would have been interesting to analyze the lipid profile of in vivo-produced embryos. In the same way, dietary rumen protected PUFA rich in LA induced an increase in PCe(38:2) only in vitrified embryo but did not affect the development rate and the re-expansion after devitrification

[71]. Modification of the diet seems sufficient to modify FA content in the follicular fluid, but in vitro supplementation should be considered when embryos are produced by OPU-IVF.

To conclude, we have provided interesting insights on the parameters leading to variations in the lipid profiles of in vitro-produced bovine embryos. The most important finding was that physiological stage as well as the whole freezing protocol strongly impacted the embryonic lipid profile unlike the addition of n-3 PUFA or n-6 PUFA to the donor diet.

Supplementary material

Supplementary material is available at *BIOLRE* online.

Conflict of interest

None of the authors declares any conflict of interest.

Data availability statement

All data described in the present paper are located within the manuscript and the supplementary data.

Authors' contributions statement

P.S., D.L.B., L.S., T.J., S.B., and P.E. conceived the experiment(s); S.J.I., D.L.B., O.D., L.L.B., and A.L. conducted the experiment(s); and S.J.I. analyzed the results. S.J.I. and S.E. wrote the main manuscript text. S.I.J., S.E., P.S., D.L.B., L.S., P.E., L.L.B., O.D., A.L., S.F., T.J., and S.B. reviewed the manuscript.

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