Dynamic changes and importance of plasma concentrations of ether phospholipids, of which the majority are plasmalogens, in postpartum Holstein dairy cows

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ABSTRACT

Context: Ethanolamine plasmalogens (EPls) and choline plasmalogens (CPls) are classes of ethanolamine ether phospholipids (ePE) or choline ether phospholipids (ePC), respectively. EPls play crucial roles in maternal and breastfed infant bodies and stimulate gonadotropin secretion by gonadotrophs.

Aims: To estimate changes in and importance of plasma concentrations of EPls and CPls, utilizing newly developed enzymatic fluorometric assays for ePE and ePC in postpartum Holstein cows.

Methods: Plasma samples were collected 3 weeks before expected parturition until approximately 8 weeks after parturition (16 primiparous and 38 multiparous cows) for analysis.

Key results: Plasma concentrations of ePE and ePC, most of which are plasmalogens, declined before and increased after parturition and stabilised near the day of the first postpartum ovulation (1stOV). From weeks 2 to 3 after parturition, third-parity cows exhibited ePE concentrations that were higher than those of other parity cows. The days from parturition to 1stOV correlated with days from parturition to conception. On the day of 1stOV, milk yield correlated with plasma concentration of both ePE and ePC, while ePC concentration correlated negatively with milk fat percentage. At the early luteal phase after 1stOV, plasma ePE concentration correlated with plasma anti-Müllerian hormone concentration ($r = 0.39$, $P < 0.01$), and plasma ePC concentration correlated with plasma follicle-stimulating hormone concentration ($r = 0.43$, $P < 0.01$).

Conclusion: The concentrations of ePE and ePC changed dramatically around parturition and 1stOV, and then the concentrations correlated with the important parameters.

Implications: The blood plasmalogen may play important roles in postpartum dairy cows.

Keywords: aging, anti-Müllerian hormone, choline plasmalogen, conception, ethanolamine plasmalogens, first postpartum ovulation, functional lipids, FSH, G protein-coupled receptor 61, GPR61, LH, parity, phospholipase A_1, postpartum Holstein dairy cows, ruminant.

Introduction

Early re-establishment of postpartum ovarian activity is an important prerequisite for high fertility in dairy cows (Senatore *et al.* 1996; Darwash *et al.* 1997; Galvão *et al.* 2010). Especially, cows with postpartum ovulation by three weeks postpartum have been shown to be more fertile than other cows (Galvão *et al.* 2010). The most important factor in delaying the first postpartum ovulation (1stOV) is negative energy balance, and various metabolic protein hormones may be a molecular link between active milk production and suppressed reproductive function (Kadokawa *et al.* 2000, 2006; Rhoads *et al.* 2008; Subramaniam *et al.* 2016; Banuelos and Stevenson 2021). However, there could be other important molecular links between active milk production and suppressed reproductive function, because diet containing rumen-protected fish oil supplement, rich in n-3 polyunsaturated fatty acid, improves fertility via unknown mechanisms in postpartum dairy cows (Elis *et al.* 2016). Sufficient data explaining the whole mechanism is not available because of the lack of information on functional lipids (Almsherqi 2021).

Ethanolamine plasmalogens (EPls; alkenyl-acyl-phosphatidylethanolamines) are a class of ethanolamine ether phospholipids (ePE), and choline plasmalogens (CPls; alkenyl-acyl-phosphatidylcholines) are a class of choline ether phospholipid (ePC). Recent studies revealed that EPls play various crucial roles, including as endogenous antioxidants, immune modulators, and neuronal protectors (Almsherqi 2021). The maternal body supplies EPls to new-born animals through milk (Moukarzel *et al*. 2016; Liu *et al.* 2020). Orally supplied plasmalogens are adsorbed from the intestine via various mechanisms to rapidly increase plasmalogen in both the blood and brain (Nishimukai *et al*. 2003; Takahashi *et al.* 2020; Smith *et al.* 2022) for the various crucial roles, including normal neurodevelopment (Hiratsuka *et al.* 2013; Li *et al.* 2022). However, little is known about the plasmalogens in domestic animals.

Brain EPls have another novel role as ligands of G protein-coupled receptor 61 (GPR61), a recently discovered receptor that stimulates gonadotropin secretion by bovine gonadotrophs in the anterior pituitary (Pandey *et al.* 2017; Kereilwe *et al.* 2018a; Kadokawa *et al.* 2022a). The secretion of gonadotropins, luteinizing hormone (LH**)**, and follicle-stimulating hormone (FSH) from gonadotrophs is the key driver of the resumption of ovulation during the postpartum period (Beam and Butler 1997; Kadokawa *et al.* 2000). Therefore, the blood EPl concentration may be related to the 1stOV in postpartum dairy cows.

We recently reported the age-related quality degradation of bovine brain EPls; EPls extracted from the brains of aged (approximately 90 months old) cows could not stimulate gonadotrophs to secrete FSH in the absence of gonadotropin-releasing hormone (GnRH) unlike EPls extracted from the brains of young (approximately 26 months old), healthy heifers (Kadokawa *et al.* 2021). However, no data on age-related differences in blood concentrations in domestic animals are available.

After aging, patients with neuronal diseases (e.g., Alzheimer's and Parkinson's) have decreased plasma EPl levels (Fujino *et al.* 2017). Plasma EPl and choline plasmalogens (CPl) levels were studied using thin-layer or liquid chromatography with

tandem mass spectrometry (Mawatari *et al.* 2018; Morita *et al.* 2020). However, it is difficult to measure a large number of samples using these methods. Recently, enzymatic fluorometric assays have been developed to measure the plasma concentrations of ePEs or ePCs to monitor human blood levels in in vivo and in vitro studies (Mawatari *et al.* 2018; Morita *et al.* 2020). These enzymatic fluorometric assays are more sensitive than those using thin-layer or liquid chromatography with tandem mass spectrometry (Morita *et al.* 2020).

Various organs in prepartum dairy cows begin to prepare for lactation, and the organs in postpartum cows must produce high amounts of milk for new-born calves. To the best of our knowledge, blood plasmalogen concentrations have not yet been measured in domestic animals. Although, there are no previous studies on the direct effect of CPls on gonadotrophs, it is known that EPls and CPls are remodelled to each other by enzymatic polar head group remodelling (Dorninger *et al*. 2022). Therefore, we estimated changes and importance of plasma concentrations of EPls and CPls, utilizing newly developed enzymatic fluorometric assays for ePE and ePC in postpartum Holstein cows. We also evaluated a hypothesis that higher parity cows have lower plasma concentrations of ePE and ePC. We also evaluated whether milk production or reproduction parameters correlate with plasma concentrations of ePE and ePC.

Materials and methods

Animals and treatments

All experiments were performed according to the Guiding Principles for the Care and Use of Experimental Animals in the Field of Physiological Sciences (Physiological Society of Japan) and approved by the Committee on Animal Experiments of Yamaguchi University and the Hokkaido Research Organization. A total of 54 Holstein dairy cows (first-parity cows [n = 16], second-parity cows [n = 16], third-parity cows [n = 11], and fourth- or higher-parity cows [n = 11]) were housed at the Animal Center of the Hokkaido Research Organization's Dairy Research Center. Care and sampling were carried out following the Guide for the Care and Use of Agricultural Animals in Agricultural Research and Teaching (Consortium for Developing a Guide for the Care and Use of Agricultural Animals in Agricultural Research and Teaching, 1995).

The calves were housed individually in calf stalls and reared using the feeding regimen to meet the growth requirements per the Japanese Feeding Standard for Dairy Cattle (Agriculture, Forestry and Fisheries Research Council Secretariat, 2017). After weaning 42 days after birth, they were moved to free stalls and fed concentrate and grass silage per the Japanese Feeding Standard for Dairy Cattle. Their body weight and withers height were measured monthly. Artificial insemination was conducted when the body weight and withers height of heifers exceeded 350 kg and 125 cm, respectively (the average age of heifers was 13.4 ± 0.4 months). Heifers were also reared in free stalls after conception.

Pregnant heifers and cows [gestation period of nine months (Pajohande *et al.* 2023)] were fed either a prepartum or early lactation period total mixed ration of grass

silage (mainly timothy), corn silage, flaked corn, soybean meal, precipitated calcium carbonate, and dicalcium phosphate, based on the Japanese Feeding Standards for Dairy Cattle (Agriculture, Forestry and Fisheries Research Council Secretariat, 2017). The formulation and chemical composition of the total mixed ration are listed in Table 1. Feed intake was measured daily using automated feeders (Roughage Intake Control, Insentec BV, Marknesse, Netherlands). Water and mineral blocks (Koen-S, Nippon Zenyaku Kogyo, Co., Ltd., Fukushima, Japan) were freely available. The daily metabolizable energy (ME) intake was calculated from the dry matter intake and ME of the total mixed ration. Cows were milked twice daily at 09:00 and 19:00. Milk yield and feed intake were measured daily, body weight and body condition score on a 5-point scale (Ferguson *et al.* 1994) were measured weekly, and milk components (fat %, protein %, and lactose %) were measured weekly using an infrared milk analyser (MilkoScan FT2, Foss Electric, Hillerød, Denmark). The averages of morning and evening measurements were used as the daily values. Energy balance was calculated weekly as the difference between the ME intake and the ME requirement according to the Japanese Feeding Standard. This was the sum of the ME requirements for maintenance (0.1163 × metabolic body weight) and lactation, based on the equation: (0.0913 × milk fat content + 0.3678) × milk yield × 1.613.

Jugular blood was sampled into heparinised tubes once per week at 10:00–11:00 from 3 weeks before expected parturition until approximately 8 weeks after parturition. Samples were centrifuged at 1,000 × *g* for 30 min at 4 °C and stored at −35 °C until analysis.

All animals were subjected to a routine health examination, and their reproductive tracts were palpated at least thrice per week using a real-time linear array ultrasound scanner (HS-1600V; Honda Electronics, Aichi, Japan) equipped with a 7.5-MHz rectal probe (HLV-875M; Honda Electronics), beginning on days 6–8 postpartum and continuing until the second postpartum ovulation.

After 50 days of a voluntary waiting period, cows received artificial insemination only after apparent oestrus. Hormonal treatment was not performed until 70 days after parturition for cows with an ovarian disease, including follicular cysts, luteal cysts, ovarian quiescence, and retained corpus luteum. Although dairy farmers want their cows to give birth once every year, it is difficult because they must get pregnant only 85 days after parturition, especially for cows with high parity (Terawaki and Ducrocq, 2009). Hence, a total of 48 Holstein dairy cows [first-parity cows (n = 14), second-parity cows (n = 14), third-parity cows (n = 10), and fourth- or higher-parity cows (n = 10)] were pregnant by 200 days after parturition.

Preparation of ePE standard

All organic solvents were of HPLC grade and purchased from Fujifilm Wako Pure Chemical Corporation (Osaka, Japan). As reported previously, highly purified brain lipids were used to prepare ePE assay standards (Kadokawa *et al.* 2021). The extracted lipids were treated with phospholipase A_1 to remove diacyl phosphatidylethanolamine and diacyl phosphatidylcholine. Briefly, five whole brains of fertile young Japanese Black heifers were minced in a food processor (DLC-NXJ2PS,

Conair Japan G. K., Tokyo, Japan), pooled, frozen at −80 °C, and vacuum-dried (ADP200, Yamato Scientific Co. Ltd., Tokyo, Japan). Vacuum-dried brain tissue was extracted by incubation in ethanol (brain tissue/ethanol, 1:10 (v/v)) at 40 °C for 8 h with shaking. After centrifugation at 10,800 × *g* for 1 h at 25 °C, the supernatant was collected and dried using a rotary evaporator (N2110; Tokyo Rikakikai Co. LTD., Tokyo, Japan). The remaining lipids were collected in several 50 mL glass centrifuge tubes and dissolved in diluted acetone (acetone/water, 2:1 (v/v); lipids/diluted acetone, 1:10 (v/v)). After incubating at 4 °C for 1 h, the solutions were centrifuged at 1,200 × *g* for 20 min at 4 °C, and the supernatant was removed. The remaining precipitates were dissolved in diluted acetone (acetone/water, 1:1 (v/v); precipitate/diluted acetone, 1:10 (v/v)). The solutions were centrifuged at 1,200 × *g* for 20 min at 4 °C to collect the precipitates, which were subsequently mixed with cold acetone (precipitate/acetone, 1:10 (v/v)) and stored at –20 °C overnight. Thereafter, the acetone-treated precipitates were centrifuged at 1,200 × *g* for 20 min at 4 °C, and the supernatant was removed. The remaining precipitates were dissolved in a hexane and acetone mixture (hexane/acetone, 7:3 (v/v); precipitate/mixture, 1:10 (v/v)). This solution was subjected to centrifugation at 1,200 × *g* for 30 min at 4 °C, and the supernatant was collected into a flask and dried using a rotary evaporator. After evaporation, the remaining lipids were treated with 20 mg/mL phospholipase A_1 (Enzyme commission number 3.1.1.32; 10,000–13,000 units/g; Mitsubishi Kagaku and Foods Co., Tokyo, Japan) dissolved in 0.1 M citric acid buffer (pH 4.5) at a volume ratio of 1:10, under a low oxygen atmosphere (air pressure, 50 kPa) and at 50 °C in a rotary evaporator flask for 2 h. Subsequently, the enzyme-treated sample was mixed with a 1:1 (v/v) mixture of hexane and acetone (sample/mixture, 1:6 (v/v)) and transferred to a separating funnel for collection of the upper layer. This extraction was repeated two more times. The upper layer was transferred to a flask for drying using a rotary evaporator. After evaporation, the residual lipids were dissolved in acetone (lipids/acetone, 1:10 (v/v)) in glass tubes and stored at –20 °C overnight. Subsequently, the solutions were centrifuged at 1,200 × *g* for 20 min at 4 °C to collect the precipitates, which were washed with acetone (precipitate/acetone, 1:10 (v/v)), and recentrifuged at 1,200 × *g* for 20 min at 4 °C. The resulting precipitates were dissolved in a hexane and acetone mixture (hexane/acetone, 7:3 (v/v); precipitate/mixture, 1:10 (v/v)). The solutions were centrifuged at 1,200 × *g* for 20 min at 4 °C, and the supernatant was collected for evaporation. After evaporation, the remaining lipids were dissolved in a mixture of hexane, acetone, and water [hexane/acetone/water, 3:3:1 (v/v/v); lipids/mixture, 1:10 (v/v)] and transferred to a separating funnel. After shaking the separating funnel, the upper layer was collected. This extraction was repeated two more times. The combined upper layer extracts were evaporated using a rotary evaporator to obtain ePE-rich lipids. Aliquots of these lipids were vacuum-packed and stored at –35 °C prior to analysis. The lipids were dissolved at a final concentration of 1.0 mg/mL in dilution buffer (0.75 mM $CaCl_2$, 0.5% Triton X-100, 50 mM NaCl, and 50 mM Tris-HCl, pH 7.4), then sequentially diluted in assay buffer (0.75 mM $CaCl_2$, 0.1% Triton X-100, 50 mM NaCl, and 50 mM Tris-HCl, pH 7.4) to prepare the 0.5–120 μg/mL ePE standards.

Preparation of ePC standard

Purified plasma ePC from heifers was used to prepare ePC assay standards, according to Mawatari *et al.* (2018). The extracted lipids were treated with phospholipase A_1 to remove diacyl phosphatidylethanolamine and diacyl phosphatidylcholine. Briefly, 40 mL of plasma (mixture of plasma from 20 heifers) were diluted in 40 mL of 0.1 M citrate buffer (pH 4.5), mixed with 20 mL of 0.1 M citric acid buffer (pH 4.5) containing 50 mg/mL of the phospholipase A_1, and incubated at 45 ℃ for 1 h. The phospholipase A_1 treated plasma was mixed well with 800 mL of hexane and isopropanol mixture (hexane/isopropanol, 3:2 (v/v)). After adding 400 mL of 66.6 mg/mL anhydrous sodium sulfate solution, 400 mL of the upper hexane layer was harvested. The remaining lower layer was mixed with 400 mL of hexane and isopropanol mixture (hexane/isopropanol, 7:2 (v/v)) for re-extraction and harvested. The combined hexane layer was aliquoted, dried under nitrogen gas, and stored at −35 °C until use. The lipids were dissolved at a final concentration of 1.0 mg/mL in the dilution buffer, then sequentially diluted in the assay buffer to prepare the 0.5–120 μg/mL ePC standards.

HPLC analysis for ePE and ePC standards

The ePE and ePC standards were analysed using a previously reported normal-phase HPLC system with a charged aerosol detector to identify the lipids (Takahashi *et al.* 2018). The separation was performed using a YMC-Pack PVA-Sil (250 mm length (L) × 4.6 mm internal diameter, 5-μm column; YMC Co. Ltd., Kyoto, Japan). The HPLC separation temperature and flow rate were set to 30 °C and 1.0 mL/min, respectively. The lipid sample was prepared at a concentration of 5 mg/mL in chloroform/methanol (2:1 (v/v)), and a 0.02 mL aliquot was injected into the HPLC system. Mobile phases, A, B, and C were hexane, 2-methoxy-2-methylpropane, and methanol, respectively. The solvent gradient program was as follows: 0–7 min A/B/C 88%/10%/2%; 7–12 min A/B/C 2%/88%/10%; 12–22 min A/B/C 2%/28%/70%; 22–32 min A/B/C 2%/28%/70%; and 32–35 min A/B/C 88%/10%/2%. The separation profile was monitored at 210 nm using a variable-wavelength detector. The charged aerosol detector's acquisition range and nitrogen gas pressure were 500 pA and 241.3 kPa, respectively. Each sample was analysed in triplicate, and each relative standard deviation of the retention time and peak area was less than 0.05% and 0.93%, respectively.

Extraction of lipids from plasma samples

Lipid samples were extracted from the plasma samples according to Mawatari *et al.* (2018) and treated with phospholipase A_1. Briefly, 80 μL of plasma was diluted in 80 μL of 0.1 M citrate buffer (pH 4.5) and mixed with 40 μL of 0.1 M citric acid buffer (pH 4.5) containing 50 mg/mL of the phospholipase A_1 and incubated at 45 ℃ for 1 h. The phospholipase A_1 treated plasma was mixed well with 1.6 mL of hexane/isopropanol (3:2 [v/v]). Then 0.8 mL of 66.6 mg/mL anhydrous sodium sulfate

solution was added. The upper hexane layer was then harvested. The remaining lower layer was mixed with 0.8 mL of hexane and isopropanol (hexane/isopropanol, 7:2 [v/v]) for re-extraction and harvesting. The combined hexane layer was dried under nitrogen gas and stored at −35 °C until analysis.

Estimation of the EPl:ePE or CPl:ePC ratios in plasma lipids

To estimate the EPl:ePE or CPl:ePC ratios, the ePE or ePC concentrations between lipid samples that underwent only phospholipase A_1 hydrolysis and those that underwent both phospholipase A_1 and HCl hydrolysis were compared (Mawatari *et al.* 2007; Kadokawa *et al.* 2022b). Briefly, dried lipid samples extracted from either a mixture of six randomly selected plasma samples collected on the day of 1stOV or a mixture of six randomly selected plasma samples collected 7 weeks after parturition were treated with the phospholipase A_1 treatment and dissolved in 4 mL of methanol/chloroform/water (2:1:1 [v/v/v]). Half of the dissolved lipids were mixed with 4 mL of methanol/water (1:1 [v/v]) in a glass tube, while the remaining half of dissolved lipids were mixed with 4 mL of methanol/1 N HCl (1:1 [v/v]) in another glass tube. After vigorous mixing, the mixture was incubated at room temperature for 1 h. After 2 mL of chloroform and 2 mL of water were added, the tubes were centrifuged at 1,200 × *g* for 10 min. The chloroform layer was harvested for drying under nitrogen gas.

Before injection into the HPLC system, the plasma lipid samples were reconstituted with chloroform/methanol (2:1 [v/v]). The separation was performed using a LiChrospher 100Diol (250 mm length (L) × 4 mm internal diameter, 5-μm column) (No. 937855, Merck Millipore Co. Ltd., Tokyo, Japan). The HPLC separation temperature and flow rate were set to 50 ℃ and 1.0 mL/min, respectively. Mobile phase A was hexane/isopropanol/acetic acid (82:17:1 [v/v/v]), and mobile phase B was isopropanol/water/acetic acid (85:14:1 [v/v/v]) with 0.2% TEA. Mobile phase A was 95% at 0 min and decreased linearly to 60% at 23 min. The solvent gradient program was as follows: 0–1 min A/B 95%/5%; 1–24 min A/B 60%/40%; 24–25.5 min A/B 60%/40%; and 25.5–28 min A/B 100%/0%. The charged aerosol detector's acquisition range and nitrogen gas pressure were 500 pA and 241.3 kPa, respectively. Each sample was analysed in triplicate, and each relative standard deviation of the retention time and peak area was less than 0.05% and 0.93%, respectively.

The HPLC method can differentiate ether-phosphatidylethanolamines from diacyl-phosphatidylethanolamines and ether-phosphatidylcholine from diacyl phosphatidylcholine in a single chromatography run. However, the HPLC method cannot distinguish alkenyl-acyl-phosphatidylethanolamines (EPls) from alkyl-acyl-phosphatidylethanolamines, or alkenyl-acyl-phosphatidylcholines (CPls) from alkyl-acyl-phosphatidylcholines. The peak remaining after the HCl hydrolysis are alkenyl-acyl-phospholipids (Mawatari *et al.* 2007). Sphingolipids are not hydrolysed by either phospholipase A1 or HCl (Murphy *et al.* 1993). Therefore, we calculated the ratios as follows:

EP:S ratio after phospholipase A1 hydrolysis: ratio of peak area of total ePE after

phospholipase A1 hydrolysis to the peak area of sphingomyelin after phospholipase A1 hydrolysis

EP:S ratio after phospholipase A1 and HCl hydrolysis: ratio of peak area of total ePE after phospholipase A1 and HCl hydrolysis to the peak area of sphingomyelin after phospholipase A1 and HCl hydrolysis

CP:S ratio after phospholipase A1 hydrolysis: ratio of peak area of total ePC after phospholipase A1 hydrolysis to the peak area of sphingomyelin after phospholipase A1 hydrolysis

CP:S ratio after phospholipase A1 and HCl hydrolysis: ratio of peak area of total ePC after phospholipase A1 and HCl hydrolysis to the peak area of sphingomyelin after phospholipase A1 and HCl hydrolysis

Therefore, EPl and CPl purities were calculated as follows:

EPl purity = 100 − (100 × "EP to S ratio after phospholipase A1 and HCl hydrolysis" / "EP to S ratio after phospholipase A1 hydrolysis)"

CPl purity = 100 − (100 × "CP to S ratio after phospholipase A1 and HCl hydrolysis" / "CP to S ratio after phospholipase A1 hydrolysis)".

Enzymatic fluorometric assays for ePE and ePC

Recently developed enzymatic assays for ePE and ePC (Mawatari *et al*. 2018; Morita *et al.* 2020) was used with minor modifications. The principle of the assay is as follows: (1) glycerophospholipid-specific phospholipase D is used to hydrolyse ePE or ePC to produce ethanolamine or choline; (2) amine oxidase or choline oxidase is used to oxidise ethanolamine or choline to produce hydrogen peroxide; (3) the amount of hydrogen peroxide produced was measured by a fluorescence microplate reader after reaction with peroxidase and 10-acetyl-3,7-dihydroxyphenoxazine (Amplex Red; A12222, Thermo Fisher Scientific, Waltham, MA, USA) to produce fluorescent resorufin.

The dried lipid extract sample was dissolved in 500 μL of 0.5% Triton X-100 dissolved in assay buffer immediately before use.

To evaluate parallelism and sample volume for the ePE assay, triplicate 50 μL volumes of 10 ePE standards (0 μg/mL and 0.5–120 μg/mL) and various volumes of plasma lipid samples diluted with assay buffer (6.25–100 μL of lipid samples extracted from a mixture of 20 randomly selected plasma samples; brought to a final volume of 50 μL, except for the case of 100 μL sample) were added into the wells of a fluorescence grade black microplate (MS-8596K, Sumitomo Bakelite Co. Ltd., Tokyo, Japan). Each well received 50 μL of assay buffer containing 15.2 U of phospholipase D (from *Streptomyces chromofuscus*; Enzyme commission number 3.1.4.4; T-222, Asahi Kasei Pharma, Tokyo, Japan). The microplate was sealed, shaken briefly, spun down, and incubated at 37 °C for 30 min. Thereafter, 100 μL of assay buffer containing 8 U/mL of tyramine oxidase (from *Arthrobacter* sp.; Enzyme commission number 1.4.3.4; T-25, Asahi Kasei Pharma), 5 U/mL horseradish peroxidase (Enzyme commission number 1.11.1.7; 303-50991, Oriental Yeast Co. Ltd., Tokyo, Japan), and 50 μL Amplex Red (A12222, Thermo Fisher Scientific) were added to each well. The microplate was sealed, shaken briefly, spun down, and incubated at 37 °C for 30 min. Lastly, 20 μL Amplex

Red/UltraRed Stop Reagent (A33855, Thermo Fisher Scientific) was added to each well. Fluorescence intensity was measured using a fluorescence microplate reader (Arvo X4; Perkin Elmer Japan, Tokyo, Japan). The excitation and emission wavelengths were set to 540 and 615 nm, respectively. In this assay, other amine-containing phospholipids, phosphatidylcholine, and phosphatidylserine did not increase fluorescence (Morita *et al*. 2020). After developing the ePE assay, duplicate 50 μL volumes of eight standards (0 ng/mL and 1.9–120 μg/mL) or plasma lipid samples were measured in routine assays.

To evaluate parallelism and determine the sample volume for the ePC assay, triplicate 50 μL volumes of 10 ePC standards (0 ng/mL and 0.5–120 μg/mL) and various volumes of plasma lipid samples diluted with assay buffer (3.1–50 μL of lipid samples extracted from a mixture of 20 randomly selected plasma samples; brought to a final volume of 50 μL) were added to wells of the 96-well microplates. Then, each well received 87.5 μL assay buffer containing 1.0 U of phospholipase D (from *Streptomyces* sp.; Enzyme commission number 3.1.4.4; T-138, Asahi Kasei Pharma). The microplate was sealed, shaken briefly, spun down, and incubated at 37 °C for 30 min. Then, 100 μL of assay buffer containing 1 U/mL of choline oxidase (from *Arthrobacter globiformis*; Enzyme Commission Number 1.1.3.17; T-05, Asahi Kasei Pharma), 5 U/mL horseradish peroxidase, and 50 μM Amplex Red were added to each well. The microplate was sealed, shaken briefly, spun down, and incubated at 37 °C for 30 min. Lastly, 20 μL of Amplex Red/Ultrared stop reagent was added to each well. The fluorescence intensity was measured using a fluorescence microplate reader at the same excitation and emission wavelengths mentioned above. In this assay, other choline-containing phospholipids, sphingomyelin, and lysophosphatidylcholine did not increase the fluorescence (Morita *et al.* 2020). After developing the ePC assay, duplicate 50 μL volumes of 8 standards (0 ng/mL and 1.9–120 μg/ mL) or 12.5 μL of plasma lipid samples plus 37.5 μL assay buffer were measured in routine assays.

For the ePE and ePC assays, sample concentrations were determined by analysing the fluorescence intensity data with appropriate software (Microplate Manager Software Version 6.3, Bio-rad, Hercules, CA, USA). Lipid samples were extracted from 80 μL of plasma and dissolved in 500 μL of assay buffer. Thus, the ePE concentration was shown to increase by 6.25 times from the calculated concentration. In the ePC assay, the volume of the standards was 50 μL, whereas the volume of the samples was 12.5 μL. Thus, the ePC concentration needed to be increased four times; then, the ePC concentration was shown to increase by 25 times from the calculated concentration.

Assays for hormones and metabolites

We also measured plasma concentrations of LH, FSH, anti-Müllerian hormone (AMH), and metabolites at the early luteal phase (a few days after ovulation). This phase was selected for the following reasons: (1) both LH and FSH secretion are active (Walters *et al.* 1984), and (2) blood AMH concentration is not changed during oestrus cycles (Rico *et al.* 2011; Koizumi and Kadokawa 2017).

Plasma LH concentrations were assayed in duplicate using a double antibody radioimmunoassay (RIA) with ^{125}I-labelled bLH and anti-oLH-antiserum (AFP11743B

and AFP192279, National Hormone and Pituitary Program of the National Institute of Diabetes and Digestive and Kidney Diseases [NIDDK], Bethesda, CA, USA). The limit of detection was 0.40 ng/mL. At 2.04 ng/mL, the intra- and inter-assay coefficient of variation (CV) were 3.6% and 6.2%, respectively.

Plasma FSH concentrations were assayed in duplicate using a double antibody RIA with ^{125}I-labelled bFSH, reference grade bFSH, and anti-oFSH antiserum (AFP5318C, AFP5346D, and AFPC5288113, NIDDK). The limit of detection was 0.20 ng/mL. At 4.00 ng/mL, the intra- and inter-assay CVs were 4.3% and 7.1%, respectively.

Plasma AMH concentrations were assessed using a bovine AMH ELISA kit (Ansh Labs, TX, USA). The detection limit was 11 pg/mL, and the intra- and inter-assay CVs were 4.3% and 8.6%, respectively.

An autoanalyzer (Model 3100, Hitachi High-Tech Corporation, Tokyo, Japan) was used to measure total protein, aspartate transaminase (AST), total cholesterol, blood urea nitrogen (BUN), and glucose plasma concentrations using commercial kits (LSIMedience Corporation, Tokyo, Japan). The autoanalyzer was also used to measure plasma concentrations of non-esterified fatty acids and beta-hydroxybutyric acid using commercial kits (Fujifilm Wako Pure Chemical Corporation).

Statistical analyses

Data were analysed using StatView version 5.0 for Windows (SAS Institute, Inc., Cary, NC, USA). Analysis of variance (ANOVA) followed by the Tukey–Kramer test was performed to evaluate the effect of parity (first-, second-, third-, or higher-parity) on the number of days from parturition to 1stOV and 305-day milk yield. This analysis used a total of 54 cows [first-parity cows (n = 16), second-parity cows (n = 16), third-parity cows (n = 11), and fourth- or higher-parity cows (n = 11)].

The Shapiro–Wilk's W test or Kolmogrov–Smirnov Lilliefors test were used to evaluate the normality or log-normality of the distribution of each variable. All variables were normally distributed, and there were no outliers in any variables (Grubb's test).

Repeated-measure ANOVA was performed to evaluate the effect of time, parity, and the interaction between time and parity on body weight, daily milk yield, milk fat percentage, dry matter intake, energy balance, and body condition scores from parturition to 8 weeks after parturition in the 54 cows.

Repeated-measures ANOVA was also performed to evaluate the effect of time, parity, and the interaction between time and parity on plasma ePE or ePC concentrations from 2 weeks before to 8 weeks after parturition in the 54 cows. Additionally, ANOVA followed by the Tukey–Kramer test was performed to evaluate the effect of parity (first-, second-, third-, or higher-parity) on ePE or ePC at each time point.

Repeated-measures ANOVA was performed to evaluate the effect of time, parity (first-, second-, third-, or higher-parity), and the interaction between time and parity on plasma EPl or CPl concentrations from 2 weeks before to 2 weeks after the 1stOV (period around 1stOV). This analysis excluded cows not showing the 1stOV by the 5th postpartum week. Thus, this analysis used a total of 42 cows [first-parity cows (n = 12), second-parity cows (n = 10), third-parity cows (n = 10), and fourth- or higher-parity cows

(n = 10)]. ANOVA followed by the Tukey–Kramer test was also performed to evaluate the effect of parity (first, second, third, or higher-parity) on ePE or ePC at each time point.

Pearson's correlation analysis was used to evaluate the relationship between the plasma ePE or ePC concentration on the day of the 1stOV and the age in months on the day of the 1stOV. This analysis excluded cows that did not show the 1stOV by the 8th postpartum week, because pre-ovulation values are not consider as representative of each individual owing to the unstable metabolic condition in the pre-ovulation period (Kadokawa *et al.* 2000, 2006; Rhoads *et al.* 2008; Subramaniam *et al.* 2016; Banuelos and Stevenson 2021). Thus, this analysis used a total of 51 cows [first-parity cows (n = 16), second-parity cows (n = 15), third-parity cows (n = 10), and fourth- or higher-parity cows (n = 10)]. Additionally, analysis of covariance (ANCOVA) was performed to evaluate the effect of parity; the effect of either body weight, daily milk yield, milk fat percentage, dry matter intake, energy balance, or plasma concentration of metabolites; and the interaction in the 51 cows.

ANCOVA was performed to evaluate the effect of parity, the effect of plasma ePC concentration, and the interaction on plasma ePE concentration at the early luteal phase after the 1stOV. Pearson's correlation analysis was used to evaluate the relationship between the both concentrations at the early luteal phase in the 51 cows.

ANCOVA was performed to evaluate the effect of parity, the effect of plasma ePE or ePC concentration, and the interaction on plasma LH, FSH, or AMH concentration at the early luteal phase in the 51 cows. Pearson's correlation analysis was used to evaluate the relationship between the plasma ePE or ePC concentration and plasma LH, FSH, or AMH concentration at the early luteal phase in the 51 cows.

ANCOVA was performed to evaluate the effect of parity, the effect of days from parturition to 1stOV, and the interaction on days from parturition to conception in the 48 Holstein dairy cows that became pregnant by 200 days after parturition [first-parity cows (n = 14), second-parity cows (n = 14), third-parity cows (n = 10), and fourth- or higher-parity cows (n = 10)]. Pearson's correlation analysis was used to evaluate the relationship between both days in the 48 cows.

The level of significance was set at $P < 0.05$. Data are expressed as mean ± standard error of the mean.

Results

HPLC analyses for the lipids extracted from bovine brain and plasma

Figure 1 presents examples of the HPLC profile of (1) extracted bovine brain lipids, which were diluted for use as ePE standards, (2) the extracted plasma lipids, which were diluted for use as ePC standards, and (3) 12 lipid standards.

As shown in Table 1, the most prevalent major lipid in the brain was cholesterol; the ratio of the peak area to the total peak area was 27.9%. The ePEs were the second most detected major lipids; the ratio of the peak area to total peak area was 17.6%. The ratio of ePC peak area to total peak area was 2.2%.

The most prevalent major lipid in the plasma was triacylglyceride (TAG); the ratio of the peak area to the total peak area was 36.7%. The ePC was the fourth most

492 detected major lipid; the ratio of its peak area to total peak area was 6.5%. The ratio of
493 ePE peak area to total peak area was 2.4%.
494 In the mixture of randomly selected plasma samples collected on the day of
495 1stOV, 100.0 ± 0.0 % of ePE were EPls, and 57.4 ± 0.5 % of ePC were CPls; the
496 remaining 42.6 ± 0.5 % were alkyl-acyl-cholineglycerophospholipids. In the mixture of
497 randomly selected plasma samples collected at 7 weeks after parturition, 100.0 ± 0.0 %
498 of ePE were EPls, and 60.3 ± 0.4 % of ePC were CPls; the remaining 39.7 ± 0.4 % were
499 alkyl-acyl-cholineglycerophospholipids.
500
501 *Enzymatic fluorometric assays developed to measure plasma ePE or ePC*
502 *concentrations*

503 Figure 2 depicts the good parallelism between the ePE or ePC standard curve and
504 serially diluted plasma lipids. The detection limit of the ePE assay was 2.49 μg/mL. The
505 intra- and inter-assay CVs at 30 μg/mL were.3% and 0.7%, respectively. The detection
506 limit of the ePC assay was 2.18 μg/mL. The intra- and inter-assay CVs at 30 μg/mL were
507 0.2% and 0.3%, respectively.

508
509 *Conditions of cows and the days from parturition to 1stOV*
510 Figure 3 shows the effect of time and parity on other parameters. The effect of
511 time was significant on body weight (Figure 3A), daily milk yield (Figure 3B), milk fat
512 percentage (Figure 3C), dry matter intake (Figure 3D), energy balance (Figure 3E), and
513 body condition score (Figure 3F). The effect of parity was significant on body weight
514 (Figure 3A), daily milk yield (Figure 3B), dry matter intake (Figure 3D), and body
515 condition score (Figure 3F), but not milk fat percentage (Figure 3C) or energy balance
516 (Figure 3E). There were no differences among parities in the days from parturition to the
517 1stOV (Figure 3G). The first- and second-parity cows had a lower 305-day milk yield
518 than the higher-parity cows (Figure 3H).
519
520 *Changes in plasma concentrations of ePE and ePC in cows*
521 Figure 4 shows the changes in the plasma concentrations of ePE and ePC in two
522 representative cows that had their 1stOV on either day 16 or 58. The cow that ovulated
523 on day 58 developed follicular cysts after parturition, recovered spontaneously, and then
524 ovulated. The plasma concentrations of ePE were lower around the day of parturition
525 than day of 1stOV in the normal cows (Figure 4A, 4C). They remained low for a variable
526 length of time and then increased in all normal cows. However, both ePE and ePC
527 concentrations remained low in the cows which developed postpartum follicular cysts
528 (Figure 4B, 4D).
529 Figure 5 shows changes in mean ePE and ePC levels across all cows, with timing
530 synchronised around parturition. Both levels were low around parturition, then increased.
531 The effect of parity on ePE and ePC was significant. The third-parity cows had higher
532 ePE concentrations 2 to 6 weeks after parturition than other parity cows. Although the

effect of parity on plasma ePC concentrations was not significant based on repeated-measures ANOVA, the ANOVA followed by the Tukey–Kramer test for each time point revealed that the second-parity cows had higher ePC concentrations than the first-parity cows in the 1 week before to 5 weeks after parturition, except for the 4th week.

Figure 6 shows changes in mean ePE and ePC levels across all cows, with timing synchronised around the 1stOV. The effects of time and parity on ePE and the effect of time on ePC were significant.

Relationships between plasma ePE or ePC concentrations and age, body weight, milk production, and nutritional condition

As shown in Figures 7A and 8A, there was no significant relationship between age in months and plasma concentrations of ePE or ePC on the day of 1stOV.

On the day of 1stOV, the effect of daily milk yield on the plasma ePE concentrations was significant (Figure 7C). The plasma ePE concentration was correlated positively with daily milk yield (Figure 7C) and dry matter intake (Figure 7E). The effect of plasma concentrations of total cholesterol (Figure 7G) and BUN (Figure 7H) were significant. The concentrations of total cholesterol and BUN were positively correlated with the plasma ePE concentration. Other metabolites had no significant effects on the plasma ePE concentrations and did not correlate with the plasma ePE concentrations (data not shown).

On the day of 1stOV, the effect of milk fat percentage on the plasma ePC concentration was significant (Figure 8D). The milk fat percentage was negatively correlated with the plasma ePC concentration (Figure 8D). The total cholesterol concentrations were positively correlated with the ePC concentration (Figure 8G). The effect of AST concentrations on the ePC concentrations was significant (Figure 8H). The AST concentrations were negatively correlated with the ePC concentrations. Other metabolites had no significant effects on the plasma ePC concentrations and did not correlate with the plasma ePC concentrations (data not shown).

Relationships between plasma ePE or ePC concentrations and plasma concentrations of LH, FSH, and AMH

At the early luteal phase after 1stOV, plasma ePE concentration was correlated positively with plasma ePC concentrations (Figure 9A).

The days from parturition to 1stOV were positively correlated with the days from parturition to conception (Figure 9B).

The plasma ePE concentration was correlated positively with plasma AMH concentrations (Figure 9E) but not with LH and FSH. The effect of plasma ePE concentration on plasma AMH concentration was significant (Figure 9E).

The plasma ePC concentration was correlated positively with blood FSH concentration (Figure 9F) but not with LH and AMH. The effect of plasma ePC concentration on plasma FSH concentration was significant (Figure 9F).

The effect of parity on the plasma concentrations of LH, FSH, and AMH was not

significant.

Discussion

In the present study, we developed enzymatic fluorometric assays with sufficient sensitivity and reliable performance to measure ePE and ePC concentrations in bovine plasma samples. The shape and dynamic range of the standard curves were similar to those reported in previous studies (Mawatari *et al.* 2018; Morita *et al.* 2020). We presume that the observed changes in plasma concentrations of ePE and ePC indicate changes in plasma concentrations of EPls and CPls for the following two reasons. Firstly, the plasma samples were treated with phospholipase A_1 to remove diacyl phosphatidylethanolamine and diacyl phosphatidylcholine. Secondly, 100 % of ePE were EPls, and about 60 % of ePC were CPls. The concentrations of ePE and ePC in bovine plasma are shown as weight per volume of brain or plasma lipid standards. However, if the ratio of EPls or CPls to the total peak area is considered, the estimated EPl and CPl concentrations were similar to the values reported in humans measured using traditional methods (Fujino *et al.* 2020; Mawatari *et al.* 2020a; Fujino *et al.* 2022). This study revealed the significant differences in plasma concentrations of ePE and ePC, of which the majority were EPl or CPl, before and after parturition, up to the 1stOV. While no previous studies for any species, including humans, are available for comparison, we discuss the possible mechanisms that reduce plasma ePE and ePC levels after parturition, affect 1stOV, and reduce plasma ePE and ePC levels in cows with higher parity. We also discuss the significant relationship with the important parameters of production and reproduction. Finally, we discuss ways to manage the plasma concentration of ePE or ePC based on recent studies in human medicine.

In this study, we found that plasma concentrations of ePE and ePC were reduced during the peripartum period. There are three possible reasons for this: (1) increased consumption of EPls in dairy cows' bodies, (2) decreased synthesis of EPls and CPls in dairy cows' bodies, and (3) increased usage of EPls and CPls in milk production.

Regarding the first possible explanation, it must be noted that EPls play various crucial roles, including as endogenous antioxidants and immune modulators (Almsherqi 2021). Oxidation stress increases during late gestation to early lactation due to increased metabolic activity (Gong and Xiao 2018; Urh *et al.* 2019). In this period, the local and systemic immune systems also changed drastically, as reviewed by Velázquez *et al.* (2019). Therefore, consumption of EPls may increase in postpartum dairy cows.

Regarding the second possible explanation, both EPls and CPls are synthesised in various organs, including the brain, heart, kidneys, and liver (Arthur and Page 1991). A previous study showed that patients with non-alcoholic steatohepatitis have decreased blood concentrations of EPls and CPls, probably because of suppressed plasmalogen biosynthesis in the liver (Ikuta *et al.* 2019). More than 50% of postpartum dairy cows have fatty liver disease (McFadden 2020), indicating a probable suppression of plasmalogen biosynthesis in the liver of cows. The negative correlation between ePC and AST supported this hypothesis.

Regarding the third possible explanation, the maternal body supplies EPls via

milk to new-born animals to facilitate their normal neurodevelopment and other crucial functions (Hiratsuka *et al.* 2013; Moukarzel *et al.* 2016; Liu *et al.* 2020; Li *et al.* 2022). Full cream commercial milk obtained from the supermarket contains EPls and CPls (Liu *et al.* 2020). Thus, another potential reason is the increased amount of EPls and CPls supplied to the milk from the blood. Further studies are needed to elucidate how the concentrations of EPls and CPls change in fresh milk at various stages, including in colostrum.

Darwash et al. (1997) analysed approximately 1700 dairy cows and reported that the interval between parturition and 1stOV correlated with measures of fertility, and every day delay in the interval to 1stOV causes an average delay of 0.24 days in the interval to first service ($P < 0{\cdot}001$) and 0.41 days in the interval to conception ($P < 0{\cdot}001$). This study showed a positive correlation between the days from parturition to 1stOV and the days from parturition to conception in the cows, as shown in Figure 9B. Therefore, this study supported the previous studies reporting the importance of early re-establishment of postpartum ovarian activity for high fertility in dairy cows (Senatore *et al.* 1996; Darwash *et al.* 1997; Galvão *et al.* 2010). At the early luteal phase after 1stOV, plasma ePE concentration correlated with plasma AMH concentration, and plasma ePC concentration correlated with plasma FSH concentration. Both AMH and FSH are important hormones to control folliculogenesis (Visser and Themmen 2014). Insulin and IGF-1 are important hormones reported to affect reproductive functions (Rhoads *et al.* 2008; Subramaniam *et al.* 2016; Banuelos and Stevenson 2021). However, both insulin and IGF-1 plasma concentrations did not corelate with the ePE and ePC plasma concentrations (data not shown). Therefore, any direct effect of ePE and ePC on the hypothalamus-pituitary-gonadal axis are discussed.

GnRH controls gonadotrophs via the GnRH receptor on the gonadotroph surface. However, even in the absence of GnRH, EPls extracted from the brains of young (approximately 26 months old), healthy bovines, but not from aged (approximately 90 months old) bovines, strongly stimulate gonadotropins to secrete FSH via GPR61 (Kereilwe *et al.* 2018a; Kadokawa *et al*. 2021). Moreover, a chemosynthetic EPl activates the cytoplasmic Smad and ERK pathways and stimulates FSH and LH secretion from cultured bovine anterior pituitary cells (Kadokawa *et al.* 2022a). Therefore, EPls may be involved in the mechanisms of the 1stOV in gonadotrophs via GPR61. There are no previous studies on the direct effect of CPls on gonadotrophs. Therefore, further studies are required to clarify whether CPl also stimulates FSH and LH secretion from anterior pituitary cells.

The plasma AMH concentration is a good biomarker for ovarian reserve, ovarian function, and fertility in dairy cows (Mossa and Ireland 2019). Ribeiro *et al.* (2014) found a positive correlation between pregnancy rates and plasma AMH concentrations in dairy cows. Therefore, the observed positive correlation between ePE and AMH suggested the importance of ePE. Although AMH is primarily produced by the preantral and small antral follicles in humans and animals (Monniaux *et al.* 2012), bovine gonadotroph and GnRH neurons express both AMH and AMH receptors (Kereilwe *et al.* 2018b, 2019; Kereilwe and Kadokawa 2020). AMH stimulates FSH synthesis in LβT2 cells

(Tumurbaatar *et al.* 2021), and AMH stimulates FSH and LH secretion from gonadotrophs (Kereilwe *et al.* 2019; Tumurbaatar *et al.* 2021). Thus, small follicles, the hypothalamus, and gonadotrophs secrete AMH to regulate reproductive functions in endocrine, autocrine and paracrine manners. Because GnRH receptor colocalises with both GPR61 and AMH receptor type 2 on lipid-raft in the surface of bovine gonadotrophs (Kereilwe *et al.* 2018b; Pandey *et al.* 2017; Kadokawa 2020; Kadokawa *et al.* 2022a), plasma EPls may stimulate AMH secretion from gonadotrophs.

Plasma EPl and CPl concentrations in elderly humans (approximately 66 years of age) are lower than those in younger humans (approximately 24 years of age) (Maeba *et al.* 2007). Low levels of plasmalogen is associated with various diseases in aged humans, as reviewed by Dorninger *et al.* (2022). In this study, we observed decreased plasma ePE and ePC levels in higher-parity cows. Possible reasons are the balance between production and consumption for oxidation stress and a changed immune system (Gong and Xiao 2018; Velázquez *et al.* 2019). The key enzymes for plasmalogen synthesis are glyceronephosphate O-acyltransferase, alkylglycerone phosphate synthase, and fatty acyl-CoA reductase 1, expressed in various organs including the liver and brain (Honsho and Fujiki, 2017; Dorninger *et al.* 2022). Therefore, further studies are required to clarify whether hepatic plasmalogen biosynthesis decreases in higher-parity cows. We recently reported age-related differences in the quality of bovine brain EPl based on different EPl molecular species (Kadokawa *et al.* 2021). Weisser and Spiteller (1996) reported that plasmalogens in the brains of older bovines undergo much easier hydrolysis to corresponding aldehydes than in the brains of young bovines, and the brains of the older animals contain higher amounts of free aldehydes and plasmalogen epoxides than young animals. Therefore, the molecular structure of plasmalogens in the plasma of cows with higher parity may undergo easier hydrolysis.

In a recent study on dairy cows in New Zealand, third-parity cows showed better reproductive performance than lower- and higher-parity cows (Jayawardana *et al*. 2022). The number of primordial follicles in cows remains relatively constant until they reach the age of approximately four years and declines thereafter (Erickson 1966). In addition, antral follicle counts in beef ovaries increase until 5 years of age and decrease thereafter (Cushman *et al*. 2009). However, the mechanisms underlying these differences among parities have yet to be fully clarified. The third-parity cows in this study showed plasma ePE concentrations higher than those of other parity cows. Therefore, further studies are necessary to determine the relationship between the plasma concentrations of ePE and ePC within the context of age-related infertility.

This study also showed a positive correlation between daily milk yield and plasma concentrations of both ePE and ePC on the day of 1stOV. We could not find any previous studies related to these discoveries. However, this data suggests that there is no negative impact of milk yield on both ePE and ePC concentration from 1stOV. Therefore, further studies are required to develop strategies for the early resumption of plasma ePE and ePC concentration after parturition.

Orally supplied EPls and CPls are adsorbed from the intestine via various mechanisms to rapidly increase both blood and brain levels (Nishimukai *et al.* 2003;

Takahashi *et al.* 2020; Smith *et al.* 2022). Therefore, oral administration of plasmalogens (Fujino *et al*. 2020; Mawatari *et al.* 2020a; Fujino *et al.* 2022) or their precursors (Sibomana *et al.* 2019; Sultanov *et al.* 2021) has been studied in aged humans to improve or prevent brain diseases. These continuous oral administration strategies seem unlikely to be successful in domestic ruminants. However, intestinal microbes, such as *Bifidobacterium longum* (Mawatari *et al*. 2020b), produce EPls and CPls to supply to the host as postbiotics (Hernández-Granados and Franco-Robles 2020). Thus, a new concept is to manage the intestinal microbiota to increase postbiotics in human medicine (Hernández-Granados and Franco-Robles 2020). Therefore, further studies are required to determine the microbiota-host body axis in domestic animals.

The plasma ePE concentration of third-parity cows was higher than that of others in early postpartum but later became equal to that of others. These plasma concentration results may be attributed to the balance between biosynthesis and consumption, but the specific reasons for the differences and changes in the plasma concentration could not be explained at the present time. The abundance of some groups of intestinal microbes such as Bifidobacteria decreases with age in humans (Hopkins *et al*. 2002; Odamaki *et al*. 2016). Therefore, further studies are required to clarify the differences in the intestinal microbiota in relation to plasmalogen biosynthesis during lactation and aging in cows.

Notably, as shown in Figures 7A and 8A, the variables of age at either calving or 1stOV are not continuous, because Japanese dairy farmers want their heifers to be pregnant at approximately 13.5 months of age and also want their cows to give birth once every year. Pearson's correlation coefficient was not significant between age and EPl or CPl concentration among the 51 cows. Even when our calculations considered all the 54 cows, the correlation between age and EPl or CPl level was not significant (EPl vs. age is r= 0.167, P=0.243, and CPl vs. age is r= 0.267, P=0.061), indicating that age may not have an effect on EPl and CPl. However, we could not conclude that parity has a stronger effect than age on postpartum lactating dairy cows because of the special relationship between age and parity in dairy cows. Therefore, caution needs to be exercised in using the present data to fully infer the relationship between age and EPl or CPl concentration in other species.

In conclusion, the concentrations of ePE and ePC, most of which are EPls or CPls, changed dramatically around parturition and 1stOV, and then the concentrations correlated with the important parameters of milk production and reproduction. Therefore, blood plasmalogen may play important roles in postpartum dairy cows.

Data availability

The data that support this study will be shared upon reasonable request to the corresponding authors.

Conflicts of interest

The authors declare no conflicts of interest.

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991 plasmalogens in the brain of aged cattle. *Chemistry and Physics of Lipids* **82**, 173-
992 178. doi:10.1016/0009-3084(96)02588-1

993 **Table 1.** Formulation and chemical composition of the total mixed ration

	Late dry period (%)	**Early lactation period** (%)
Ingredients (% dry matter)		
Grass silage	70.6 ± 0.3	34.1 ± 0.2
Corn silage	0.0	22.0 ± 0.1
Rolled corn	19.4 ± 0.2	25.0 ± 0.1
Soybean meal	10.0 ± 0.1	17.3 ± 0.1
Calcium carbonate	0.0	1.6 ± 0.01
Chemical composition		
Dry matter (%)	27.0 ± 0.3	34.4 ± 0.2
Metabolizable energy (Mcal/kg)	2.62 ± 0.01	2.77 ± 0.003
Crude protein (% dry matter)	14.7 ± 0.04	16.6 ± 0.02
Neutral detergent fibre (% dry matter)	51.0 ± 0.1	37.8 ± 0.1
Non-fibre carbohydrate (% dry matter)	23.8 ± 0.1	35.0 ± 0.1
Organic matter (% dry matter)	93.6 ± 0.02	93.2 ± 0.01
Ether extract (% dry matter)	4.5 ± 0.03	4.2 ± 0.02

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995

Table 2. Ratio of the peak area of each lipid class to the total peak area of all lipids in the brain and plasma

Lipid class **Full name (abbreviation)**	**Brain area (%)[a]**		**Plasma area (%)[a]**	
	Mean	**SEM**	**Mean**	**SEM**
Triacylglycerol (TAG)	0.1	0.0	34.2	0.1
Free fatty acid (FFA)	4.9	0.0	20.5	0.1
Cholesterol (Chol)	18.9	0.1	15.9	0.1
Glucosylceramide (GlcCer)	10.3	0.0	0.1	0.0
Ethanolamine ether phospholipids (ePE)	16.5	0.0	0.6	0.0
Lysophosphatidylethanolamine (LPE)	1.7	0.0	0.1	0.0
Choline ether phospholipids (ePC)	2.9	0.1	13.4	0.1
Sphingomyelin (SPM)	8.5	0.1	9.4	0.1
Lysophosphatidylcholine (LPC)	1.2	0.1	2.1	0.0
Others	35.0	0.1	3.7	0.0
Total	100.0	-	100.0	-

[a] Ratio of the peak area of each lipid class to the total peak area.
Analyses were performed in triplicate for each lipid.
SEM, standard error of the mean

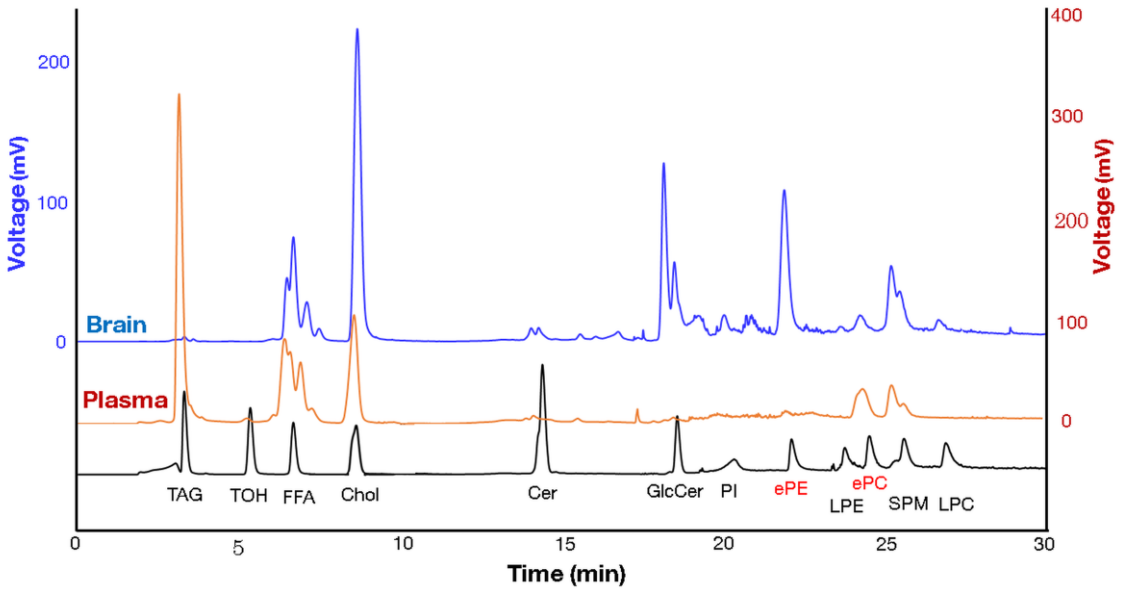

Fig. 1. Chromatograms depicting an example of an HPLC (normal-phase HPLC and a charged aerosol detector) profile of the lipids extracted from bovine brains, plasma, and 12 lipid standards. The brain lipids were diluted as the standard for ethanolamine ether phospholipids (ePE) assay, and plasma lipids were diluted as the standard for choline ether phospholipid (ePC) assay. The primary Y-axis indicates the voltage values of the samples from the brain, and the secondary Y-axis indicates the plasma samples' voltage values. The chromatogram of the plasma was shifted down for clarity, and there was no difference in the baseline values between the brain and plasma samples.
HPLC, high-performance liquid chromatography; mV, milli-voltage; TAG, Triacylglycerol; TOH, D-α-tocopherol; FFA, Free fatty acids; Chol, Cholesterol; Cer, Ceramide; GlcCer, Glucosylceramide; PI, Phosphatidylinositol; ePE, Ethanolamine ether phospholipids; LPE, Lysophosphatidylethanolamine; ePC, Choline ether phospholipids; SPM, Sphingomyelin; LPC, Lysophosphatidylcholines.

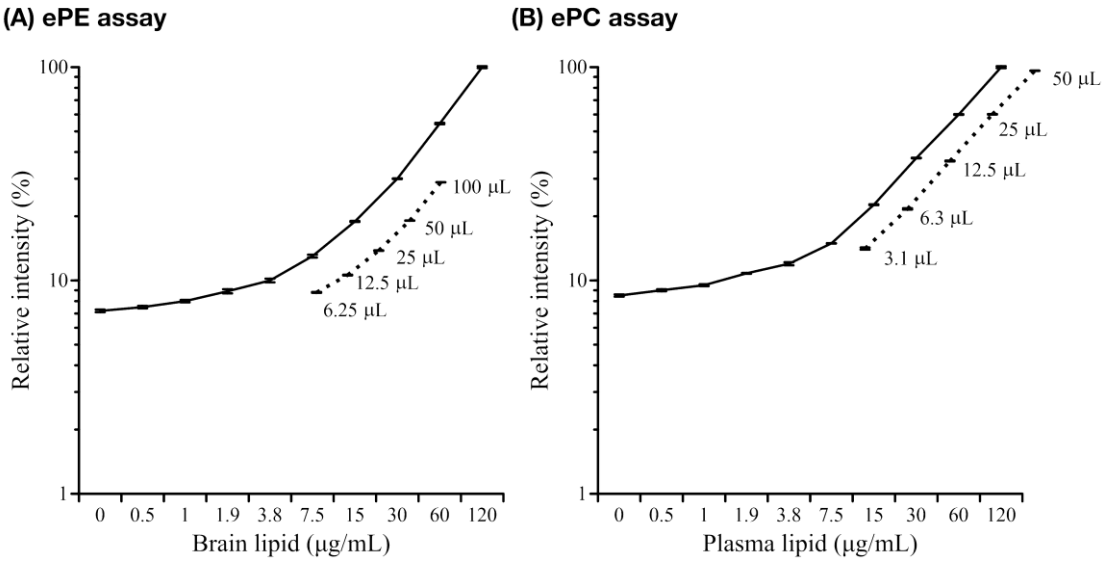

Fig. 2. The solid lines indicate the standard curve of ethanolamine ether phospholipid (ePE) assay (A) or choline ether phospholipid (ePC) assay (B). Both standards were prepared by diluting either the brain or plasma lipids; thus, the X-axis values are the concentrations (in μg/mL) of lipids (the details are shown in Figure 1), not as 100% purified ePE or ePC. The dashed lines indicate the serially diluted plasma lipid samples (in μL) used to evaluate parallelism with the standard curve.

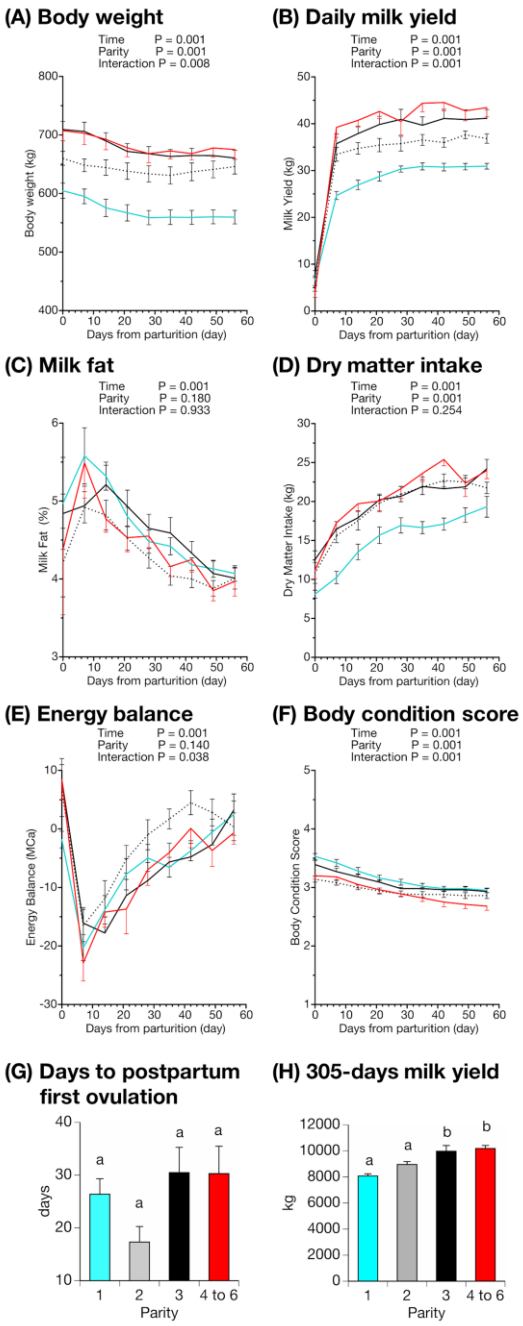

Fig. 3. Postpartum Changes in body weight (A), daily milk yield (B), milk fat percentage (C), dry matter intake (D), energy balance as metabolizable energy (E), and body condition scores on a five-point scale (F), the days from parturition to first postpartum ovulation (G), and 305-day milk yield in the used cows (H). The header of graphs A to F represents the results of the repeated-measure ANOVA model, including the effects of time, parity [first- (light blue), second- (grey), third- (black), or fourth- and higher-parity (red)], and interaction between time and parity on measurements as shown as the label of each Y-axis. Different letters (a or b) in graphs G and H indicate significant differences among the different parity cows (one-way ANOVA followed by Tukey–Kramer test).

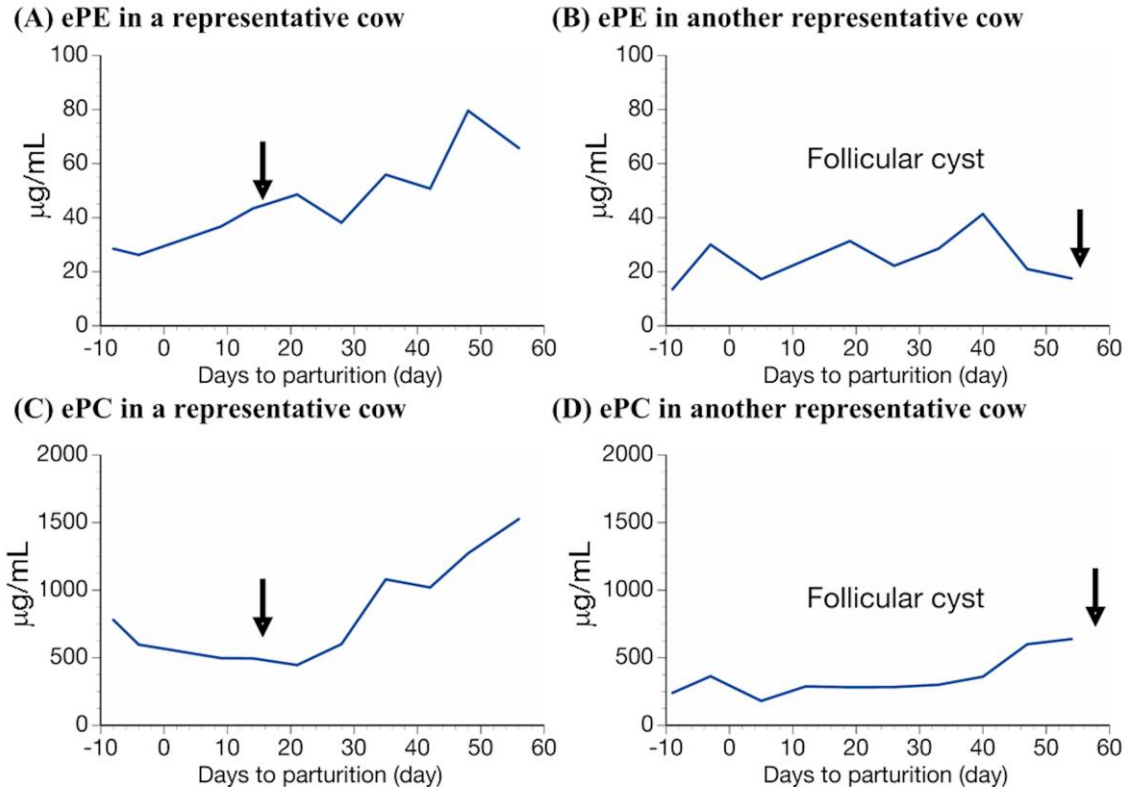

Fig. 4. Changes in the plasma concentrations of ethanolamine ether phospholipids (ePE) (A, B) and choline ether phospholipid (ePC) (C, D) around parturition in representative second-parity dairy cows. One cow free of ovarian diseases had the first postpartum ovulation (1stOV) on day 16 (A, C). Another cow developed a follicular cyst, healed naturally, and then had the 1stOV on day 58 (B, D). Black arrows indicate the 1stOV.

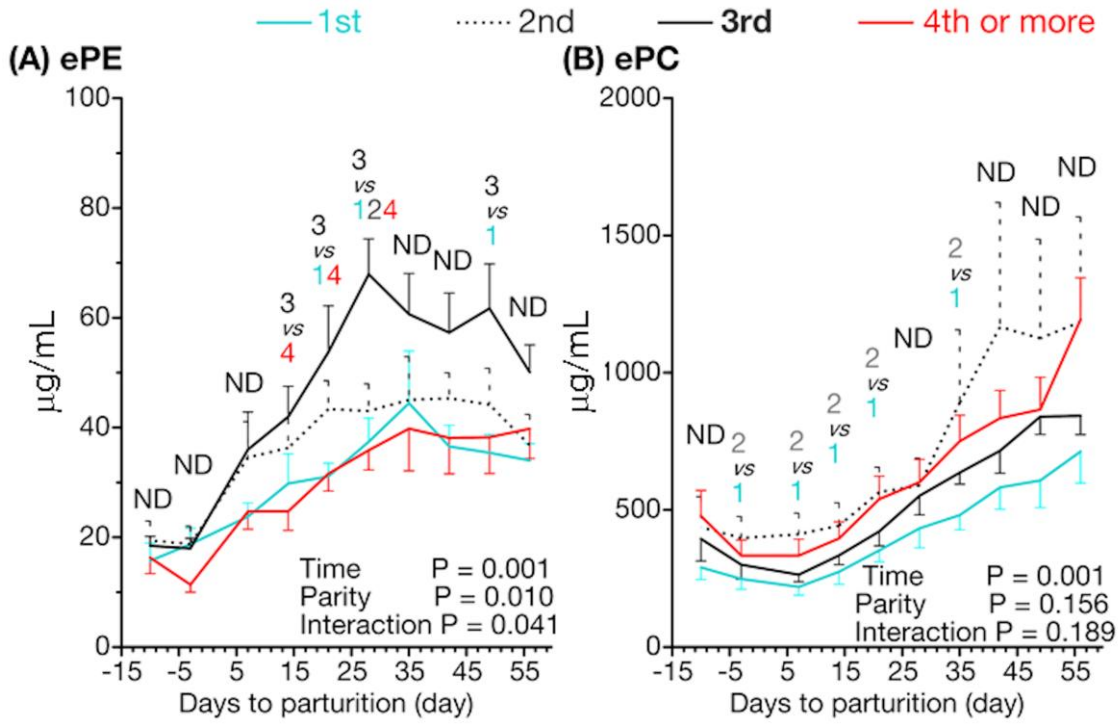

Fig. 5. Changes in plasma concentrations of ethanolamine ether phospholipids (ePE) (A) and choline ether phospholipid (ePC) (B) before and after parturition in first-parity cows (n = 16; light blue line); second-parity cows (n = 16; dotted black line); third-parity cows (n = 11; black line); and fourth- or higher-parity cows (n = 11; red line). The footer in the lower right corner of each graph represents the results of the repeated-measure ANOVA model, including the effects of time, parity (first-, second-, third-, or fourth- and higher-parity), and interaction between time and parity on plasma ePE or ePC concentrations from 2 weeks before to 7 weeks after parturition. The letters above each time point represent the results of the Tukey–Kramer test to evaluate the effect of parity; for example, "3 vs. 124" indicates a difference ($P < 0.05$) between third-parity cows and first, second, and fourth or more parity cows, whereas "ND" indicates no difference among the different parities.

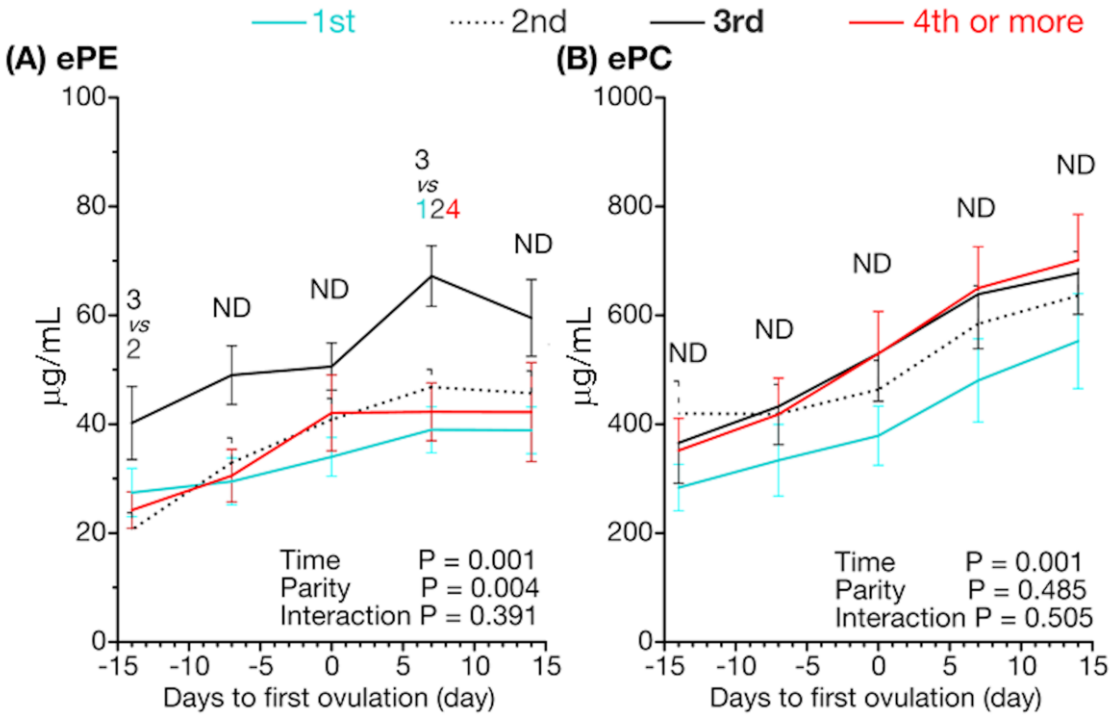

Fig. 6. Changes in plasma concentrations of ethanolamine ether phospholipids (ePE) (A) and choline ether phospholipid (ePC) (B) before and after first postpartum ovulation in first-parity cows (n = 12; light blue line); second-parity cows (n = 10; dotted black line); third-parity cows (n = 10; black line); and fourth- or higher-parity cows (n = 10; red line). The footer in the lower right corner of each graph represents the results of the repeated-measure ANOVA model, including the effects of time, parity (first-, second-, third-, or fourth- and higher-parity), and interaction between time and parity on plasma ePE or ePC concentrations from 2 weeks before to 2 weeks after ovulation. The letters above each time point represent the results of the Tukey–Kramer test to evaluate the effect of parity in each time; for example, "3 vs 124" indicates a difference ($P < 0.05$) between third-parity cows and first-, second-, and fourth- or higher-parity cows, whereas "ND" indicates no difference among the different parities.

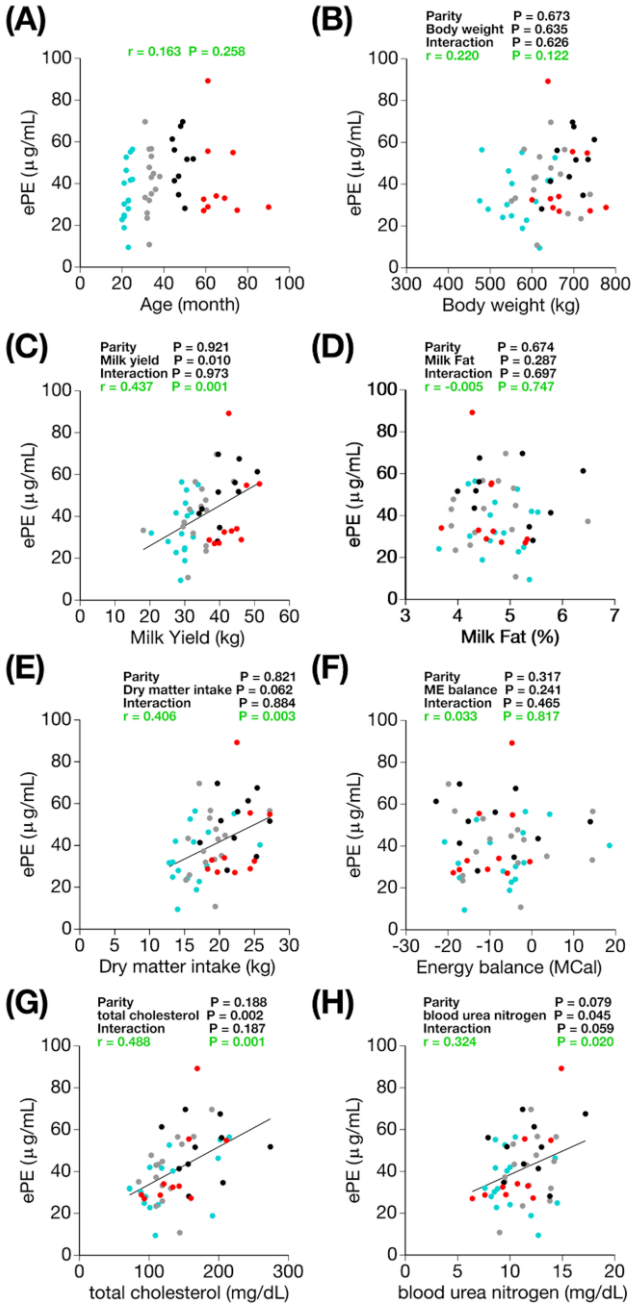

Fig. 7. Relationships of the plasma ethanolamine ether phospholipid (ePE) concentration on the day of first postpartum ovulation with either the age in months (A), body weight (B), daily milk yield (C), milk fat percentage (D), dry matter intake (E), energy balance (F), blood cholesterol concentration (G), and blood urea nitrogen concentration (H). The header of each graph represents the results of the Pearson correlation coefficient (A) or ANCOVA model, including the effects of parity, measurements as shown as the label of each X-axis, and the interaction on plasma ePE concentrations. The light blue, grey, black, and red dots indicate first-, second-, third-, fourth,- or higher-parity cows. Regression lines shown for the pair indicates significant correlation.

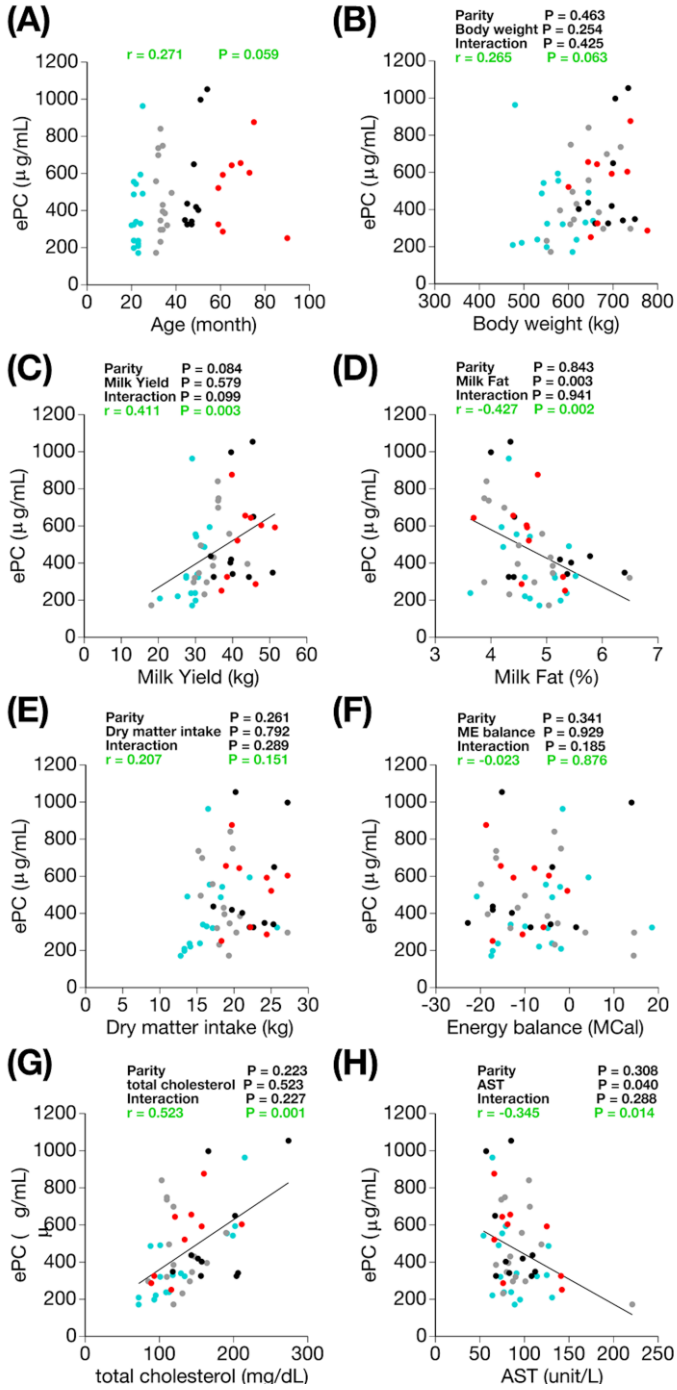

Fig. 8. Relationships of the plasma choline ether phospholipid (ePC) concentration on the day of first postpartum ovulation with either the age in months (A), body weight (B), daily milk yield (C), milk fat percentage (D), dry matter intake (E), and energy balance (F). The header of each graph represents the results of the Pearson correlation coefficient (A) or ANCOVA model, including the effects of parity, measurements as shown as the label of each X-axis, and the interaction on plasma ePC concentrations. The light blue, grey, black, and red dots indicate first-, second-, third-, fourth-, or higher-parity cows. Regression lines shown for the pair indicate significant correlation.

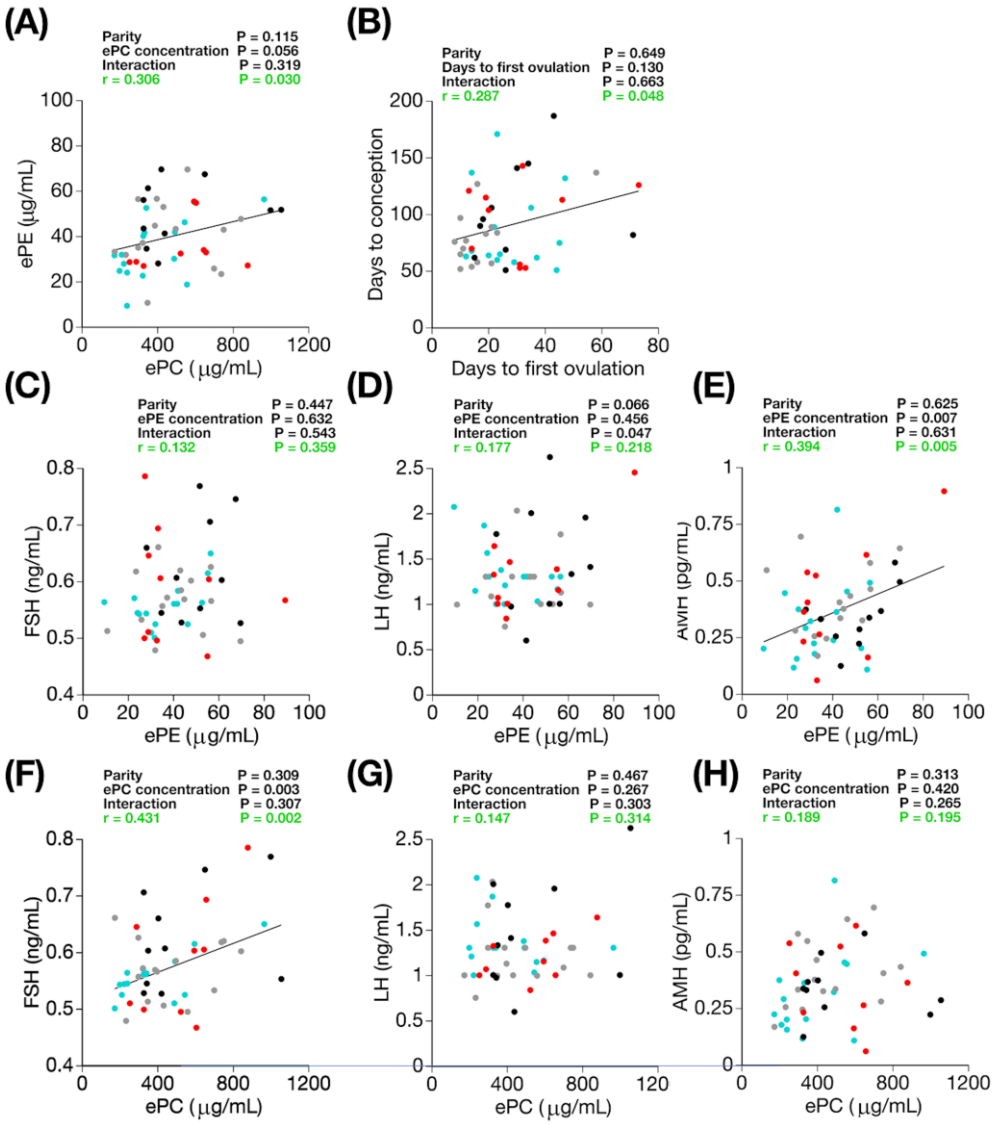

Fig. 9. Relationships of the plasma concentration of ethanolamine ether phospholipid (ePE) with the plasma concentration of choline ether phospholipid (ePC) at the early luteal phase after first postpartum ovulation (A). Relationships of the days from parturition to postpartum first ovulation and the days from parturition to conception (B). Relationships of the plasma concentration of ePE or ePC with either plasma concentration of FSH (C and F), LH (D and G), or AMH (E and H) at the early luteal phase after the first postpartum ovulation. The header of each graph represents the results of the Pearson correlation coefficient or ANCOVA model, including the effects of parity, measurements as shown as the label of each X-axis, and the interaction on plasma LH, FSH, or AMH concentrations. The light blue, grey, black, and red dots indicate first-, second-, third-, fourth-, or higher-parity cows. Regression lines shown for the pair indicate significant correlation.