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Original Research Article

Developmental changes in lipid and fatty acid metabolism and the inhibition by in ovo feeding oleic acid in Muscovy duck embryogenesis

Xiufen Zhang [a], Qilin Wu [a], Wenxuan Zheng [a], Chuang Liu [b], Liang Huang [a], Xin Zuo [b], Wenquan Xiao [b], Xiaofeng Han [b], Hui Ye [a], Wence Wang [a], Lin Yang [a, *], Yongwen Zhu [a, *]

[a] Guangdong Provincial Key Laboratory of Animal Nutrition and Regulation, College of Animal Science, South China Agricultural University, Guangzhou 510642, China
[b] Wen's Food Group Co., Ltd, Yunfu 52740, China

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ABSTRACT

Hepatic lipid and fatty acid (FA) metabolism are critical for regulating energetic homeostasis during embryogenesis. At present, it remains unclear how an exogenous FA intervention affects embryonic development in an avian embryo model. In Exp. 1, 30 fertilized eggs were sampled on embryonic days (E) 16, 19, 22, 25, 28, 31 and the day of hatch (DOH) to determine the critical period of lipid metabolism. In Exp. 2, a total of 120 fertilized eggs were divided into two groups (60 eggs/group) for in ovo feeding (IOF) procedures on E25. Eggs were injected into the yolk sac with PBS as the control group and with oleic acid (OA) as the IOF-OA treatment group. Samples were collected on E28 and E31. In Exp. 1, hepatic triacylglycerol (TG) and cholesterol (CHO) contents increased while serum TG content decreased from E16 to DOH ($P < 0.05$). Both serum and liver displayed an increase in unsaturated FA and a decrease in saturated FA ($P < 0.05$). There was a quadratic increase in the target gene and protein expression related to hepatic FA de novo synthesis and oxidation ($P < 0.05$), whose inflection period was between E22 and E28. In Exp. 2, compared with the control embryos, IOF-OA embryos had an increased yolk sac TG content on E28 and E31, and a decreased serum TG and CHO content on E28 ($P < 0.05$). The IOF-OA embryos had less OA in the yolk sac and liver on E28, and less unsaturated FA in the serum and liver on E31 than did the control embryos ($P < 0.05$). Hepatic gene mRNA expression related to FA uptake, synthesis, and oxidation on E28 was lower in IOF-OA than in control embryos ($P < 0.05$), not on E31 ($P > 0.05$). Maximal metabolic changes in lipid and FA metabolism occurred on E22-E28 in Muscovy duck embryogenesis, along with the altered target gene and protein expression related to lipogenesis and lipolysis. IOF-OA intervention on E25 could inhibit the target gene expression related to FA uptake, synthesis, and oxidation, which may influence the normal FA metabolism on E28 during embryogenesis.

1. Introduction

The avian embryo is a multifaceted model system for studying developmental mechanisms, such as organ morphogenesis, nervous system development and maternal effects on embryogenesis (Clayton and Emery, 2015; Groothuis et al., 2019; Hirst and Marcelle, 2015; Kulesa et al., 2013). During incubation, the development of poultry embryos depends on the nutrient composition of the eggs, and these functional molecules exert their effects systematically (Meng et al., 2021a; Moran, 2007). Lipid distribution in the yolk sac varies at different embryonic stages and is transferred to embryos through the highly vascularized yolk sac membrane or directly into the intestine via the yolk stalk (Meng et al., 2021b; van der Wagt et al., 2020). The liver is responsible for lipogenesis and lipolysis during the embryonic and neonatal periods (Alves-Bezerra and Cohen, 2017; Cai et al., 2017; Wang et al., 2015). Plasma lipoprotein particles are produced by very-low-density lipoprotein

* Corresponding authors.
E-mail addresses: ylin898@scau.edu.cn (L. Yang), 408034085@qq.com (Y. Zhu).
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(VLDL) in the yolk sac's endodermal epithelial cells and then transported to the liver via vitelline circulation (Hermann et al., 2000; Yadgary et al., 2010). As lipids are transferred gradually from the yolk sac into the embryo, liver lipid deposition progressively accumulates in the form of lipid droplets, typically with a core of triacylglycerols (TG) and other neutral lipids (e.g., cholesteryl and retinyl esters) surrounded by a phospholipid monolayer (Noble et al., 1984; Speake et al., 1998; Wang et al., 2017a).

The rapid growth of genetically improved birds can make the quantity of nutrients contained in the egg insufficient for the complete development of embryonic tissues (Grodzik et al., 2013). Moreover, the practice of post−hatch fasting until the chicks are housed can further limit the nutritional reserves contained in the yolk. In ovo feeding (IOF) as a tool is being developed to deliver growth-promoting compounds and nutrients during the embryonic stage to improve the performance and gut health of poultry (Das et al., 2021; Peebles, 2018; Wong and Uni, 2021). For example, IOF of carbohydrates and amino acids could improve the energy status of the embryos by increasing glycogen storage and enhancing jejunal nutrient uptake and digestion (Foye et al., 2006). Besides, IOF vitamin C and vitamin D_3 resulted in improved bone characteristics (tibia breaking strength and bone mineralization, etc.) of birds at post−hatch (Bello et al., 2014; Santos et al., 2019). However, there was little available information on the effect of IOF of fatty acids (FA). FA is a major source via enhancing mitochondrial β-oxidation for ATP generation when energy demand increases during embryonic development (Bradley and Swann, 2019; Noble and Cocchi, 1990). Moreover, FA and their metabolites are involved in cell growth and development, cell signaling, and modulating the structural and functional processes at each stage of embryonic development (Duttaroy and Basak, 2020). Furthermore, the egg yolk is the sole source of n-6 and n-3 polyunsaturated FA (PUFA) in the chick embryo due to the absence of desaturases that insert double bonds beyond the δ-9 carbon (Cherian, 2015). Oleic acid (OA) is the most abundant FA in the yolk of developing chick embryos, which is key for energy supply during incubation (Şahan et al., 2014; Su et al., 2020). We hypothesized that IOF-OA could achieve a beneficial effect on embryonic development by altering FA profile and lipid metabolism and modifying the regulation of transcription of different genes.

In this study, the dynamic patterns of lipid metabolism, FA profile, and target gene and protein expression in tissues were investigated to determine the critical period of lipid metabolism during the embryonic development of Muscovy duck. Then, based on the critical period of lipid metabolism, the effect of IOF-OA on the alteration of lipid metabolism and FA profile during embryonic development was investigated using a Muscovy duck embryo model.

2. Materials and methods

2.1. Animal ethics statement

The animal care and use protocol was approved by the Animal Care and Use Committee of South China Agricultural University (SCAU-10564), and the study was conducted following the Regulations for the Administration of Affairs Concerning Experimental Animals.

2.2. Animals and incubation

In Exp. 1, a total of 350 Muscovy duck eggs were obtained from a commercial hatchery (Wen's Food Group Co., Ltd, Yunfu, Guangdong, China). These eggs were incubated in an automatically controlled incubator (Dezhou Keyu Hatching Equipment Co., Ltd, Dezhou, China) at an incubation temperature of 37.5 ± 0.5 °C and relative humidity of 55% ± 5% until the 31st day of incubation (E31). Eggs were turned at a 90-degree angle every 90 min from the beginning of incubation through to E31. After that, all eggs were transferred to hatching crates and moved to hatchers set at a temperature of 37.0 ± 0.5 °C, which declined to 36.0 ± 0.5 °C by the end of incubation. All eggs were candled on E15, and any unfertilized and unviable eggs were discarded. A total of 180 viable eggs (75.2 ± 1.8 g) and 30 newly-hatched ducklings (43.2 ± 0.7 g) were used for testing. Thirty eggs were selected for sampling on E16, E19, E22, E25, E28 and E31, and 30 ducklings on the day of hatch (DOH, within 24 h after external piping), consisting of 6 replicates each with 5 embryos.

In Exp. 2, a total of 200 Muscovy duck eggs were incubated and 120 viable eggs (78.9 ± 1.6 g) were selected and divided into two groups (60 eggs each) on E25, each group consisting of six replicates of 10 embryos per replicate. The control and treatment groups were injected with a 100 μL volume of phosphate-buffered saline (PBS, #G4202, Servicebio, Wuhan, China) and OA (≧ 99%, #75090, Sigma−Aldrich, Wyoming, USA) into the yolk sac, respectively. To do this, a sterile disposable 25.0-mm × 0.6-mm needle was attached to a 1.0-mL syringe, which was replaced after each egg injection. Eggs were sanitized with 75% ethanol in the needle insertion region before their injection. Immediately after injection, the hole was sealed with medical adhesive tape (1.0 cm × 1.0 cm), and the eggs move into an incubator. Two embryos of each treatment per replicate were selected for sampling on E28 and E31, respectively. The number of unviable embryos was recorded during E26 to DOH, and the embryonic mortality in the control group and IOF-OA group was 16.67% and 21.67%, correspondingly.

2.3. Sample collection

To obtain sufficient samples for analysis, it was necessary to pool the serum and liver samples together, and likewise for the yolk sac samples, so that the results would be comparable (Noble and Moore, 1964). Samples of the yolk sac, serum and liver from 5 or 2 embryos were pooled together for each replicate in Exp. 1 or Exp. 2, respectively. Yolk sac (without membrane) samples were collected and stored at −20 °C for their biochemical index and FA analyses. Blood samples from E16 to E31 were collected from the umbilical vein by using glass Pasteur pipettes (7 mm × 150 mm). The tip of a glass Pasteur pipette was melted on the outer flame of an alcohol lamp and then drawn out with tweezers to make a needle with a diameter of < 0.3 mm. Blood samples on DOH were collected from the jugular vein using disposable syringes (16.0-mm × 0.45-mm needle attached to a 1.0-mL syringe). The serum was separated via centrifuging at 664 ×*g* for 10 min at room temperature and stored at −20 °C until the biochemical index and FA analyses. From Exp.1, approximately 0.5 to 1 cm^3 of each liver sample was fixed in 4% formaldehyde for its hematoxylin-eosin (H&E) and oil-red O staining histological analysis. The rest of the liver samples were rinsed with ice-cold PBS, frozen in liquid nitrogen and stored at −80 °C for later analyses of biochemical index, FA composition and relative gene mRNA and protein expression levels.

2.4. Morphological examination

After fixing, paraffin sections and cryosections were taken from the liver and these were used for H&E staining and oil-red O staining, respectively. The procedure consisted of dehydration, paraffin embedding, sectioning and staining. The sections were observed and photographed under a microscope (Eclipse E100 and DS-U3, Nikon, Tokyo, Japan). The ratio of red-stained area to picture

area in oil-red O-stained sections was calculated in Image J (National Institutes of Health, Maryland, USA).

2.5. Biochemical index detection

Biochemical indices were measured according to the manufacturer's instructions for each assay kit used (Nanjing Jiancheng Bioengineering Institute, Nanjing, China), including TG, cholesterol (CHO), high-density lipoprotein cholesterol (HDLC), low-density lipoprotein cholesterol (LDLC), lipoprotein lipase (LPL), hepatic lipase (HL) and total lipase (TL). VLDL was measured with an ELISA assay kit according to the manufacturer's instructions (Shanghai Enzyme-linked Biotechnology Co., Ltd. Shanghai, China).

2.6. Fatty acid analysis

Lipid extraction and FA analysis of yolk sac, serum and liver samples were conducted as previously reported (Li et al., 2020). In brief, lipids from the yolk sac and liver tissues were extracted with chloroform and methanol (2:1, vol/vol) and FA methyl esters (FAME) were prepared by transesterification with boron trifluoride etherate. The lipids present in serum samples were directly converted to FAME by transesterification. Next, the FAME were determined by a gas chromatography system (GC-7890A, Agilent Technologies, Santa Clara, CA, USA) equipped with a hydrogen flame ionization detector. The GC system was fitted with a capillary column (60 m × 0.25 mm CP7487, 0.20-μm film thickness, Agilent). Next, the FAME were identified by comparing the FAME profiles of samples with those of FAME standards (#CRM47885, Sigma−Aldrich). FA profiles of the yolk sac, serum, and liver tissues are reported here as percentages of total FA.

2.7. Relative gene mRNA expression analysis

Total RNA was extracted from liver tissue with Trizol reagent (Invitrogen, Carlsbad, CA, USA) according to protocols for using this reagent. Reverse transcription was performed as described in the Primer Script RT Reagent Kit (TaKaRa, Dalian, China). Primer sequences used in the current study were obtained from GenBank (Table 1) and synthesized by the Sangon Biotechnology Co., Ltd. (Shanghai, China). The samples were analyzed in duplicate by real-time quantitative reverse transcription polymerase chain reaction (qRT-PCR) performed on a detection system (Applied Biosystems QuantiStudio 7 Flex, Life Technologies, Carlsbad, CA, USA). Each qRT-PCR reaction consisted of 5 μL of SYBR Green Realtime PCR Master Mix kit (#QPK-201, TOYOBO, Osaka, Japan), 0.4 μL of each forward primer and reverse primer, 3.2 μL of ddH_2O, and 1.0 μL of template cDNA into a total volume of 10 μL. The PCR program went as follows: 95 °C for 1 min, followed by 40 cycles each at 95 °C for 15 s, then 60 °C for 15 s, and finally 72 °C for 45 s. The amplification specificity was verified by a melting curve analysis done at the end of every PCR run. Beta-actin was highly expressed in the liver with a stable Cq value and was selected as the reference gene. Gene expression was normalized to the reference gene and the mRNA transcripts were quantified using the comparative CT method ($2^{-\Delta\Delta CT}$). The data were analyzed using Cq, where $\Delta\Delta C_T = (C_{T,Target} - C_{T,Actin})_{Time\ x} - (C_{T,Target} - C_{T,Actin})_{Time\ 0}$. Time x is the time point ranging from E19 to DOH and Time 0 represents the 1 × expression of the target gene on E16 normalized to β-actin (Livak and Schmittgen, 2001).

2.8. Western blotting analysis

Liver samples collected on E16, E22, E28 and DOH in Exp. 1 were used for western blotting analysis. Each tissue sample was homogenized with ice-cold RIPA lysis buffer (#AWB0136, Abiowell, Changsha, China) containing 1 mmol/L phenylmethylsulfonyl fluoride (#AWH0650, Abiowell). The mixture was placed on ice for 10 min and the homogenate was centrifuged at 12,000 × g for 5 min at 4 °C. Protein concentration in the collected supernatant was measured as described in the BCA assay kit (Beyotime Biotechnology, Shanghai, China). The protein supernatant was mixed with a loading buffer, boiled for 5 min and then stored at 4 °C for Western blotting analysis. Antigenic proteins were separated via electrophoretic SDS-PAGE on 10% gel for 130 min at 75 V (DYY-6C, Liuyi, Beijing, China). After transferring to a nitrocellulose filter membrane at 300 mA with Trans-Blot (DYCZ-40D, Liuyi) in a transfer buffer (#AWC0114, Abiowell), the membrane was blocked for 1.5 h at room temperature in a 5% skim milk powder solution (#AWB0004, Abiowell), then incubated overnight at 4 °C with rabbit monoclonal antibodies for SREBP1(#ab28481, Abcam, Cambridge, England), FASN (#10624-2-AP, Proteintech, Chicago, USA), PPARα (#ab24509, Abcam), CPT1 (#15184-1-AP, Proteintech) and β-actin (#66009-1-Ig, Proteintech). The membrane was then incubated with HRP goat anti-rabbit IgG (#SA00001-2, Proteintech)

Table 1
Forward and reverse primer sequences for PCR analysis.

Target genes	Forward primer (5′→3′)	Reverse primer (5′→3′)	GenBank accession No.	Product size (bp)
CD36	TATCGTTTCGCAGTTCCTCGTGAAG	AGTTCTGGGATATGACCTCCTCTGTAC	XM_038183702.1	94
SLC27A4	GCCTGATGACGTGATGTACGACTG	AGAACTTCTTGCGGATGACGATGG	XM_027470622.2	113
FABP1	TGGGAATAAGTTCAAGGTTACCGTCAC	GGTCTTGGCTTTCTCTCCTGTCAG	XM_005023289.5	112
Lpin 1	ACAAAGCAAGAACACAAATGACACAGG	GAGAAATGGCAATGGAGGGCAAATC	XM_038176802.1	137
SREBP1	TGGTGGTGGACGCCGAGAAG	TCGTTGATGGAGGAGCGGTAGC	XM_038187023.1	133
ACC1	CACAGATCCAGAGCACAGCACTTC	GGCAGGCAGTATCCGTTCATCAC	XM_038165892.1	100
FASN	TCTCTGCCATCTCCCGAACTTCC	TTAGCCACTGTGCCAACTCAAGC	XM_027471234.2	96
SCD1	AGTTCTCCTCCGCTTCCAGC	TTCTCCATGACGGCATCCCC	XM_027460089.2	82
ELOVL2	ACCGGAAAGCACCTTCAAGAACAG	TTCAGGAATCCATTGGCAGCAGTG	XM_038175034.1	106
ELOVL3	TCCTGGAACTGGGCGACACC	GCGTAGATGAGAGTGGCGATGTG	XM_038181654.1	94
PPARα	ACCATCCTGATGATACCTTCCTCTTCC	AAGTTGAGCATGTTCTGTGACAAGTTG	NM_001310383.1	86
RXRα	TGCGAGCCATTGTCCTCTTCAAC	GATGCGTACACCTTCTCCCGTAAC	XM_027471073.2	88
CPT1A	CCGCCATCTGTTCTGCCTCTATG	TGTGTTGCTGTGGTGTCTGACTTG	XM_027457809.2	119
ACADL	TGGTGCCATTGCCATGACAGAAC	CTCCCGTTAAGAATCCAGTCACTTCC	XM_027461394.2	99
ACOX3	GAAGGAGAAGCAGTCAGGGCAAAG	GCAATGGCTAGTGACCGACAGTAG	XM_038178270.1	81
β-actin	TACGCCAACACGGTGCTG	GATTCATCATACTCCTGCTTG	NM_00131042.1	215

CD36 = CD36 molecule; *SLC27A4* = solute carrier family 27 member 4; *FABP1* = fatty acid-binding protein 1; *Lipin 1* = phosphatidate phosphatase; *SREBP1* = sterol regulatory element-binding transcription factor 1; *ACC1* = acetyl-CoA carboxylase alpha; *FASN* = fatty acid synthase; *SCD1* = stearoyl-CoA desaturase; *ELOVL2* = elongation of very-long-chain fatty acids protein 2; *ELOVL3* = elongation of very-long-chain fatty acids protein 3; *PPARα* = peroxisome proliferator-activated receptor alpha; *RXRα* = retinoid X receptor alpha; *CPT1A* = carnitine palmitoyltransferase 1 A; *ACADL* = long-chain-acyl-CoA dehydrogenase; *ACOX3* = acyl-CoA oxidase 3.

diluted in PBST buffer (#AWI0130, Abiowell) and also with ECL chemiluminescence solution (#AWB0005, Abiowell) for 1 min. Ensuing bands were detected by a chemiluminescence imaging system (ChemiScope6100, Qinxiang, Shanghai China) and their density was determined using Image J software.

2.9. Statistical analysis

Data were expressed as mean ± standard error of the mean (SEM). Data from Exp. 1 were analyzed with a one-way analysis of variance (ANOVA) using the PROC GLM procedure in Statistical Analysis System (SAS) v9.2 (SAS Inst. Inc., Cary, NC, USA). The differences in means were considered significant at $P < 0.05$, and notable differences between groups were identified using Duncan's multiple comparisons test. Orthogonal polynomial contrasts were used to identify the form of the effect (linear or quadratic) over time (embryonic development days). The data for each sampling time point from Exp. 2 were analyzed with a *t*-test, using the PROC TTEST procedure in SAS v9.2, for which differences were considered significant at $P < 0.05$.

3. Results

3.1. Hepatic morphological change

Representative hepatic H&E stained and oil-red stained sections in the course of Muscovy duck embryogenesis are presented in Fig. 1A and B, respectively. With morphological post-processing of liver tissue, boundaries of indicative fat droplets were identified as white cavities and red dots for quantitative analysis by H&E and oil-red O staining, respectively. The H&E staining results showed that fat shapes increasingly erased small regions by filling holes and gaps and eventually separated adjacent fat droplets as the incubation period progressed. The red-stained area percentage of the hepatic oil-red O-stained sections increased ($P < 0.05$) linearly from E16 through to DOH (Fig. 1C).

3.2. Developmental changes in biochemical indices related to lipid metabolism

During embryogenesis, the TG content of the yolk and liver increased ($P < 0.05$) linearly or quadratically, while the serum TG content decreased ($P < 0.05$) linearly or quadratically during the embryonic period (Fig. 2A and B). The HDLC level of yolk increased ($P < 0.05$) linearly, while the LDLC level of yolk decreased ($P < 0.05$) linearly (Fig. 2A). There were significant increases in hepatic content of CHO and LDLC and a decrease in hepatic TL activity ($P < 0.05$) in a linear or quadratic manner with prolonged incubation (Fig. 2B). The level of HDLC and HL both increased ($P < 0.05$) in serum yet decreased ($P < 0.05$) in liver linearly or quadratically in response to the longer incubation period (Fig. 2B). The VLDL content and the TL activity in serum increased ($P < 0.05$) in a quadratic manner and hepatic VLDL content increased ($P < 0.05$) in a linear manner (Fig. 2B). Quadratic changes ($P < 0.05$) were evident for the LPL activity of liver and serum, which plateaued in periods corresponding to E19 to E25 and E28 to E31, respectively (Fig. 2B).

3.3. Developmental changes in fatty acid profiles in serum and tissues

The OA varied in a linear ($P < 0.05$) or quadratic ($P < 0.05$) manner in the yolk sac (Table 2), serum (Table 3), and liver (Table 4), with its level peaking on DOH, E25, and E31, respectively. Similarly,

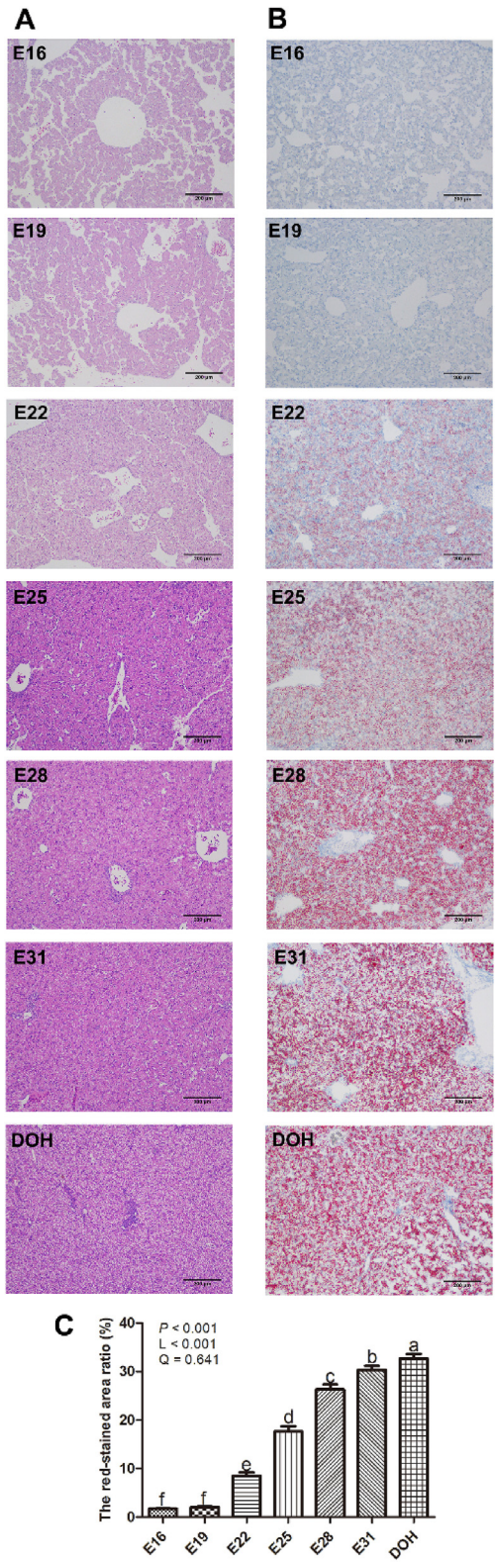

Fig. 1. Representative hepatic H&E stained sections and oil-red O stained sections in Muscovy duck embryogenesis. (A) H&E stained sections. (B) Oil-red O stained sections. The pictures are at 200 × magnification and the ruler in the lower right corner of the picture (A, B) is 200 μm. (C) The percentage of red-stained area to picture area in the oil red-stained O section was calculated using software Image J. [a-f] Data are expressed as mean ± SEM ($n = 6$), and bars with different lowercase letters indicate statistically significant differences (one-way ANOVA, $P < 0.05$). L, linear; Q, quadratic.

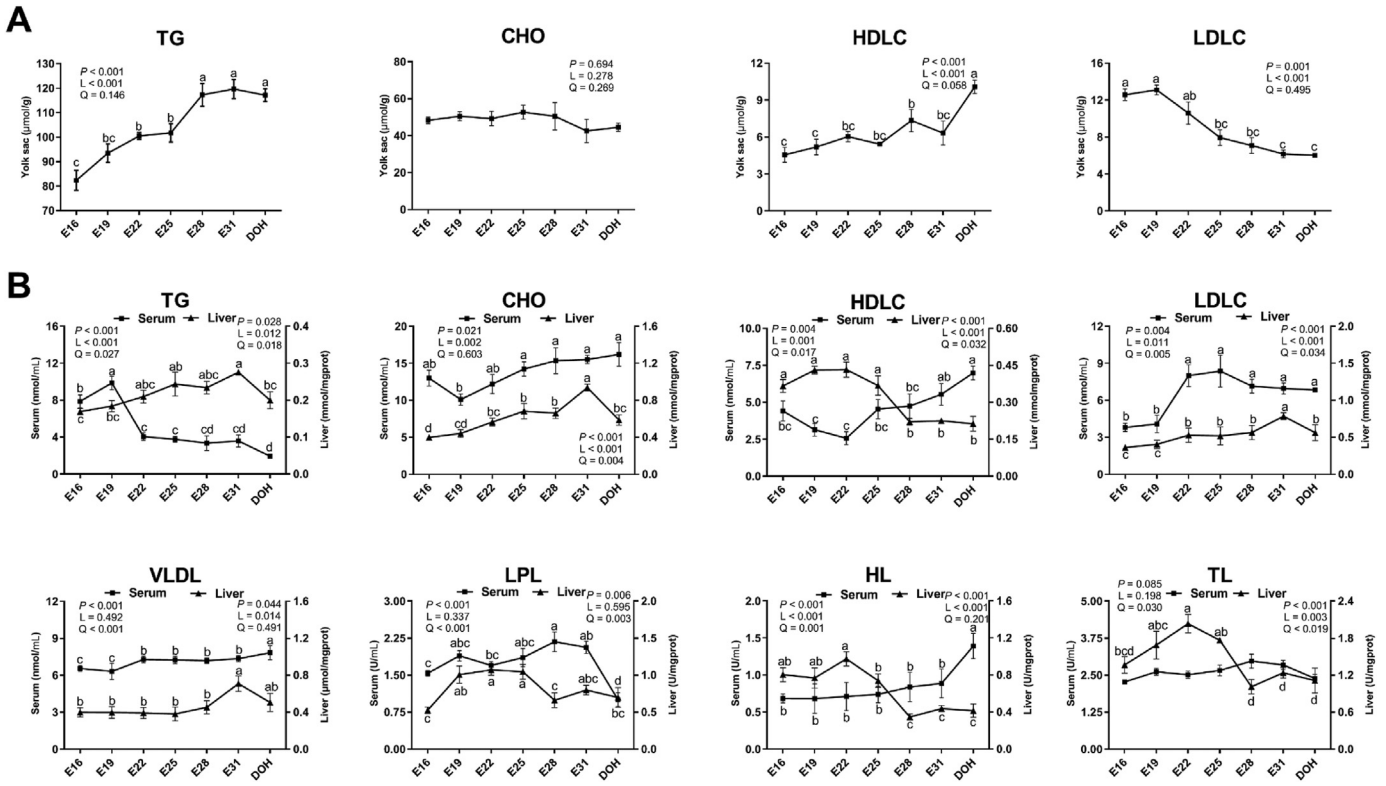

Fig. 2. Dynamic changes of the yolk, serum and hepatic biochemical index in Muscovy duck embryogenesis. (A) yolk sac, as a fresh basis. (B) serum and liver. TG = triacylglycerol contents; CHO = total cholesterol contents; HDLC = high-density lipoprotein cholesterol contents; LDLC = low-density lipoprotein cholesterol contents; VLDL = very-low-density lipoprotein contents; LPL = lipoprotein lipase activity; HL = hepatic lipase activity; TL = total lipase activity. [a-d] Data are expressed as mean ± SEM ($n = 6$), and values on the same line with different lowercase letters indicate statistically significant differences (one-way ANOVA, $P < 0.05$). L, linear; Q, quadratic.

monounsaturated FA (MUFA) increased ($P < 0.05$) linearly or quadratically, attaining their highest level on E31 to DOH, E25 and E31 in the yolk sac, serum and liver, respectively. The PUFA increased ($P < 0.05$) linearly from E16 to DOH in serum, but no differences ($P > 0.05$) in PUFA were observed in either the yolk sac or liver. Unsaturated FA (UFA) increased ($P < 0.05$) linearly or quadratically, whereas saturated FA (SFA) decreased ($P < 0.05$) linearly or quadratically in the yolk sac, serum and liver from E16 to DOH.

3.4. Developmental changes in hepatic gene and protein expression levels related to fatty acid metabolism

Hepatic mRNA expression of the genes *CD36, SLC27A4, Lipin1, FASN, SCD1, ELOVL2, PPARα, RXRα, CPT1A, ACADL* and *ACOX3* increased ($P < 0.05$) all linearly or quadratically from E16 to DOH, for which the maximal values were observed between E25 and E28 (Fig. 3A). Hepatic mRNA expression levels of *FABP1, SREBP1, ACC1* and *ELOVL3* genes decreased ($P < 0.05$) from E16 to E31 but significantly increased ($P < 0.05$) on DOH (Fig. 3A). Hepatic protein expression of SREBP1, FASN, PPARα and CPT1 increased ($P < 0.05$) linearly or quadratically, with maximal values arising between E22 and E28 (Fig. 3B and C).

3.5. Effects of IOF-OA on biochemical indices of lipid and fatty acid profiles

Compared with the control group, the IOF-OA group had a greater ($P < 0.05$) content of TG content in its yolk sac on E28 and E31 (Fig. 4A). On E28, the IOF-OA group also showed augmented ($P < 0.05$) serum activities of LPL and TL, in addition to a greater hepatic HDLC content as well as HL and TL activities (Fig. 4B and C). The IOF-OA group had a reduced ($P < 0.05$) serum content of TG and CHO on E28 (Fig. 4B), and a lower ($P < 0.05$) hepatic VLDL content on E31 vis-à-vis the control group (Fig. 4C). Regarding their FA profiles, relative to the control group, the IOF-OA group was characterized by lower ($P < 0.05$) OA percentages in its yolk sac and liver on E28 (Tables 5 and 7), less ($P < 0.05$) n-6 PUFA in its serum on E28 and E31 (Table 6), and reductions ($P < 0.05$) in UFA in both its serum and liver on E31 (Tables 6 and 7).

3.6. Effects of IOF-OA on hepatic gene expression related to fatty acid metabolism

The hepatic mRNA expression of genes related to FA uptake, synthesis and oxidation, namely *CD36, SLC27A4, Lipin 1, SREBP1, ACC1, FASN, SCD1, ELOVL2, ELOVL3, PPARα, RXRα, CPT1* and *ACOX3* were all lower ($P < 0.05$) in the IOF-OA group than the control group on E28 (Fig. 5). No significant differences ($P > 0.05$) were found in the above gene mRNA expression between the control group and the IOF-OA group on E31.

4. Discussion

Hepatic lipids accumulate progressively during embryogenesis in our study, as evinced by the greater number and area of fat droplets present in the histological sections and the higher hepatic contents of TG and CHO, which were consistent with previous studies of chicken embryos (Guedes et al., 2014; Kim et al., 2017; Liu et al., 2020; Zhao et al., 2007). There were similar developmental patterns between serum and liver in the changes of their CHO, LDLC, VLDL, LPL and TL. Greater hepatic CHO biosynthesis leads to

Table 2
Dynamic changes of the yolk sac fatty acid composition in Muscovy duck embryogenesis (%).

Item		E16	E19	E22	E25	E28	E31	DOH	SEM	*P*	Linear	Quadratic
Myristic acid	C14:0	0.69^{a}	0.60^{ab}	0.56^{b}	0.66^{ab}	0.57^{b}	0.55^{bc}	0.45^{c}	0.032	0.001	<0.001	0.304
Palmitic acid	C16:0	30.27^{ab}	28.51^{abc}	31.44^{a}	28.38^{abc}	30.79^{ab}	26.72^{bc}	25.94^{c}	0.017	0.024	0.011	0.088
Palmitoleic acid	C16:1	2.36	2.39	2.48	2.55	2.19	2.46	2.11	0.533	0.547	0.325	0.245
Stearic acid	C18:0	7.43	6.91	6.93	7.60	6.86	7.54	7.40	0.063	0.171	0.408	0.306
Oleic acid	C18:1n-9	40.59^{d}	43.82^{bc}	40.77^{d}	42.43^{cd}	40.58^{d}	44.97^{ab}	46.48^{a}	0.099	<0.001	<0.001	0.003
Linoleic acid	C18:2n-6	11.67	11.36	11.73	11.54	10.46	11.15	11.29	0.433	0.872	0.379	0.708
Linolenic acid	C18:3n-3	0.58	0.39	0.59	0.55	0.51	0.58	0.61	0.220	0.335	0.310	0.510
Eicosatrienoic acid	C20:3n-6	0.42^{ab}	0.31^{bc}	0.30^{c}	0.46^{a}	0.36^{abc}	0.37^{abc}	0.42^{ab}	0.027	0.023	0.343	0.235
Eicosatrienoic acid	C20:3n-3	3.19^{a}	3.11^{ab}	2.82^{bc}	2.90^{abc}	2.63^{c}	2.80^{bc}	2.62^{c}	0.015	0.009	<0.001	0.253
Nervonic acid	C24:1n-9	0.37^{a}	0.32^{ab}	0.30^{ab}	0.28^{b}	0.29^{b}	0.38^{a}	0.37^{a}	0.050	0.034	0.303	0.002
Docosahexaenoic acid	C22:6n-3	0.63^{a}	0.55^{a}	0.42^{b}	0.41^{b}	0.39^{b}	0.40^{b}	0.45^{b}	0.011	<0.001	<0.001	<0.001
MUFA		43.31^{b}	46.53^{ab}	43.55^{b}	44.84^{b}	43.06^{b}	47.81^{a}	48.97^{a}	0.455	<0.001	<0.001	0.007
PUFA		16.49	15.72	15.85	15.86	14.26	15.21	15.22	0.268	0.557	0.094	0.476
n-6 PUFA		12.09	11.67	12.03	12.00	10.73	11.52	11.71	0.017	0.834	0.402	0.628
n-3 PUFA		4.40^{a}	4.05^{ab}	3.82^{b}	3.87^{b}	3.53^{b}	3.69^{b}	3.50^{b}	0.225	0.010	<0.001	0.211
n-6/n-3		2.75	2.89	3.14	3.11	3.04	3.14	3.41	0.075	0.125	0.007	0.918
UFA		59.80^{bc}	62.25^{ab}	59.39^{bc}	60.70^{abc}	57.31^{c}	63.02^{ab}	64.18^{a}	0.063	0.007	0.048	0.017
SFA		38.38^{a}	36.02^{ab}	38.93^{a}	36.64^{ab}	38.21^{a}	34.93^{ab}	33.60^{b}	0.527	0.039	0.012	0.122
UFA/SFA		1.57^{c}	1.73^{abc}	1.54^{c}	1.66^{bc}	1.55^{c}	1.81^{ab}	1.92^{a}	0.522	0.004	0.003	0.030

E16 to E31 = embroynic day 16 to 31; DOH = day of hatch.
MUFA is the sum of monounsaturated fatty acids that include C16:1, C18:1n-9 and C24:1n-9. PUFA is the sum of polyunsaturated fatty acids that include C18:2n-6, C20:3n-6, C18:3n-3, C20:3n-3 and C22:6n-3. N-6 PUFA is the sum of n-6 polyunsaturated fatty acids that include C18:2n-6 and C20:3n-6. N-3 PUFA is the sum of n-3 polyunsaturated fatty acids that include C18:3n-3, C20:3n-3 and C22:6n-3. UFA is the sum of unsaturated fatty acids that include MUFA and PUFA. SFA is the sum of saturated fatty acids that include C14:0, C16:0 and C18:0.
[a-d] Data are expressed as mean ± SEM ($n = 6$), and data on the same line with different lowercase letters indicate statistically significant differences (one-way ANOVA, $P < 0.05$).

more VLDL being secreted into the blood, thereby increasing the total plasma CHO and LDLC concentrations (Wang et al., 2017b). The liver secretes TG-rich VLDL that is delivered to peripheral tissues for oxidation or storage (Xiao et al., 2011). The HDLC content, regulated by HL activity, underwent a pronounced increase in serum from E22 to DOH, but a sharp decline in the liver from E28 to DOH. It has been suggested that HL serves as a ligand to facilitate lipoprotein uptake by hepatic cell surface receptors and proteoglycans in the middle period of embryogenesis (E16-E25) (Thuren, 2000). In addition, HL functions as a lipolytic enzyme to hydrolyze TG and phospholipids in circulating plasma lipoproteins in the late period of embryogenesis (E28-DOH) (Santamarina-Fojo et al., 2004). Plasma TG is emulsified and packaged into chylomicrons and then delivered to the liver, where they are taken up via receptor-mediated endocytosis, releasing FA when the lysosomes process these particles (Kawano and Cohen, 2013). Interestingly, the hepatic TG content increased during embryogenesis yet the serum TG content declined markedly from E19 to DOH. The LPL activity varies with the changes to its main substrates, TG and VLDL (Li et al., 2020; Olivecrona, 2016). Additionally, relative expression levels of hepatic *LPL* mRNA increased from E9 through E19 in chick embryos (Zhao et al., 2010). It is presumed that TG is hydrolyzed by LPL to release FA for β-oxidation to satisfy the energy demands arising in mid-to-late embryogenesis.

Hepatic FA metabolism is a key step in the regulation of energy homeostasis in the course of embryogenesis. Parallel changes in FA composition were found between the liver and serum in the current study. As the incubation proceeded, hepatic and serum UFA increased and SFA decreased, which was consistent with the FA changes reported in chicken and turkey embryos (Ding and Lilburn, 1996; Su et al., 2020). In contrast to the FA composition of the liver or serum, that of the yolk sac stayed constant throughout embryogenesis, especially distinguished by a steady level of PUFA. In liver and serum, the most abundant FA (OA) was remarkably increased going from E16 to E31 but exhibited a notable decrease on DOH. Except for de novo synthesis, the rising hepatic OA percentage was affected by the accumulation within hepatocytes of large lipid droplets consisting mainly of cholesteryl oleate transferred from the yolk sac (Decrock et al., 2001; Noble and Moore, 1964; Speake et al., 1998). It has been suggested that OA acts as fuel in FA oxidation to generate ATP during mid-to-late embryonic development (Wajner and Amaral, 2015). As the parent compound for the family of n-6 PUFA, C18:2n-6 was the predominant n-6 PUFA present in the yolk sac, serum and liver during embryogenesis, whose content rose from E16 to E28 and fell from E31 to DOH in our study. This parallel change of n-6 PUFA and C18:2n-6 also occurs in chick embryos (Ding and Lilburn, 1996; Kuksis, 1992; Su et al., 2020; Xiao et al., 2020). Generally, C18:2n-6 can be esterified to form neutral and polar lipids such as phospholipids, a structural component of membranes, or they can be elongated and desaturated to produce other bioactive n-6 PUFA (Das, 2006; Whelan and Fritsche, 2013). The main n-3 PUFA are transferred from the yolk sac to the liver, and the developmental maturation of hepatic n-3 PUFA metabolism may supply DHA to the retina and brain during embryogenesis (Cherian et al., 1997; Cherian and Sim, 1992; Martin et al., 1994). This explains why the main n-3 PUFA, composed of C20:3n-3 and C22:6n-3 (DHA), decreased in the serum and liver of duck embryos. A similar changing pattern of n-3 PUFA was also observed in king penguin embryos between E33 and E55 (Decrock et al., 2001). Hepatic UFA increased with the absorption of the yolk sac as well as their de novo synthesis, and serum UFA changed dynamically in parallel with hepatic UFA in response to vital requirements for embryonic development, such as energy demands, organogenesis and cellular homeostasis regulation.

The energy derived from FA β-oxidation is essential for maintaining the normal development and growth of poultry embryos. This process involves the expression of key genes related to FA uptake, de novo biosynthesis and oxidation within the cell. Hepatic FA de novo synthesis increased during E25-DOH, and this might be regulated by the gene and protein expression of SREBP1 and FASN. In addition, ACC1, FASN and SCD1 were subjected to SREBP1 which coordinates the synthesis of FA (Khesht and Hassanabadi, 2012). ACC1 catalyzes the rate-limiting step of the FA biosynthesis pathway by converting acetyl-CoA to malonyl-CoA (Salie and Thelen, 2016). Both acetyl-CoA and malonyl-CoA serve as substrates for FASN to produce C16:0 (Jensen-Urstad and Semenkovich, 2012). SCD1 is the enzyme responsible for the synthesis of MUFA, especially OA (Lounis and Bergeron, 2017; Piccinin et al., 2019). The

Table 3
Dynamic changes of the serum fatty acid composition in Muscovy duck embryogenesis (%).

Item		E16	E19	E22	E25	E28	E31	DOH	SEM	*P*	Linear	Quadratic
Myristic acid	C14:0	0.77	0.53	0.53	0.58	0.62	0.91	0.72	0.041	0.115	0.231	0.088
Palmitic acid	C16:0	27.51^{a}	27.37^{a}	24.92^{b}	23.86^{bc}	23.91^{bc}	23.65^{bc}	23.33^{c}	0.270	<0.001	<0.001	0.003
Palmitoleic acid	C16:1	1.90^{a}	1.08^{b}	1.15^{b}	1.34^{b}	1.00^{b}	1.08^{b}	1.14^{b}	0.058	<0.001	0.001	0.003
Stearic acid	C18:0	12.73^{a}	11.79^{ab}	11.64^{ab}	10.12^{bc}	10.54^{bc}	9.83^{bc}	9.52^{c}	0.280	0.011	<0.001	0.482
Oleic acid	C18:1n-9	30.78^{e}	33.08^{d}	34.14^{cd}	38.60^{a}	36.57^{ab}	37.01^{ab}	35.39^{bc}	0.413	<0.001	<0.001	<0.001
Linoleic acid	C18:2n-6	8.62^{e}	9.6^{de}	11.67^{ab}	11.89^{a}	11.01^{abc}	10.13^{cd}	10.78^{bc}	0.189	<0.001	0.001	<0.001
Eicosatrienoic acid	C20:3n-6	0.64	0.82	0.68	0.73	0.67	0.71	0.61	0.032	0.807	0.535	0.402
Eicosatrienoic acid	C20:3n-3	10.73^{c}	9.25^{de}	9.55^{d}	8.43^{e}	11.00^{bc}	12.37^{a}	11.81^{ab}	0.217	<0.001	<0.001	<0.001
Nervonic acid	C24:1n-9	1.07^{ab}	0.88^{bc}	0.70^{cd}	0.55^{d}	0.74^{cd}	0.69^{cd}	1.16^{a}	0.042	<0.001	0.838	<0.001
Docosahexaenoic acid	C22:6n-3	3.53^{a}	3.30^{bc}	3.01^{cd}	2.63^{d}	2.01^{d}	2.00^{d}	3.10^{ab}	0.110	<0.001	<0.001	<0.001
MUFA		33.74^{e}	35.05^{de}	35.87^{cd}	40.50^{a}	38.11^{b}	38.77^{ab}	37.56^{bc}	0.382	<0.001	<0.001	<0.001
PUFA		23.41^{bcd}	22.97^{cd}	24.91^{abc}	22.26^{d}	24.69^{abc}	25.20^{ab}	26.02^{a}	0.300	0.002	0.002	0.138
n-6 PUFA		9.15^{d}	10.42^{c}	12.35^{a}	12.62^{a}	11.68^{ab}	10.84^{bc}	11.12^{bc}	0.197	<0.001	0.004	<0.001
n-3 PUFA		14.26^{a}	12.55^{b}	12.56^{b}	11.21^{c}	13.01^{b}	14.37^{a}	14.90^{a}	0.222	<0.001	0.007	<0.001
n-6/n-3		0.64^{e}	0.83^{cd}	0.99^{ab}	1.12^{a}	0.91^{bc}	0.76^{de}	0.75^{de}	0.025	<0.001	0.730	<0.001
UFA		57.15^{c}	58.02^{c}	60.78^{b}	62.07^{ab}	62.80^{ab}	63.98^{a}	63.59^{a}	0.414	<0.001	<0.001	0.017
SFA		40.76^{a}	39.60^{ab}	37.09^{b}	33.30^{c}	33.77^{c}	32.86^{c}	33.40^{c}	0.576	<0.001	<0.001	0.019
UFA/SFA		1.40^{c}	1.47^{c}	1.64^{b}	1.82^{a}	1.80^{a}	1.87^{a}	1.88^{a}	0.030	<0.001	<0.001	0.002

E16 to E31 = embroynic day 16 to 31; DOH = day of hatch.
MUFA is the sum of monounsaturated fatty acids that include C16:1, C18:1n-9 and C24:1n-9. PUFA is the sum of polyunsaturated fatty acids that include C18:2n-6, C20:3n-6, C20:3n-3 and C22:6n-3. N-6 PUFA is the sum of n-6 polyunsaturated fatty acids that include C18:2n-6 and C20:3n-6. N-3 PUFA is the sum of n-3 polyunsaturated fatty acids that include C20:3n-3 and C22:6n-3. UFA is the sum of unsaturated fatty acids that include MUFA and PUFA. SFA is the sum of saturated fatty acids that include C14:0, C16:0 and C18:0.
[a-e] Data are expressed as mean ± SEM ($n = 6$), and data on the same line with different lowercase letters indicate statistically significant differences (one-way ANOVA, $P < 0.05$).

mRNA expression levels of *FASN* and *SCD1* increased during E25-DOH, which was consistent with the increased OA in the liver. FA elongation occurs predominantly in microsomes, with the rate-limiting step of condensation catalyzed by the ELOVL family (Zhang et al., 2016). ELOVL2 prefers PUFA, and ELOVL3 prefers SFA or MUFA as substrates, respectively (Guillou et al., 2010). The mRNA expression levels of *ELOVL2* and *ELOVL3* were higher on E28-DOH and DOH, which respectively coincided with an increase in PUFA and a decrease in SFA and MUFA. The hepatic uptake rate of FA is regulated by FA transport proteins, namely FA translocase (CD36), FA transport protein (FATP/SLC27A4) and FA-binding protein (FABP) (Canbay et al., 2007; Gimeno, 2007; Kazantzis and Stahl, 2012; Pepino et al., 2014). There were increases in hepatic mRNA expression levels of *CD36* and *SLC27A4* on E25, and *FABP1* on DOH, indicating the uptake of hepatic FA from serum was enhanced, resulting in the reduced serum TG content. There was rapid uptake of yolk in avian embryos during the late term of incubation, especially on DOH (Liu et al., 2020). FABP1 is associated with fatty acid transportation and its dramatic increase on DOH implied the accelerated utilization of yolk fat by embryos. Lipin 1 was found to have important functions in glycerolipid biosynthesis and its enhanced expression led to stimulation of TG synthesis and secretion (Reue and Dwyer, 2009; Zhang and Reue, 2017). The greater mRNA expression of *Lipin1* on E25 and DOH could upregulate the activities of glycerolipid biosynthetic enzymes, thereby increasing the hepatic TG content. The expression levels of *PPARα*,

Table 4
Dynamic changes of the hepatic fatty acid composition in Muscovy duck embryogenesis (%).

Item		E16	E19	E22	E25	E28	E31	DOH	SEM	*P*	Linear	Quadratic
Myristic acid	C14:0	8.63^{a}	8.59^{a}	5.25^{b}	4.06^{bc}	3.29^{bc}	2.76^{bc}	2.31^{c}	0.489	<0.001	<0.001	0.075
Myristoleic acid	C14:1	0.91^{ab}	0.94^{a}	0.70^{abc}	0.47^{c}	0.56^{bc}	1.01^{a}	0.53^{c}	0.052	0.011	0.072	0.136
Palmitic acid	C16:0	19.67^{a}	18.44^{a}	15.48^{b}	14.93^{b}	14.70^{b}	13.65^{b}	14.32^{b}	0.433	<0.001	<0.001	0.008
Palmitoleic acid	C16:1	0.44^{c}	0.42^{c}	0.72^{b}	0.84^{b}	1.10^{a}	0.67^{bc}	0.64^{bc}	0.048	<0.001	0.004	<0.001
Stearic acid	C18:0	14.46^{a}	12.67^{abc}	12.95^{abc}	10.86^{bcd}	10.26^{d}	12.09^{cd}	13.50^{ab}	0.335	0.002	0.086	<0.001
Oleic acid	C18:1n-9	20.07^{e}	22.98^{e}	32.61^{d}	36.79^{bc}	39.84^{ab}	40.26^{a}	36.30^{c}	1.263	<0.001	<0.001	<0.001
Linoleic acid	C18:2n-6	5.18^{c}	5.87^{c}	8.18^{b}	9.92^{a}	10.00^{a}	9.10^{ab}	8.70^{ab}	0.330	<0.001	<0.001	<0.001
Eicosatrienoic acid	C20:3n-6	0.38^{c}	0.33^{c}	0.34^{c}	0.53^{bc}	0.88^{a}	0.61^{b}	0.46^{bc}	0.038	<0.001	0.001	0.010
Eicosatrienoic acid	C20:3n-3	17.96^{a}	15.23^{abc}	16.10^{ab}	13.05^{cd}	11.41^{d}	14.00^{bcd}	14.42^{bc}	0.461	0.001	0.001	0.002
Nervonic acid	C24:1n-9	0.88	0.75	0.83	0.76	0.72	0.78	0.87	0.026	0.502	0.836	0.081
Docosahexaenoic acid	C22:6n-3	7.15^{a}	5.09^{b}	5.64^{b}	5.45^{b}	5.05^{b}	5.79^{b}	4.73^{b}	0.184	0.003	0.005	0.121
MUFA		22.29^{d}	24.79^{d}	34.72^{c}	38.65^{b}	42.12^{ab}	42.47^{a}	38.86^{b}	1.278	<0.001	<0.001	<0.001
PUFA		30.67	26.52	30.21	28.93	27.33	29.50	28.30	0.434	0.098	0.488	0.496
n-6 PUFA		5.56^{d}	6.19^{d}	8.47^{c}	10.44^{ab}	10.88^{a}	9.71^{abc}	9.15^{bc}	0.350	<0.001	<0.001	<0.001
n-3 PUFA		25.11^{a}	20.32^{b}	21.74^{b}	18.49^{bc}	16.45^{c}	19.79^{bc}	19.15^{bc}	0.575	<0.001	<0.001	0.002
n-6/n-3		0.22^{e}	0.31^{de}	0.41^{cd}	0.57^{ab}	0.69^{a}	0.49^{bc}	0.48^{bc}	0.029	<0.001	<0.001	<0.001
UFA		52.96^{d}	51.31^{d}	64.93^{c}	67.58^{bc}	69.45^{ab}	71.97^{a}	67.16^{bc}	1.280	<0.001	<0.001	<0.001
SFA		42.77^{a}	39.70^{a}	32.80^{b}	29.84^{bc}	28.25^{c}	28.51^{c}	30.13^{bc}	0.988	<0.001	<0.001	<0.001
UFA/SFA		1.24^{d}	1.31^{d}	1.99^{c}	2.27^{b}	2.48^{ab}	2.53^{a}	2.25^{b}	0.084	<0.001	<0.001	<0.001

E16 to E31 =embroynic day 16 to 31; DOH= day of hatch.
MUFA is the sum of monounsaturated fatty acids that include C14:1, C16:1, C18:1n-9 and C24:1n-9. PUFA is the sum of polyunsaturated fatty acids that include C18:2n-6, C20:3n-6, C20:3n-3 and C22:6n-3. N-6 PUFA is the sum of n-6 polyunsaturated fatty acids that include C18:2n-6 and C20:3n-6. N-3 PUFA is the sum of n-3 polyunsaturated fatty acids that include C20:3n-3 and C22:6n-3. UFA is the sum of unsaturated fatty acids that include MUFA and PUFA. SFA is the sum of saturated fatty acids that include C14:0, C16:0 and C18:0.
[a-e] Data are expressed as mean ± SEM ($n = 6$), and data on the same line with different lowercase letters indicate statistically significant differences (one-way ANOVA, $P < 0.05$).

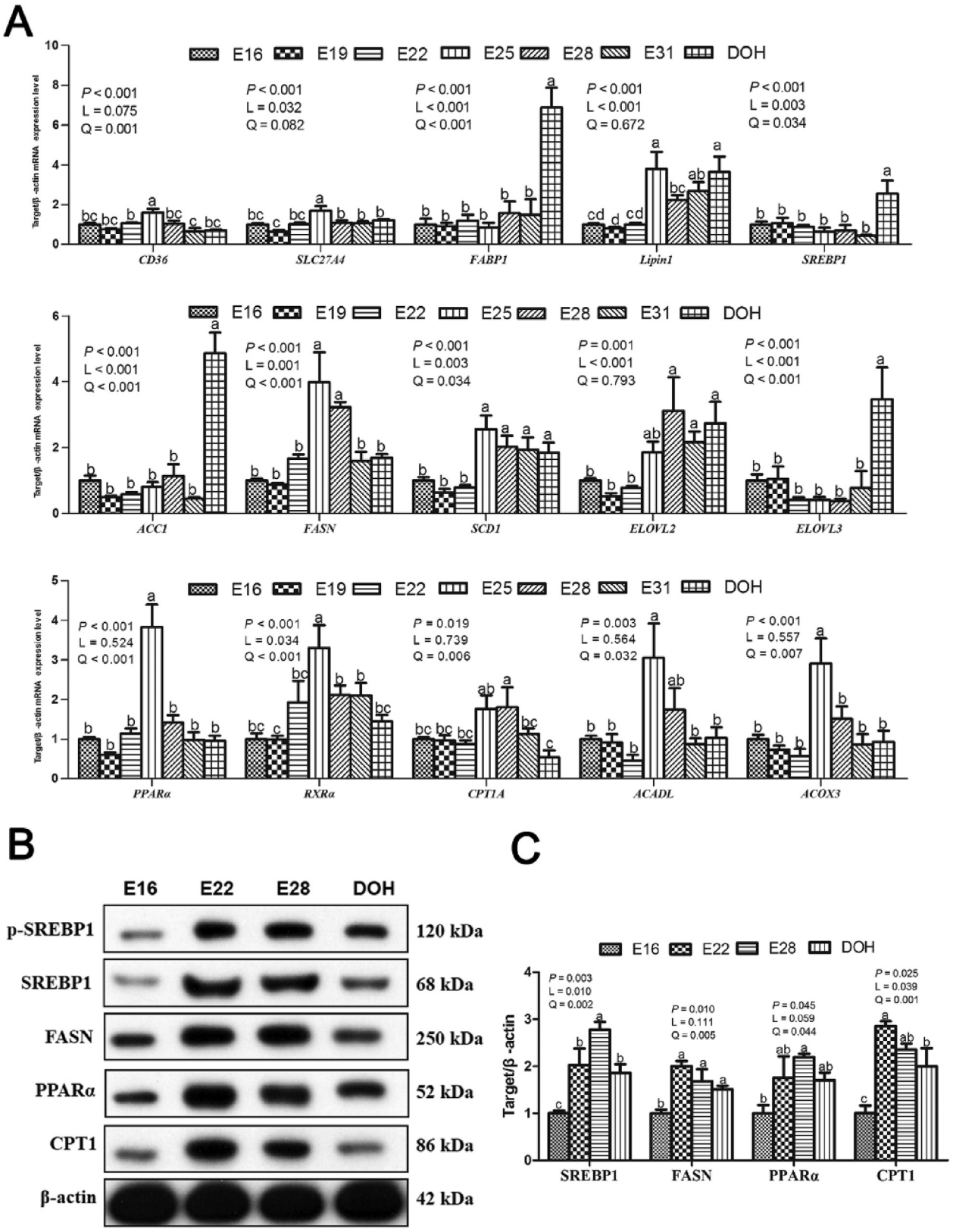

Fig. 3. Dynamic changes in hepatic gene and protein expression related to fatty acid metabolism in Muscovy duck embryogenesis. (A) Targeted gene relative expression ($n = 6$). (B, C) The band density in western blotting analysis was determined using Image J software ($n = 3$). *CD36* = CD36 molecule; *SLC27A4* = solute carrier family 27 member 4; *FABP1* = fatty acid-binding protein 1; *Lipin 1* = phosphatidate phosphatase 1; *SREBP1* = sterol regulatory element-binding transcription factor 1; *ACC1* = acetyl-CoA carboxylase alpha; *FASN* = fatty acid synthase; *SCD1* = stearoyl-CoA desaturase; *ELOVL2* = elongation of very-long-chain fatty acids protein 2; *ELOVL3* = elongation of very-long-chain fatty acids protein 3; *PPARα* = peroxisome proliferator-activated receptor alpha; *RXRα* = retinoid X receptor alpha; *CPT1A* = carnitine palmitoyltransferase 1A; *ACADL* = long-chain-acyl-CoA dehydrogenase; *ACOX3* = acyl-CoA oxidase 3. [a-d] Data are expressed as mean ± SEM, and bars with different lowercase letters indicate statistically significant differences (one-way ANOVA, $P < 0.05$). L, linear; Q, quadratic.

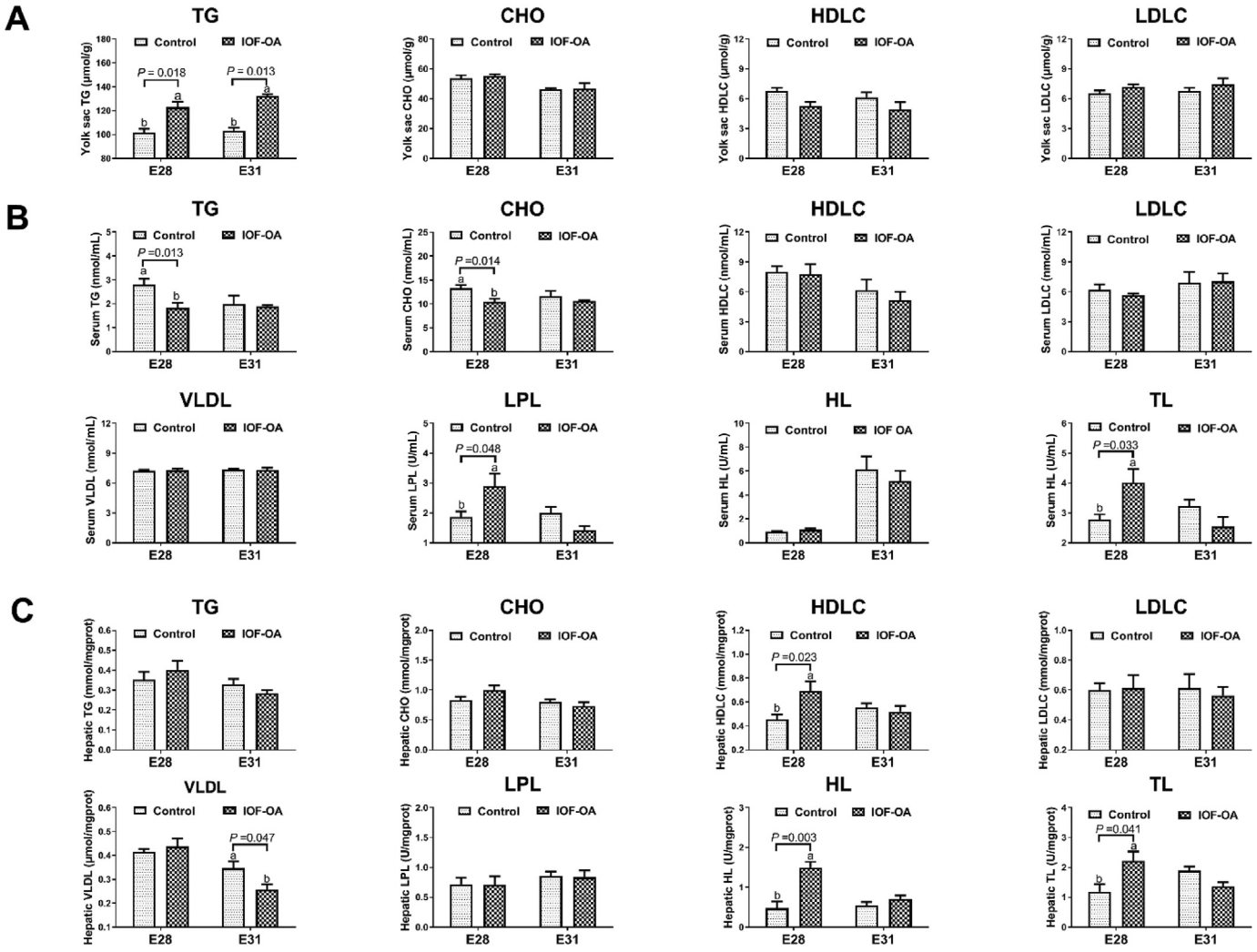

Fig. 4. Effect of in ovo feeding oleic acid on the changes in biochemical index in Muscovy duck embryogenesis. (A) yolk sac, as a fresh basis; (B) serum; (C) liver. Control group was in ovo injected with PBS, and IOF-OA was the treatment group which had in ovo feeding with oleic acid on E25. TG = triacylglycerol contents; CHO = total cholesterol contents; HDLC = high-density lipoprotein cholesterol contents; LDLC = low-density lipoprotein cholesterol contents; VLDL = very-low-density lipoprotein cholesterol contents; LPL = lipoprotein lipase activity; HL = hepatic lipase activity; TL = total lipase activity. [a-b] Data are expressed as mean ± SEM (n = 6), and bars on the same sampled day with different lowercase letters indicate statistically significant differences (t-test, $P < 0.05$).

RXRα, CPT1A, ACADL and *ACOX3* genes related to FA oxidation all increased during E25-E28, which was partly regulated by the expressions of PPAR and CPT1. PPAR is of critical importance to catabolism and clearance of FA, and retinoid X receptors are obligate heterodimeric partners for PPAR action (Cai et al., 2021; Wang et al., 2020), while CPT1A is required for the transport of long-chain FA into mitochondria (Broadway et al., 2001). Accordingly, protein expression levels of SREBP1, FASN, PPARα and CPT1 increased quadratically and peaked in the period of E22 to E28. Altogether, the results of mRNA and protein expression analyses suggest that FA synthesis and oxidation increased during E22 to E28 to meet the energy demands of embryo development.

During incubation, embryonic growth and development are dependent on nutrients deposited in the egg. However, the rapid growth of genetically improved birds can make the amount of nutrients contained in the egg insufficient for the complete development of embryonic tissues (Grodzik et al., 2013). In order to overcome these problems, IOF was developed to increase the availability of nutrients for the embryo. Previous studies have demonstrated the beneficial effects of IOF carbohydrates, amino acids, minerals, and vitamins on embryonic development but the consequences of an intervention by FA are rarely reported (Foye et al., 2006; Tangara et al., 2010; Yair et al., 2015). In our study, the IOF-OA treatment increased the TG content of the yolk sac on E28 and E31, as well as the hepatic HDLC content and HL activity on E28, but decreased the TG and CHO contents of serum on E28 and hepatic VLDL content on E31. As a key component of the cell membrane, CHO is pivotal to the growth process of embryos, including the regulation of cellular signaling and production of bile acids and steroid hormones, and deficiencies in CHO during embryogenesis and organogenesis cause severe abnormalities (Connor et al., 1969; Roux et al., 2000; Woollett, 2008; Yoshida and Wada, 2005). In cell models, OA inhibits the synthesis of CHO and reduces its accumulation by decreasing the CHO biosynthesis rate and the influx of low-density lipoprotein while increasing the high-density lipoprotein-mediated efflux of CHO (Natali et al., 2007; Priore and Gnoni, 2017; Rosenblat et al., 2016). Hepatic FA metabolism is typically associated with energy homeostasis in growth and development, such that metabolic disorders of FA often lead to dysfunction and adverse physiological effects (Hodson, 2019; Migrenne et al., 2007). In avian embryo models, the FA composition in embryonic tissues could be affected by the maternal FA supply

Table 5
Effect of in ovo feeding oleic acid on changes in yolk sac fatty acid composition in Muscovy duck embryogenesis (%).

Item		E28				E31			
		Control	IOF-OA	SEM	*P*	Control	IOF-OA	SEM	*P*
Myristic acid	C14:0	0.97	0.99	0.082	0.782	0.96[b]	1.30[a]	0.151	0.049
Palmitic acid	C16:0	27.45	29.30	1.357	0.202	27.12	26.37	0.755	0.346
Palmitoleic acid	C16:1	2.79[b]	4.35[a]	0.860	0.010	3.51[a]	2.29[b]	0.512	0.038
Stearic acid	C18:0	4.78	4.09	0.326	0.060	4.90	4.27	0.751	0.421
Oleic acid	C18:1n-9	46.27[a]	43.61[b]	1.051	0.022	45.42	46.78	1.606	0.417
Linoleic acid	C18:2n-6	12.37	12.39	0.571	0.977	12.79	13.61	1.211	0.515
Linolenic acid	C18:3n-3	0.35	0.33	0.018	0.527	0.35	0.37	0.039	0.736
Eicosatrienoic acid	C20:3n-6	0.25	0.23	0.028	0.425	0.23	0.22	0.028	0.561
Eicosatrienoic acid	C20:3n-3	3.07	3.13	0.180	0.756	3.22[b]	3.52[a]	0.125	0.038
Nervonic acid	C24:1n-9	0.40	0.40	0.046	0.972	0.38	0.35	0.039	0.428
Docosahexaenoic acid	C22:6n-3	0.56[a]	0.46[b]	0.034	0.016	0.49[b]	0.58[a]	0.014	<0.001
MUFA		49.40	48.36	1.649	0.544	49.31	49.35	1.673	0.981
PUFA		16.60	16.51	0.641	0.885	17.08	18.19	1.269	0.401
n-6 PUFA		12.62	12.58	0.575	0.939	13.02	13.82	1.216	0.526
n-3 PUFA		3.98	3.93	0.204	0.811	4.05	4.37	0.192	0.134
n-6/n-3		3.19	3.22	0.197	0.876	3.20	3.20	0.313	0.998
UFA		66.00	64.87	1.641	0.506	66.39	67.54	1.285	0.393
SFA		33.20	34.39	1.567	0.466	32.98	31.73	1.286	0.353
UFA/SFA		2.01	1.90	0.137	0.447	2.02	2.14	0.126	0.363

Control group was in ovo injected with PBS, and IOF-OA was the treatment group which had in ovo feeding with oleic acid on embryonic day 25 (E25). MUFA is the sum of monounsaturated fatty acids that include C16:1, C18:1n-9 and C24:1n-9. PUFA is the sum of polyunsaturated fatty acids that include C18:2n-6, C20:3n-6, C18:3n-3, C20:3n-3 and C22:6n-3. N-6 PUFA is the sum of n-6 polyunsaturated fatty acids that include C18:2n-6 and C20:3n-6. N-3 PUFA is the sum of n-3 polyunsaturated fatty acids that include C18:3n-3, C20:3n-3 and C22:6n-3. UFA is the sum of unsaturated fatty acids that include MUFA and PUFA. SFA is the sum of saturated fatty acids that include C14:0, C16:0 and C18:0.
[a,b] Data are expressed as mean ± SEM ($n = 6$), and data on the same sampled day on the same line with different lowercase letters indicate statistically significant differences (*t*-test, $P < 0.05$).

and lipid utilization (Akbari et al., 2020; Cherian et al., 1997; Speake et al., 1999). Maternal dietary conjugated linoleic acid increased the SFA in the yolk sac and decreased hepatic UFA in chick or quail embryos, resulting in their lower hatchability rate and diminished chick weight upon hatching (Aydin and Cook, 2004; Fu et al., 2019; Leone et al., 2010). Here, the IOF-OA in duck embryos on E25 decreased the UFA percentage in serum and liver and down-regulated the hepatic mRNA expression of *CD36, SLC27A4* and *FABP1* genes related to FA uptake, and *Lipin1, SREBP1, ACC1, FASN, SCD1, ELOVL2* and *ELOVL3* genes related to FA synthesis on E28, while there was no difference on these genes mRNA expression on E31. These inconsistent results between E28 and E31 implied that exogenous OA intervention may lose effectiveness on FA metabolism and target gene expression as development progresses. Additionally, the decreased hepatic mRNA expression of *PPARα, RXRα, CPT1A* and *ACOX3* genes related to FA oxidation induced by the IOF-OA treatment might generate less energy to support embryonic growth and development on E28, contributing to higher embryonic mortality (control group vs IOF-OA group: 16.67% vs 21.67%).

Table 6
Effect of in ovo feeding oleic acid on changes in serum fatty acid composition in Muscovy duck embryogenesis (%).

Item		E28				E31			
		Control	IOF-OA	SEM	*P*	Control	IOF-OA	SEM	*P*
Myristic acid	C14:0	3.71	5.02	0.860	0.159	6.32	7.58	1.244	0.344
Palmitic acid	C16:0	20.94	20.77	1.081	0.876	21.24[a]	19.47[b]	0.904	0.039
Palmitoleic acid	C16:1	0.91	1.04	0.204	0.555	0.81	0.63	0.173	0.314
Stearic acid	C18:0	8.36	8.40	0.419	0.932	8.86[b]	9.96[a]	0.511	0.046
Oleic acid	C18:1n-9	35.08	37.08	1.740	0.280	35.29	33.32	1.549	0.231
Linoleic acid	C18:2n-6	12.48	11.18	0.696	0.093	10.42[a]	8.77[b]	0.549	0.013
Eicosatrienoic acid	C20:3n-6	1.16	1.02	0.308	0.671	0.51	0.75	0.251	0.361
Eicosatrienoic acid	C20:3n-3	11.37	10.12	2.062	0.557	10.67	11.94	1.187	0.312
Nervonic acid	C24:1n-9	1.06	0.54	0.368	0.233	0.64	0.69	0.094	0.604
Docosahexaenoic acid	C22:6n-3	2.03	2.10	0.128	0.580	1.89	2.09	0.309	0.529
MUFA		36.45	38.13	1.761	0.366	36.53	34.29	1.677	0.212
PUFA		27.03	24.08	2.143	0.199	23.32	23.42	1.513	0.949
n-6 PUFA		13.64[a]	11.87[b]	0.641	0.020	10.76[a]	9.39[b]	0.633	0.047
n-3 PUFA		13.40	14.09	1.673	0.511	12.56	14.03	1.210	0.254
n-6/n-3		1.05	0.85	0.120	0.128	0.88[a]	0.68[b]	0.073	0.021
UFA		63.68	62.21	1.753	0.424	61.95[a]	59.99[b]	0.471	0.049
SFA		34.33	33.35	1.839	0.608	35.36[b]	38.00[a]	0.728	0.047
UFA/SFA		1.86	1.89	0.116	0.797	1.71[a]	1.58[b]	0.061	0.048

Control group was in ovo injected with PBS, and IOF-OA was the treatment group which had in ovo feeding with oleic acid on embryonic day 25 (E25). MUFA is the sum of monounsaturated fatty acids that include C16:1, C18:1n-9 and C24:1n-9. PUFA is the sum of polyunsaturated fatty acids that include C18:2n-6, C20:3n-6, C20:3n-3 and C22:6n-3. N-6 PUFA is the sum of n-6 polyunsaturated fatty acids that include C18:2n-6 and C20:3n-6. N-3 PUFA is the sum of n-3 polyunsaturated fatty acids that include C20:3n-3 and C22:6n-3. UFA is the sum of unsaturated fatty acids that include MUFA and PUFA. SFA is the sum of saturated fatty acids that include C14:0, C16:0 and C18:0.
[a,b] Data are expressed as mean ± SEM ($n = 6$), and data on the same sampled day on the same line with different lowercase letters indicate statistically significant differences (*t*-test, $P < 0.05$).

Table 7
Effect of in ovo feeding oleic acid on the changes of hepatic fatty acid composition in Muscovy duck embryogenesis (%).

Item		E28				E31			
		Control	IOF-OA	SEM	*P*	Control	IOF-OA	SEM	*P*
Myristic acid	C14:0	5.77	5.90	0.644	0.854	5.08[b]	8.23[a]	0.528	<0.001
Myristoleic acid	C14:1	0.57[b]	0.73[a]	0.078	0.045	0.67	0.86	0.127	0.162
Palmitic acid	C16:0	16.96[a]	14.95[b]	0.799	0.033	16.91	17.10	1.020	0.850
Palmitoleic acid	C16:1	0.59	0.46	0.098	0.244	0.47	0.40	0.092	0.454
Stearic acid	C18:0	12.65[b]	16.26[a]	0.820	0.001	16.28	14.85	1.028	0.194
Oleic acid	C18:1n-9	30.18[a]	27.47[b]	1.536	0.048	26.86	27.49	1.665	0.714
Linoleic acid	C18:2n-6	8.68	8.41	0.607	0.668	8.09	8.24	0.887	0.871
Eicosatrienoic acid	C20:3n-6	0.34	0.45	0.073	0.182	0.41	0.43	0.061	0.708
Eicosatrienoic acid	C20:3n-3	14.14	16.19	1.053	0.084	15.40	16.32	1.323	0.504
Nervonic acid	C24:1n-9	0.89	0.69	0.210	0.375	0.95	1.01	0.112	0.595
Docosahexaenoic acid	C22:6n-3	6.21	6.14	1.007	0.947	6.49[a]	3.00[b]	0.737	<0.001
MUFA		31.73	29.36	1.606	0.173	28.87	29.69	1.790	0.656
PUFA		30.01	31.19	1.001	0.267	30.39	27.98	1.567	0.156
n-6 PUFA		9.03	8.86	0.615	0.797	8.50	8.67	0.867	0.847
n-3 PUFA		20.98	22.33	1.122	0.260	21.89	19.32	1.768	0.176
n-6/n-3		0.43	0.40	0.043	0.523	0.41	0.46	0.077	0.556
UFA		61.74	60.55	0.783	0.162	59.25[a]	57.67[b]	0.552	0.017
SFA		36.16	37.10	0.624	0.163	38.26	38.81	0.871	0.543
UFA/SFA		1.71	1.63	0.048	0.156	1.55	1.49	0.045	0.196

Control group was in ovo injected with PBS, and IOF-OA was the treatment group which had in ovo feeding with oleic acid on embryonic day 25 (E25). MUFA is the sum of monounsaturated fatty acids that include C14:1, C16:1, C18:1n-9 and C24:1n-9. PUFA is the sum of polyunsaturated fatty acids that include C18:2n-6, C20:3n-6, C20:3n-3 and C22:6n-3. N-6 PUFA is the sum of n-6 polyunsaturated fatty acids that include C18:2n-6 and C20:3n-6. N-3 PUFA is the sum of n-3 polyunsaturated fatty acids that include C20:3n-3 and C22:6n-3. UFA is the sum of unsaturated fatty acids that include MUFA and PUFA. SFA is the sum of saturated fatty acids that include C14:0, C16:0 and C18:0. [a,b] Data are expressed as mean ± SEM ($n = 6$), and data on the same sampled day on the same line with different lowercase letters indicate statistically significant differences (*t*-test, $P < 0.05$).

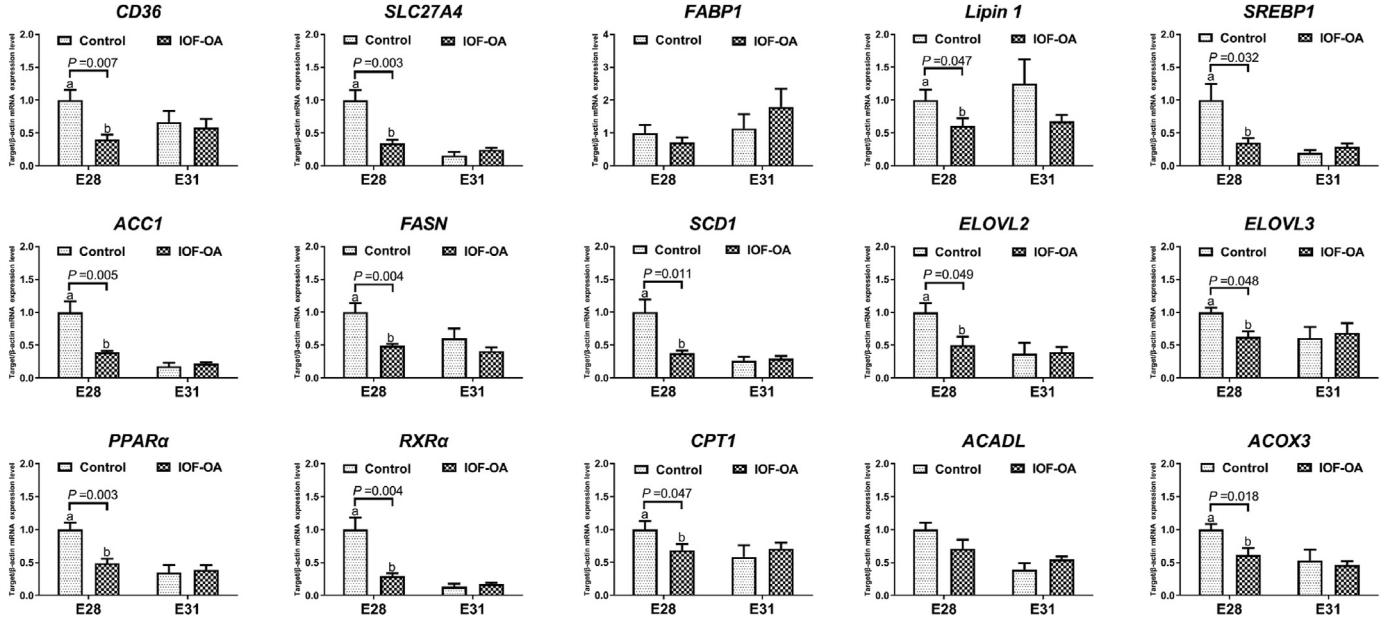

Fig. 5. Effect of in ovo feeding oleic acid on the gene expression related to hepatic fatty acid metabolism in Muscovy duck embryogenesis. Control group was in ovo injected with PBS, and IOF-OA was the treatment group which had in ovo feeding with oleic acid on E25. *CD36* = CD36 molecule; *SLC27A4* = solute carrier family 27 member 4; *FABP1* = fatty acid-binding protein 1; *Lipin 1* = phosphatidate phosphatase 1; *SREBP1* = sterol regulatory element-binding transcription factor 1; *ACC1* = acetyl-CoA carboxylase alpha; *FASN* = fatty acid synthase; *SCD1* = stearoyl-CoA desaturase; *ELOVL2* = elongation of very-long-chain fatty acids protein 2; *ELOVL3* = elongation of very-long-chain fatty acids protein 3; *PPARα* = peroxisome proliferator-activated receptor alpha; *RXRα* = retinoid X receptor alpha; *CPT1A* = carnitine palmitoyltransferase 1A; *ACADL* = long-chain-acyl-CoA dehydrogenase; *ACOX3* = acyl-CoA oxidase 3. [a-b] Data are expressed as mean ± SEM ($n = 6$), and bars on the same sampled day with different lowercase letters indicate statistically significant differences (*t*-test, $P < 0.05$).

5. Conclusion

In summary, the maximal metabolic changes in lipid metabolism in both the liver and serum of Muscovy duck embryos occurred from E22 to E28, along with altered levels of target gene and protein expression related to lipogenesis and lipolysis. In ovo feeding with oleic acid on E25 could inhibit the target gene expression related to FA uptake, synthesis and oxidation, which may influence normal FA metabolism of duck embryos on E28.

Author contributions

Xiufen Zhang: conceptualization, formal analysis, data curation, writing original draft. **Qilin Wu:** formal analysis, project

administration. **Wenxuan Zheng:** formal analysis, methodology. **Chuang Liu:** resources, software. **Liang Huang:** methodology, data curation. **Xin Zuo:** formal analysis. **Wenquan Xiao:** formal analysis. **Xiaofeng Han:** formal analysis. **Hui Ye:** data curation. **Wence Wang:** data curation. **Lin Yang:** supervision, funding acquisition, and project administration. **Yongwen Zhu:** supervision, funding acquisition, project administration, writing-reviewing, and editing.

Declaration of competing interest

We declare that we have no financial and personal relationships with other people or organizations that can inappropriately influence our work, and there is no professional or other personal interest of any nature or kind in any product, service and/or company that could be construed as influencing the content of this paper.

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