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Vitellogenin 1 is essential for fish reproduction by transporting DHA-containing phosphatidylcholine from liver to ovary

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ABSTRACT

Vitellogenins (Vtgs) are essential for female reproduction in oviparous animals, yet the exact roles and mechanisms remain unknown. In the present study, we knocked out *vtg1*, which is the most abundant Vtg in zebrafish, *Danio rerio* via the CRISPR/Cas 9 technology. We aimed to identify the roles of Vtg1 and related mechanisms in reproduction and development. We found that, the Vtg1-deficient female zebrafish reduced gonadosomatic index, egg production, yolk granules and mature follicles in ovary compared to the wide type (WT). Moreover, the Vtg1-deficient zebrafish diminished hatching rates, cumulative survival rate, swimming capacity and food intake, but increased malformation rate, and delayed swim bladder development during embryo and early-larval phases. The Vtg1-deficiency in female broodstock inhibited docosahexaenoic acid-enriched phosphatidylcholine (DHA-PC) transportation from liver to ovary, which lowered DHA-PC content in ovary and offspring during larval stage. However, the Vtg1-deficient zebrafish increased gradually the total DHA-PC content via exogeneous food intake, and the differences in swimming capacity and food intake returned to normal as they matured. Furthermore, supplementing Vtg1-deficient zebrafish with dietary PC and DHA partly ameliorated the impaired female reproductive capacity and larval development during early phases. This study indicates that, DHA and PC carried by Vtg1 are crucial for female fecundity, and affect embryo and larval development through maternal-nutrition effects. This is the first study elucidating the nutrient and physiological functions of Vtg1 and the underlying biochemical mechanisms in fish reproduction and development.

1. Introduction

Vitellogenins (Vtgs) are yolk proteins precursors in nearly all female oviparous animals such as fish, amphibians, reptiles, birds and insects. Accordingly, they are regarded as female-specific proteins [1]. The Vtgs are high molecular weight lipoproteins, which contain variable amount of nutrients, including lipids, amino acids, carbohydrates, vitamins and phosphate [2]. The of Vtgs synthesis in most female oviparous animals are stimulated by estradiol in the liver, releasing Vtgs into blood circulation, which are transported to the ovary for nourishing oocytes [3]. However, fat body tissue contributes to the majority of Vtg synthesis and exerts crucial roles for egg development and female fecundity in insects [4]. Moreover, Tingaud-Sequeira et al. found that visceral white adipose tissue (WAT)-sourced Vtgs also play the role for providing components to the ovary in female teleost fish [5].

Vtgs are known to regulate reproduction in female oviparous animals. For example, Vtgs correlated to ovarian nutrients and egg types in barfin flounder (*Verasper moseri*) through controlling free amino acids content in oocyte [6]. Moreover, Vtg knockdown damaged egg viability and embryonic development in cotton boll weevil (*Anthonomus grandis*) [7]. Interestingly, several previous studies found that Vtgs also affect immune function and antioxidant capacity in eggs and early embryos [8,9]. For instance, Vtgs possess antibacterial activity, which protects the host against bacteria and virus [8]. Furthermore, Vtgs protect cellular components from free radical damage in insects and vertebrates [9]. However, the precise underlying mechanisms on the effects of Vtgs in reproductive and antioxidant on females and eggs have not been well addressed. A general concept is that Vtgs are important for transporting nutrients to the ovary [2], thus vitellogenesis exerts high correlation to nutrient metabolism, especially lipid processes. Notably, liver

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accumulated large amounts of lipid droplets in female zebrafish (*Danio rerio*) during vitellogenin synthesis [10]. Moreover, induced vitellogenesis upregulated the expression of acyl-CoA synthetase short-chain family member 1 (*acss1*), which is related to fatty acid synthesis in European eel (*Anguilla anguilla*) [11]. A similar trend was also reported in yellowtail rockfish (*Sebastes flavidus*) with notable elevated concentrations of polar lipids, triacylglycerols (TG) and cholesterol in ovary during late vitellogenesis [12]. In our recent work, we also observed a notable lipogenesis in zebrafish liver during vitellogenin synthesis induced by estrogens [13]. These studies suggest that Vtgs play key roles in transporting various lipid components [14]. However, currently no study has intensively explored the interactions between reproduction and lipid components via the Vtgs regulations in female oviparous animals.

Vtgs are encoded by a multigene family such that different animals possess various subtypes. For instance, three *vtg* genes have been reported in chicken [15], six in nematode (*Caenorhabditis elegans*) [16], seven in zebrafish (*Danio rerio*) [17], and up to 20 in rainbow trout (*Oncorhynchus mykiss*) [18]. Among all Vtg subtypes in zebrafish, the *vtg1*, *vtg2* and *vtg3* mRNA expressions are much higher than others [17]. Accordingly, the transcription of *vtg1* mRNA expression is about 100 X and 1000 X higher than that of *vtg2* and *vtg3* mRNAs, respectively [17]. Therefore, Vtg1 is regarded as the most important Vtg among all the subtypes in zebrafish [17]. However, although many Vtg subtypes have been reported in various oviparous animals, limited studies have investigated the functions of different Vtg subtypes in animal reproduction [19]. To our knowledge, so far, only one study indicated increased egg numbers per spawn after *vtg1, 4, 5, 6* and *7* were simultaneously knocked out and *vtg3*-knockout decreased fertilization rates in zebrafish [19]. Nevertheless, the role of Vtg1 as a dominant Vtg subtype in reproduction and nutrient transportation in oviparous animals is still largely unknown.

In the present study, we used the clustered regularly interspaced short palindromic repeat-associated protein 9 (CRISPR/Cas9) technology to generate the *vtg1*-specific knockout model in zebrafish. The aim was to explore the roles of Vtg1 in reproductive process and the related mechanisms in fish. Accordingly, we compared the female reproductive activity and offspring development in different life-phases between wild type (WT) and *vtg1*-deficient zebrafish by using lipidomics assay and a series of physiological and biochemical parameters. We found that, Vtg1 plays key roles in transporting DHA-enriched phosphatidylcholine, which is essential for female fish reproduction and larval development. The knowledge generated enhances our understanding on the functions of Vtgs in fish and represents an important reference for studying other oviparous animals.

2. Materials and methods

2.1. Ethical consideration

All fish experiments were conducted under the Guidance Suggestions for the Care and Use of Laboratory Animals formulated by the Ministry of Science and Technology in China. This research was approved by the Committee on the Ethics of Animal Experiments of East China Normal University, Shanghai, China (approval NO.: F20140101).

2.2. Establishment of vtg1 knockout line

Zebrafish *vtg1* knockout line with a 7 base pairs (bp) deletion was generated by the CRISPR/Cas9 technology, which was described in details in previous study [20]. The guide RNA (gRNA), generated against the *vtg1* target sites in the fifth exon, was designed by using the online tool ZiFiT Targeter version 4.2 (http://zifit.partners.org/ZiFiT). A double strand DNA for gRNA was synthesized from the PCR products of the pMD19T-gRNA plasmid by using the primers containing the target sequence. The double strand DNA was used for single-gRNA (sgRNA) synthesis by using the TranscriptAid T7 high-yield transcription kit (K0441; Thermo Fisher Scientific, Waltham, MA). The Cas9 mRNAs were generated from the pXT7-Cas9 plasmid by using T3 mMESSAGE mMACHINE mRNA transcription synthesis kit (AM1344; Thermo Fisher Scientific). The Cas9 mRNA and sgRNA for *vtg1* were combined and injected into one-cell-stage embryos using a Picoliter Microinjector PLI-100A (Warner Instruments, Hamden, CT, USA) to produce the mutants. The mutational F0 zebrafish were mated with wild type zebrafish to generate heterozygous F1 offspring. Subsequently, the *vtg1*$^{-/-}$ homozygous mutants (F2) with 7 bp target sequence deletion were generated through self-crossing of the same *vtg1* heterozygous mutational types.

2.3. Zebrafish maintenance

Wild type (WT) and *vtg1* deficient adult zebrafish were raised and maintained in a recirculating aquaculture system with 28.5 °C water temperature, a 14 h light/10 h dark cycle. The experimental zebrafish were fed with artemia three times daily. The zebrafish embryos were obtained by natural spawning. Zebrafish embryos and larvae from 0 to 10 days post fertilization (dpf) were kept in 2 L water tanks in an incubator. The zebrafish were maintained at 37 °C with 14 h light/10 h dark cycle. The experimental zebrafish were fed twice a day with yolk homogenate from 5 to 7 dpf followed by feeding them with paramecia from 8 to 10 dpf.

2.4. Feeding trials by using the diets supplemented with docosahexaenoic acid (DHA) and/or phosphatidylcholines (PC)

To test whether DHA and PC supplementation could ameliorate the negative effects caused by *vtg1* deficiency, we formulated purified diets as detailed in supporting information (SI) Table S1. Highly purified fish oil (85 % DHA, Shouhe Biological Technology Co. LTD, Xi'an, China) and soybean lecithin (98 % PC, Taiwei Pharmaceutical Co. LTD, Shanghai, China) were used to replenish the DHA and PC in the diets, respectively. The treatments were: WT-Control (WC, containing 0.84 % DHA and 1.62 % PC); *vtg1*$^{-/-}$-Control (VC, containing 0.84 % DHA and 1.62 % PC); *vtg1*$^{-/-}$-DHA (VD, containing 2.45 % DHA and 1.59 % PC); *vtg1*$^{-/-}$- PC (VP, containing 0.8 % DHA and 4.82 % PC); and *vtg1*$^{-/-}$-DHA + PC (VDP, containing 2.55 % DHA and 4.73 % PC).

A total of 30 WT or *vtg1*$^{-/-}$ female adult zebrafish were selected randomly (three replicates, 10 fish for each) and distributed into five treatments. The fish from each treatment were cultured in a 10 L glass tank and fed with the WC, VC, VD, VP or VDP diets at a feeding rate of 5 % body weight, three times a day for three weeks. All fish in each tank were weighed on 11th day and the daily feeding amounts were adjusted accordingly. One third of the culture water by volume in each tank was replaced with fresh-dechlorinated water every day.

2.5. Zebrafish development observation and growth performance

At the end of the experiment, the zebrafish were fasted for 12 h before sampling. The survival rate in each replicate was calculated as the percentage of survived fish to the initial amount. The zebrafish were anesthetized by using MS-222 (20 mg/L, Western Chemicals, Inc., Ferndale, Washington) for liver and ovary tissues collections. The spawning cycles and oviposition amounts in mature female fish were also recorded. The larvae at various stages were randomly sampled for assessing embryo development at 1 hour post fertilization (hpf) to 7 dpf, malformation rate at 7 dpf and hatching rate at 3 dpf by observing them under a microscope (Olympus U-TV0.5XC-3, Tokyo, Japan). The malformation rate in this study was mainly reflected by curved tail and swollen-tummy. The body weight, condition factor (CF), hepatosomatic index (HSI) and gonadosomatic index (GSI) of mature female fish (120 dpf) were calculated by using the formulae: CF = 100 × (fish weight, g) / (fish length, cm)^3; HSI = 100 × (liver weight, g) / (fish weight, g); and GSI = 100 × (ovary weight, g) / (fish weight, g).

2.6. Swimming behavior assay

The zebrafish at 6 (n = 12), 15 (n = 6), 30 (n = 8) and 60 (n = 6) dpf from WT and *vtg1*$^{-/-}$ were randomly sampled and transferred into 24 - well plate, 12 - well plate, 16 - well tank and 12 - well tank, respectively. The zebrafish were prepared for swimming behavior assessment in water maintained at 28.5 °C. The swimming distance of each single fish was recorded for over eight hours by using a Zebralab and ZebraBox system (ViewPoint Life Sciences, Montreal, QC, Canada).

2.7. Food intake assay

The zebrafish at 7, 15, 30 and 60 dpf (n = 3) were randomly selected for the food intake assay, which was carried out by using fluorescently-labeled paramecia and artemia. The preparations of fluorescently-labeled paramecia and artemia were performed as described in a previous study [21]. Zebrafish were transferred into 6-well plates containing 6 ml of Milli-Q water at 12 h before food intake assay. Sufficient fluorescent paramecia or artemia were added into each well to feed the zebrafish in the dark condition. Around 200 to 250 fluorescent paramecia were added in each well for food intake assay for the zebrafish at 7 dpf. For the zebrafish at 15, 30 and 60 dpf, the fluorescent artemia amounts added in each well were 20, 50 and 200, respectively. The amounts of paramecia or artemia used in this study were sufficient for the fish. Two hours after feeding, the water with fluorescent paramecia or artemia was replaced two times with fresh Milli-Q water and the zebrafish were washed for 15 min. After washing, the zebrafish were anesthetized with 2 mg/L MS-222. The fluorescent signals in zebrafish were measured by using a fluorescent microscope (Olympus U-TV0.5XC-3, Tokyo, Japan) using a fluorescein filter set. The fluorescence intensity was defined as a proportional of the paramecia or artemia amount fed to each zebrafish.

2.8. Total lipid content and lipid composition assays

Whole zebrafish and tissue samples at various periods collected were stored at −80 °C for biochemical assays and molecular measurements. The whole fish (at 7, 60 and 120 dpf) and adult female zebrafish (120 dpf) liver and ovary tissues were collected for total lipid extraction. Total lipid was measured by using the classical methanol-chloroform method [22]. The neutral lipids such as triglyceride (TG), diglyceride (DG), monoglyceride (MG), and phospholipids (PLs) such as phosphatidylethanolamine (PE), phosphatidylinositol (PI), phosphatidylserine (PS) and PC were separated from the total lipid by using a thin layer chromatography (TLC) as described previously [23]. The separated neutral lipids and PLs were stained by using iodine for 20 min, and then measured for lipid composition by using a TLC scanner (Kezhe Biochemical Technology Co., Shanghai, China).

2.9. Fatty acid composition assay

Whole zebrafish (at 7, 60 and 120 dpf) and adult female zebrafish (120 dpf) liver and ovary tissues were collected for detecting fatty acid composition. The fatty acid measurement was done by using the methods described previously [24]. The fatty acid methyl esters were pretreated for fatty acid composition assay. Total lipid was prepared for saponification by adding of 0.5 ml methanolic KOH (2 mol/L) at 65 °C for 30 min. Subsequently, 14 % boron trifluoride-methanol was added to the fatty acid methyl esters at 75 °C for 30 min. Then 1 ml hexane and 1 ml Milli-Q water were added to the mixture. The mixture was centrifuged at 492 ×*g* for 5 min and the supernatant was collected for fatty acids analysis by using a Gas Chromatography (Shimadzu 2010, Japan) using 100 m column length (RESTEK, USA) with 0.25 mm internal diameter and 0.2 μm film thickness. The oven temperature rose from 120 °C to 240 °C at 4 °C/min with a holding time of 30 min at 240 °C. The injector and detector temperatures were set at 250 °C and 260 °C, respectively. Fatty acids were identified by retention times of standard mixtures (ANPEL Laboratory Technologies Inc., Shanghai, China).

2.10. Plasma PC and DHA detections

Sixteen adult zebrafish females (120 dpf) were sampled randomly for blood collection through tail amputation. After centrifugation, the supernatants were collected for plasma PC and DHA detection. The PC and DHA levels in diluted plasma were measured by using an enzyme-linked immunosorbent assay (ELISA) commercial kits from Jianglai Biological Technology Co. LTD, Shanghai, China according to the manufacture's protocol.

2.11. Lipidomics assay

The zebrafish (120 dpf) liver (n = 28) and ovary (n = 20) samples were randomly sampled and prepared for non-targeted lipidomics assay. The lipid extraction method and detection were performed as described previously by using a liquid chromatography/tandem mass spectrometry (LC-MS, Thermo, Ultimate 3000 LC, Orbitrap Elite) [23]. Total lipids isolation from the tissues was carried out by using chloroform and methanol (2v:1v). The lipid composition was then analyzed by using the LC-MS. The LC column used was a reversed phase 100 × 2.1 mm Kinetex C18 with 1.9 um particles [23]. The heater temperature, gas flow rate, aux gas flow rate, sweep gas flow rate, spray voltage and capillary temperature of the mass spectrometry were 300 °C, 45 arb, 15 arb, 1 arb, 3.0 KV, 350 °C, respectively [23]. The peak area obtained was defined as the proportional of the amount of each lipid.

2.12. Transcriptomic assay

A total of 120 zebrafish samples (about 40 larvae per replicate) at 7 dpf from each treatment were used for the RNA-seq transcriptomic assay. The mRNA isolation, invalid reads and unigenes assembly were performed as described in our previous study [25]. Illumina HiSeq 4000 (Illumina, Inc., San Diego, CA, USA; 2 × 150 bp read length) was used to sequence the paired-end RNA-seq sequencing library after quantification by TBS380. The differential gene expression (DGE) between WT and *vtg1* knockout zebrafish were analyzed based on fragments per kilobase of exon per million mapped reads (FRKM) method. The gene ontology (GO) and Kyoto encyclopedia of genes and genomes (KEGG) were detected for identifying DGE enriched in GO functions and signaling pathways at p value ≤0.05 compared with the whole transcriptome background [25]. To confirm the accuracy of transcriptomic data, we quantified the relative gene expression by using qPCR assay. The primers and gene expression validation are presented in Table S1 and Fig. S2, respectively.

2.13. Quantitative real-time PCR (qPCR) assay

A sample of six zebrafish livers collected randomly from *vtg1*$^{-/-}$ and WT were prepared for total RNA isolation, cDNA synthesis and qPCR assay as described in our previous study [25]. All the primer sequences of the target genes used for qPCR assay are listed in Table S2. The *β-actin* was used as a reference gene. The relative mRNA expression of the target genes were calculated by the $2^{-\Delta\Delta Ct}$ method. The qPCR efficiency was between 99 % and 103 %, and the correlation coefficient was above 0.98 for each gene.

2.14. Western blot assay

Six zebrafish livers, ovaries and adipose tissues (two fish per replicate) sampled randomly from adult female WT or mutant were homogenized on ice by using RIPA lysis buffer and 1 mM phenylmethylsulfonyl fluoride (Beyotime Biotechnology) mixture for 30 min to extract the proteins. The proteins were separated on sodium

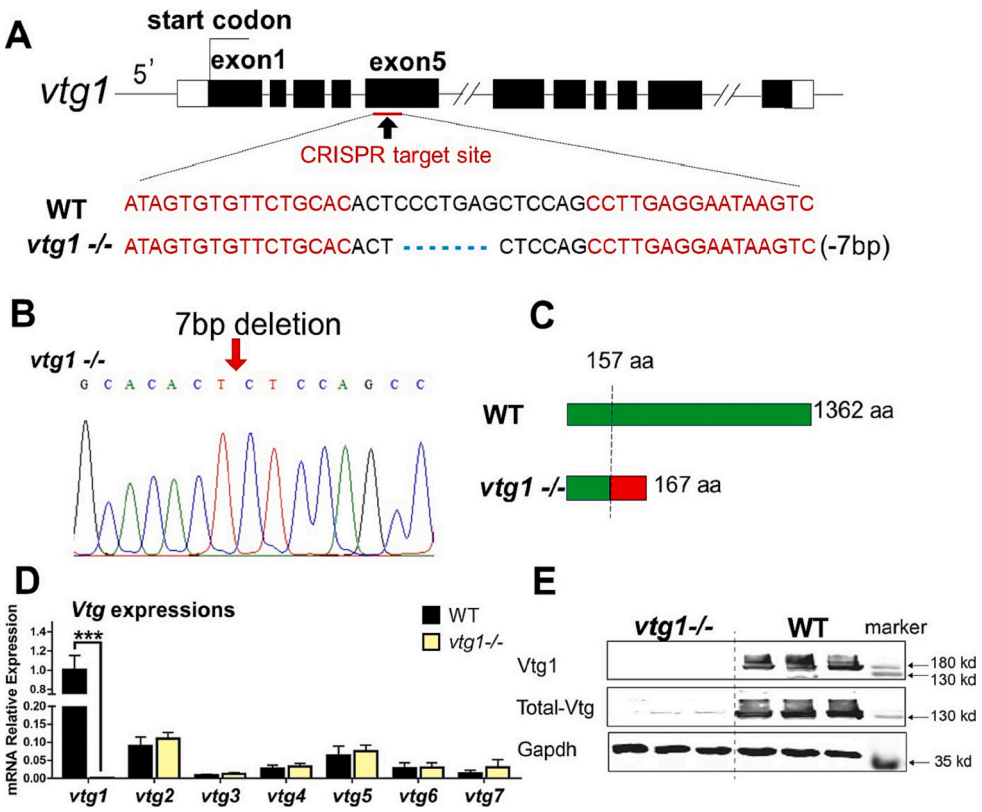


Fig. 1. Generation of *vtg1* deletion zebrafish using the CRISPR-Cas9 approach. The CRISPR target sites and deletion sequence for *vtg1* gene are presented (A). The endogenous *vtg1* gene is shown on top, and exons are depicted as black boxes. The target site aimed at the fifth exon is underlined with a red line with the target sequencing shown below in black font, and flank nucleotides are shown in red font. The blue dotted lines present the sequences of the deleted nucleotides for *vtg1*$^{-/-}$ fish. Seven nucleotides deletion in the target region (arrow) was observed in *vtg1*$^{-/-}$ fish (B). The Vtg1 proteins frame from WT and *vtg1*$^{-/-}$ (C). The Vtg1 protein in WT contains 1362 amino acids (aa), but it contains miscoding amino acids (157–167 amino acids, shown in a red box) in *vtg1*$^{-/-}$ fish. The relative expression of *vtg* genes in mature female liver (120 dpf) (n = 6) (D). Western blotting of Vtg1 and total Vtg protein in liver from WT and *vtg1*$^{-/-}$ female zebrafish (120 dpf) (E). Vtg, vitellogenin; WT, wild-type; dpf, days postfertilization; aa, amino acids. ***$P < 0.001$.

dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) gels and transferred to nitrocellulose membranes. The membranes were incubated for 1 h in the blocking solution containing 5 % bovine serum albumin (BSA) in phosphate-buffered saline (PBS) supplemented with 0.1 % Tween 20, and incubated overnight at 4 °C with the following rabbit antibodies: GAPDH (Abcam, Cambridge, MA, USA), Vtg1 and total-Vtg customized by Huaan Biotechnology Co. LTD. Antibodies were detected by using the Odyssey CLx Imager (Li-Cor, USA) with a goat anti-rabbit IgG (Li-Cor Biotechnology, Lincoln, NE, USA). The target proteins were quantified and analyzed by using ImageJ software (National Institutes of Health, Bethesda, MD, USA).

2.15. Statistical analyses

Statistical differences on measured parameters between *vtg1*$^{-/-}$ and WT fish were tested by using independent sample *t*-test ($P < 0.05$). The statistical differences in measured parameters during the dietary DHA and PC supplementation among the five treatments were tested by using one-way analysis of variance (ANOVA) followed by Duncan's multiple range test ($P < 0.05$). All statistical analyses were conducted by using SPSS 23 statistical software (IBM Corp., Armonk, NY, USA). The results are presented as mean ± standard error of the mean (SEM).

3. Results

3.1. Generation of vtg1-deficient zebrafish by using the CRISPR/Cas9 technology

The *vtg1*-depleted zebrafish were generated by using CRISPR/Cas9 technique. Target sequences against the fifth exon were designed based on the *vtg1* sequence provided in the National Center for Biotechnology Information (NCBI) (Fig. 1A). The mutant line with 7 bp deletion was obtained in black region for the fifth exon of *vtg1* by using sequence analysis (Fig. 1A and B). The WT zebrafish Vtg1 protein contained 1362 amino acids. However, the mutant zebrafish Vtg1 protein contained only 157 correct amino acids and the translation of *vtg1*-depletion gene stopped at the 167th amino acid (Fig. 1C). The qPCR results showed a significant decrease in the *vtg1* mRNA expression in mutant female zebrafish liver compared to the WT, but no significant differences for other *vtg* subtypes (Fig. 1D). Accordingly, the western-blotting results revealed that no Vtg1 protein and only slight amount of total Vtgs proteins were observed in mutant zebrafish, confirming Vtg1 deletion (Fig. 1E). Moreover, *vtg1*-depleted zebrafish showed much lower Vtg1 and total Vtgs protein contents in ovary than those in WT fish (Fig. S1A). No Vtgs bands were observed in visceral adipose tissue from either mutant or WT fish (Fig. S1B). These results confirmed that, the *vtg1* gene was deleted in zebrafish by using the CRISPR/Cas9 technique.

3.2. Genetic depletion of vtg1 disturbed female fecundity and larval development in early-phase

To fully characterize the roles played by Vtg1on fecundity and growth, we determined the reproductive capacity of female broodstock and offspring development. The body weight (Fig. 2A) and condition factor (CF) (Fig. 2B) had no significant differences between WT and *vtg1*-deficient female zebrafish. The depletion of *vtg1* increased the hepatosomatic index (HSI) (Fig. 2C), but decreased the gonadosomatic index (GSI) compared to WT females (Fig. 2D). The ovary histological pictures showed that, the *vtg1*-deficient zebrafish had higher early-phase follicles (such as primary growth follicle, PG; and primary growth follicle, PV), but fewer yolk granules and mature follicles (such as midvitellogenic follicle, MV; and full-grown follicle, FG) in ovary than those in the WT fish (Fig. 2E–G). In addition, the *vtg1*-deficient zebrafish broodstock extended spawning cycles (Fig. 2H), but decreased dramatically the oviposition amounts per clutch compared to WT fish (Fig. 2I). As for the offspring, the *vtg1*-deficient zebrafish decreased hatching rate at 72 hpf (Fig. 2J), but had significantly higher malformation rate in embryo and larvae compared to the WT (Figs. 2K–L, and S3). In fact, a

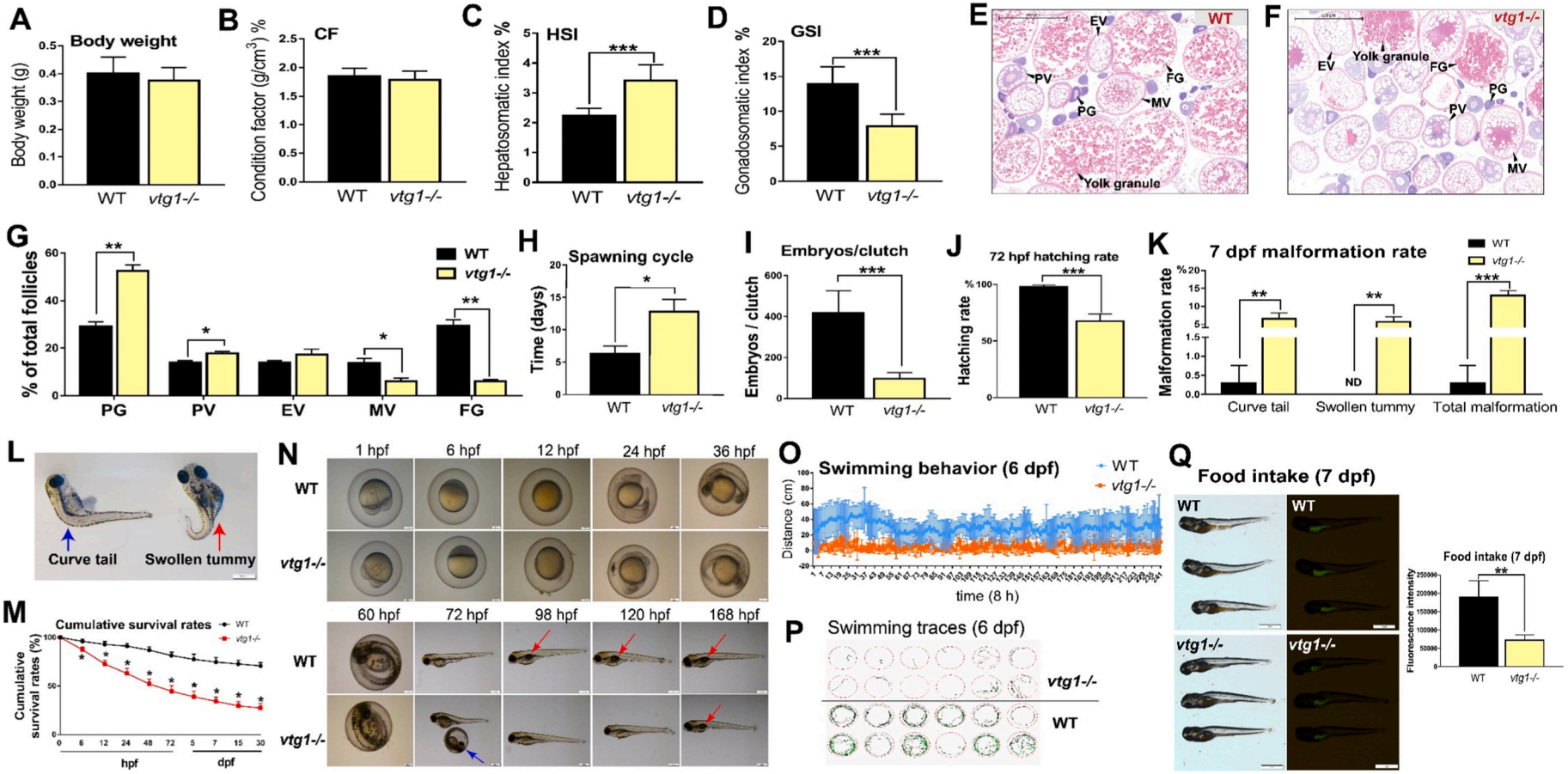


Fig. 2. Vtg1 deficiency attenuates female reproductive capacity and disturbs larval development in the early phase. Body weight (A), condition factor (CF) (B), hepatosomatic index (HSI) (C) and gonadosomatic index (GSI) (D) from zebrafish at 120 dpf (n = 15). Histological assessments of ovaries in female broodstock at 120 dpf from WT (E) and *vtg1* mutant (F). Scale bar, 500 μm. Ovarian follicular stages are labeled on top of their respective structure and abbreviated as follows: PG, primary growth follicle; PV, previtellogenic follicle; EV, early-vitellogenic follicle; MV, midvitellogenic follicle; FG, full-grown follicle. Percentages of ovarian follicles in different stages (G). Spawning cycles (H) and oviposition amounts (I) of female zebrafish. Embryo hatching rates in 72 hpf (J). The malformation rates in larval fish at 7 dpf (K). Malformation includes curve tail and swollen tummy. Swollen tummy larvae in WT fish were not detected (ND). Brightfield microscopy of larvae with curve tail (blue arrow) and swollen tummy (red arrow) (L). Scale bar, 500 μm. Cumulative survival rates of WT and *vtg1*$^{-/-}$ fish from 0 to 30 dpf (M). Brightfield microscopy of embryos and larvae development from 1 to 168 hpf (N). Blue arrow shows unhatched larvae. Red arrows represent the swim bladder in zebrafish. Scale bars, 200 μm for embryos, 500 μm for larvae. The line charts (O) and traces (P) represent larvae swimming distance (6 dpf) and locomotion pattern in a 24-well plate during 8 h (n = 12), respectively. The red, green and black traces represent the fastest, medium and the slowest speed, respectively. Food intake of larval fish at 7 dpf (Q). Scale bar, 1 mm. Bar graph (right) represent the quantified fluorescent intensities, which increase with the number of ingested fluorescent paramecia. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

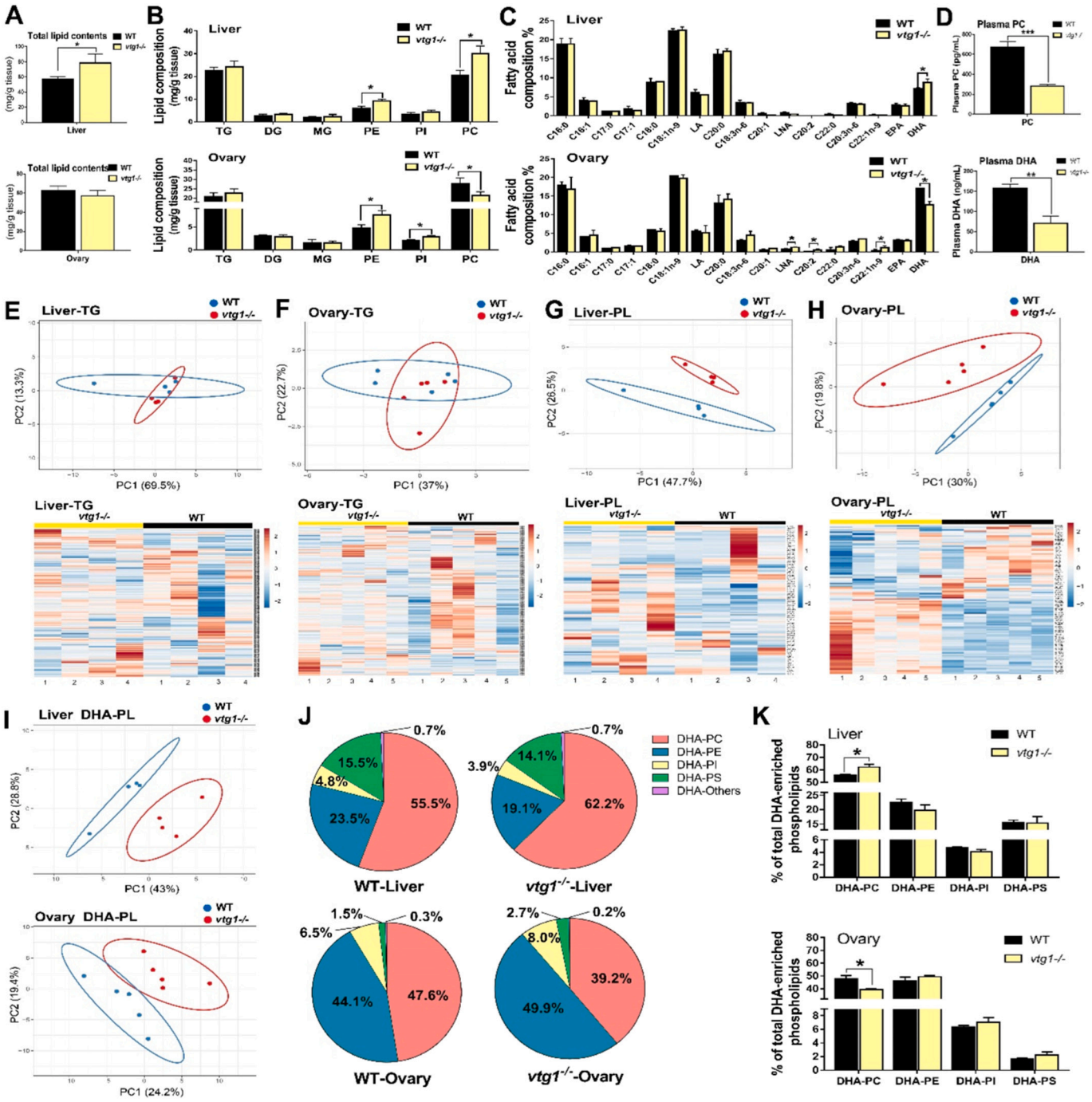


Fig. 3. Vtg1 deficiency suppresses the transportation of DHA-enriched PC (DHA-PC) from liver to ovary in mature female fish. Total lipid content (A), neutral and polar lipid composition (B) and fatty acid composition (C) in liver and ovary from mature female fish (n = 4). The DHA and PC contents in plasma (n = 3) (D). Principle component analysis (PCA, up) and heat maps (bottom) based on TG and phospholipids (PL) compositions in fish liver and ovary by the lipidomics analysis (E–H). The PCA of DHA-enriched phospholipids (DHA-PL) in liver (up) and ovary (bottom) (I). Percentages of DHA-PLs in liver and ovary (J and K). The DHA-PCs are in the major positions with red part. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. phosphatidylethanolamine, PE; phosphatidylinositol, PI; phosphatidylserines, PS. DHA-Others include DHA-enriched phosphatidylglycerols (DHA-PG), lyso-PE (DHA-LPE), lyso-PG (DHA-LPG) and lyso-PI (DHA-LPI). Liver, n = 4; Ovary, n = 5.

large number of *vtg1*-deficient larvae did not hatch at 72 hpf (Fig. 2N). Moreover, the hatched *vtg1*-deficient larvae had much slower development than the WT zebrafish. For example, the appearance of swim bladder in the *vtg1*-deficient larvae was much later (168 hpf) than in the WT fish (98 hpf) (Fig. 2N). Accordingly, the *vtg1*-deficient larvae exhibited significantly lower cumulative survival rate in larval-life phase (0 to 30 dpf) than that in the WT (Fig. 2M). The *vtg1*-deficient larvae also had much less swimming distances and traces, and lower food intake than the WT fish (detected at 6 dpf and 7 dpf) (Fig. 2O–Q). The above findings indicate that, the genetic deletion of *vtg1* impaired the female reproductive capacity and disturbed larvae development during the early phase.

3.3. Genetic depletion of vtg1 inhibited PC and DHA transportation from liver to ovary in mature female zebrafish

To further probe the influence of Vtg1 depletion on lipid transport in female zebrafish, we compared the contents of different lipid classes in

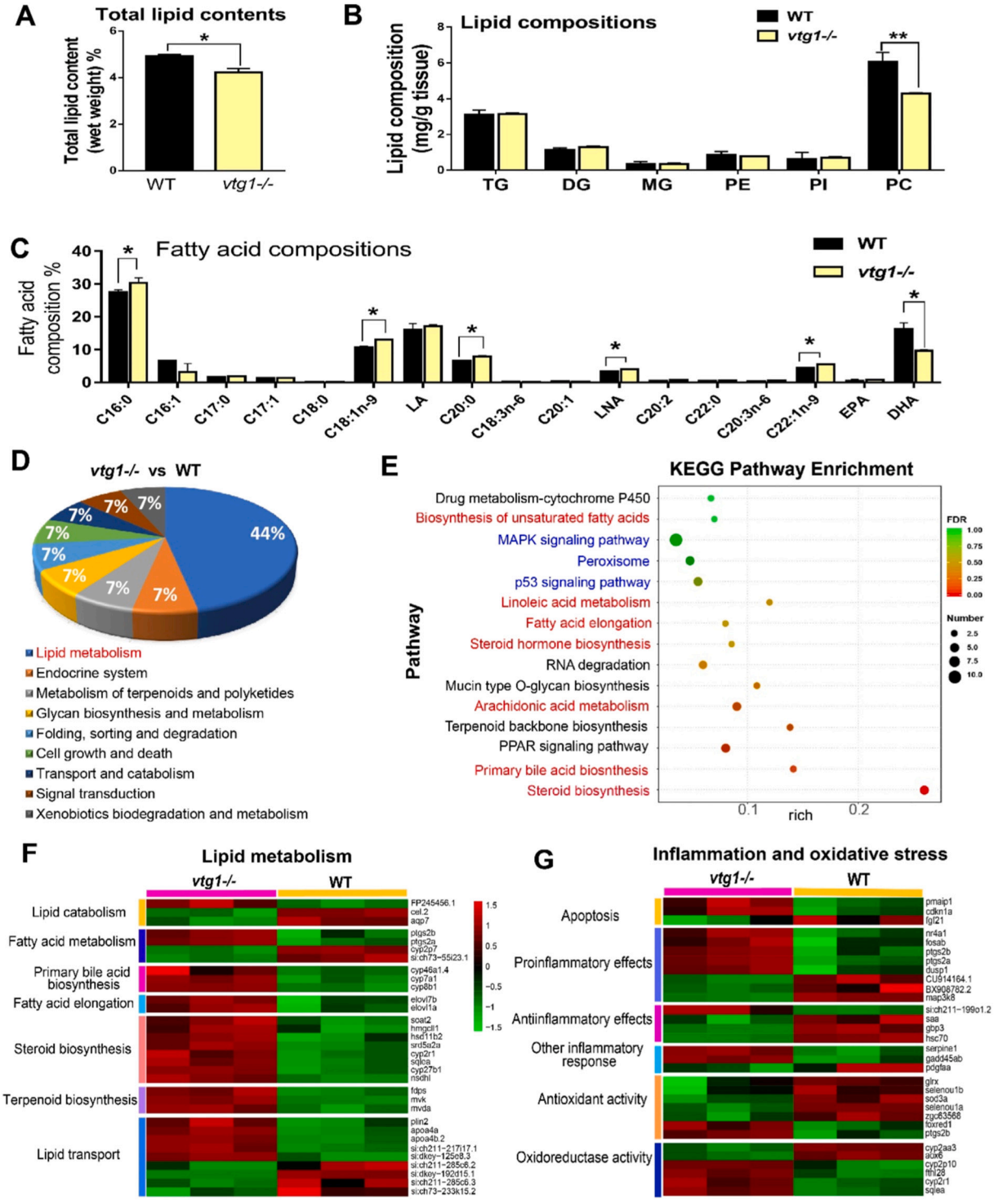


Fig. 4. The lipid composition and the transcriptomic analysis between WT and *vtg1*-deficient larvae in 7 dpf. Total lipid content (A), fatty acids composition (B) and neutral and polar lipid composition (C) in total larval fish at 7 dpf (n = 4). Pie chart represents the function analysis of different expressed genes by transcriptomics of total body fish at 7 dpf (D). Around 44 % different expressed genes are related to lipid metabolism. Top 15 differential pathways are shown as the bubble chart (E). The pathways in red font are correlated to lipid metabolism, and blue ones are immune inflammation and oxidative stress. Heat maps of changed genes in lipid metabolism (F), inflammation and oxidative stress (G). Red and green colors represent upregulation and downregulation of gene expressions, respectively. $^{*}P < 0.05$, $^{**}P < 0.01$.

adult fish liver, ovary and plasma between WT and *vtg1* mutant. The *vtg1*-deficient females had significantly higher total lipid content in the liver than the WT, and decreased significantly the lipid content in ovary compared to the WT fish (Fig. 3A). The neutral lipid classes, including TG, DG and MG were not significant different between the *vtg1*-deficient and WT zebrafish (Fig. 3B). However, the *vtg1*-deficient females had higher PE and PC amounts in the liver, and PE and PI in the ovary but lower PC than the WT fish (Fig. 3B). Of note, PC was the most dominant polar lipid class both in the liver and ovary than the other PLs (Fig. S4A). However, PS content was very low and comparable between WT and

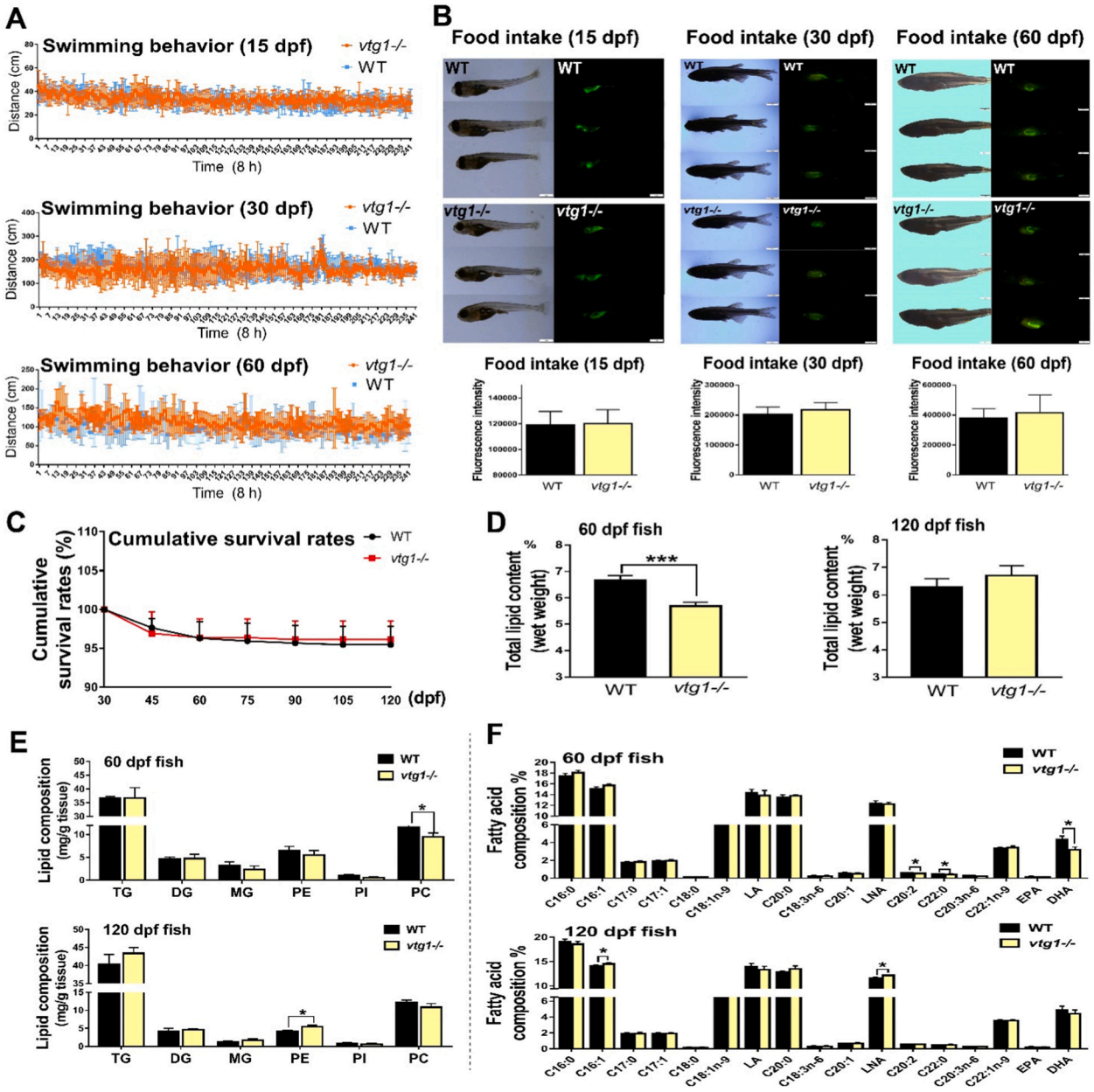


Fig. 5. The larval development and lipid composition in WT and *vtg1*-deficient zebrafish from late larvae to adult fish stages. Line charts represent the swimming distance of fish at 15, 30 and 60 dpf during 8 h (A). Food intake of zebrafish at 15 dpf (scale bar, 1 mm), 30 and 60 dpf (scale bar, 2 mm) (B). Bar graph (bottom) represent the quantified fluorescent intensities, which increase with the number of ingested fluorescent paramecia. Cumulative survival rate of zebrafish from 30 to 120 dpf (C). Total lipid content (D), neutral and polar lipid compositions (E) and fatty acids composition (F) in total fish at 60 and 120 dpf. $^{*}P < 0.05$, $^{***}P < 0.001$.

vtg1-deficient fish both in the liver and ovary (Fig. S4B). The *vtg1* mutants also increased remarkably the DHA percentage in the liver while declined in ovary compared to the WT fish (Fig. 3C), consistent with the higher PC content in the liver and lower PC content in ovary in the *vtg1*-deficient females. Accordingly, the *vtg1*-deficient females decreased significantly PC and DHA contents in the plasma than the WT fish (Fig. 3D). Together, these findings indicate that the depletion of *vtg1* reduced largely PC and DHA transportation from the liver to the ovary through blood circulation in female adult zebrafish.

3.4. *The vtg1-deficient zebrafish decreased DHA content in the ovary due to inhibited DHA-enriched PC transportation*

In order to further investigate the correlation between the changing trends in PC and DHA in the *vtg1* mutant fish, we compared the lipidomics profile in the liver and ovary between the *vtg1*-deficient female and WT fish. We did not observe significant differences in the principle component analysis (PCA) and heat maps for the TG compositions in the liver (Fig. 3E) and ovary (Fig. 3F), consistent with the results on composition analysis of neutral lipids and PLs. However, the PL composition was remarkably different between WT and *vtg1*-deficient zebrafish both in the liver (Fig. 3G) and ovary (Fig. 3H). Further analysis indicated that the major fatty acid-enriched PLs percentages, including 16:0-PL, 16:1-PL, 18:0-PL, 18:1-PL, 18:2 PL, 18:3 PL, 20:4-PL and 20:5-PL in the liver and ovary were comparable between the WT and *vtg1*-deficient females (Fig. S5). However, *vtg1*-deficient females had distinct DHA-enriched phospholipids (DHA-PLs) percentage from that in the WT fish (Fig. 3I). Deep assay showed that DHA-PC occupied the major proportion in liver (55.5 %) and ovary (47.6 %) in WT zebrafish (Fig. 3J–K) among all the DHA-PLs. In the *vtg1*-deficient females, the

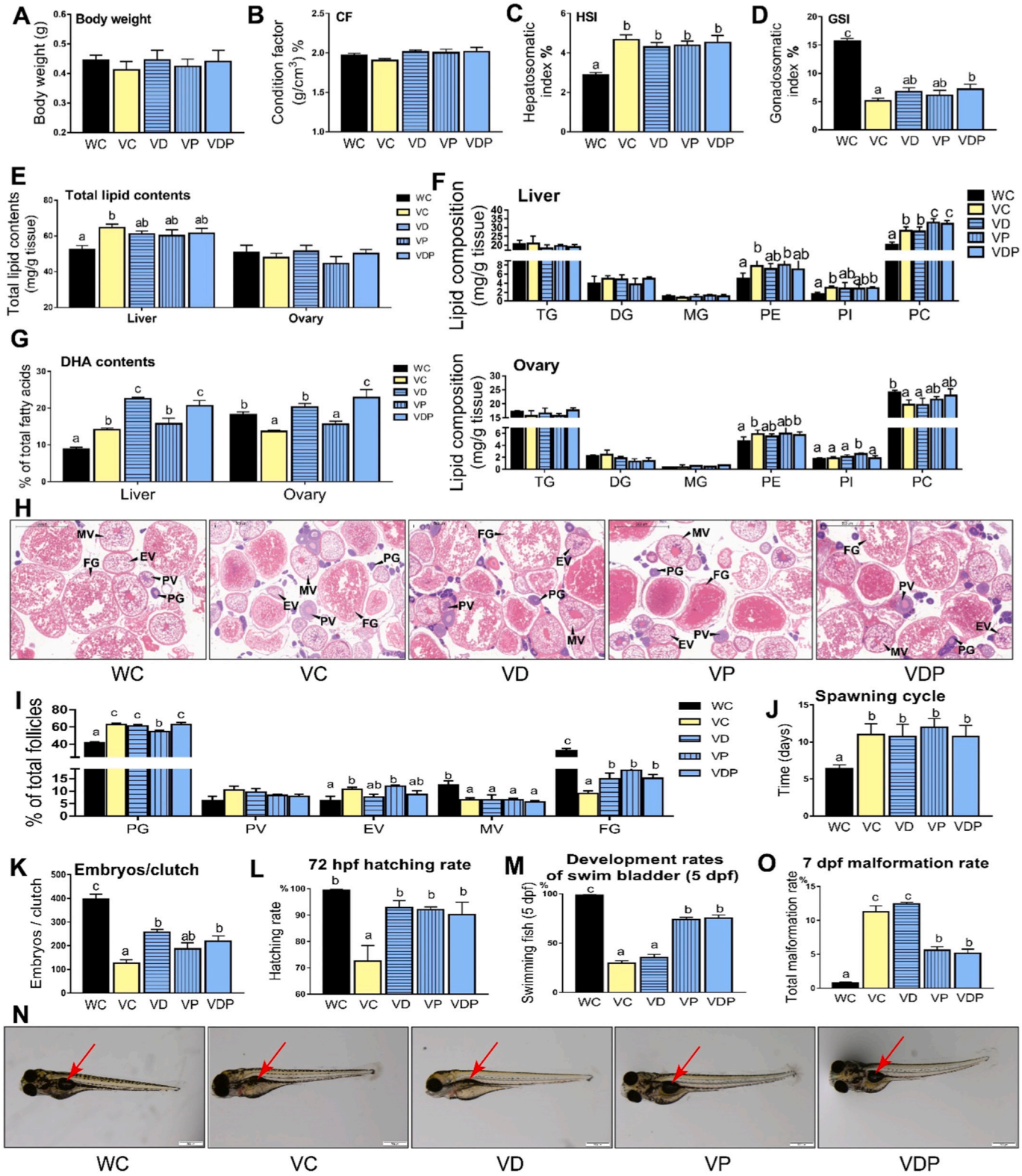


Fig. 6. Dietary supplementation of DHA and/or PC partly alleviates the negative effects of *vtg1* deficiency on female reproduction and larval development. Body weight (A), condition factor (CF) (B), hepatosomatic index (HSI) (C) and gonadosomatic index (GSI) (D) in mature females (150 dpf) from groups of WT-control (WC), *vtg1*-deficient fish (VC), VC + DHA (VD), VC + PC (VP) and VC + DHA + PC (VDP) (n = 12). Total lipid content (E), neutral and polar lipid compositions (F) and DHA content (G) in liver and ovary from mature female fish at 150 dpf (n = 4). Histological assessments of ovaries in female broodstock at 150 dpf (H). Scale bar, 500 μm. Percentages of ovarian follicles in different stages (I). Spawning cycle (J), oviposition amounts (K), hatching rates (L), swim-bladder development at 5 dpf (M) and the malformation rates at 7 dpf (O). Brightfield microscopy of larval swim bladder at 5 dpf (n = 5) (N). Red arrows represent the swim bladder. Scale bars, 500 μm. Statistical differences among treatments are marked with different lowercases ($P < 0.05$).

DHA-PC accumulated in the liver (62.2 %), but was depleted significantly in the ovary (39.2 %) (Fig. 3J–K). Altogether, these lipidomics results indicate that, PC is the main phospholipid type for carrying and transferring DHA in both WT and *vtg1*-deficient females. The decreased amounts of PC and DHA in the ovary of the *vtg1*-deficient females, were mainly due to inhibited DHA-PC transportation from the liver to the ovary.

3.5. The vtg1-deficient larvae with fewer DHA-PC accumulation exhibited lipid metabolism dysfunction, inflammation and oxidative stress

Low DHA-PC yolk might impair embryo and larvae health and development within 7 dpf, which largely depend on maternal nutrition from yolk or yolk-sac [26]. Therefore, we identified the lipid mobilization and transcriptomics in the larvae at 7 dpf. The *vtg1*-deficient larvae decreased significantly total lipid and PC contents and DHA percentage compared to the WT fish (Fig. 4A–C). The *fads2, elovl2, elovl4a* and *elovl4b* are crucial genes for DHA synthesis from alpha-linolenic acid (ALA) in zebrafish [27]. Moreover, *pcyt1a* and *pcyt1b* encode choline-phosphate cytidylyltransferase (CCT), which is the key rate-limiting enzyme for PC synthesis in the kennedy pathway [28]. Furthermore, the phosphatidylethanolamine *N*-methyltransferase (*pemt*) and lyso-phosphatidylcholine acyltransferase (*lpcat*) are also the key genes in PE methyl transferase and Lands cycle pathways for PC synthesis [29,30]. However, the mRNA expression of genes involved in PC and DHA synthesis between the two strains did not differ significantly in this study (Fig. S6A), suggesting that *vtg1* had independent PC and DHA synthetic ability in the larvae. The transcriptomic assay indicated that the lipid metabolism-related pathways were largely changed by the *vtg1* deficiency (Fig. 4D–E), including a significant increase in the expression of genes related to bile acid biosynthesis, fatty acid elongation, steroid biosynthesis, terpenoid biosynthesis and lipid transportation (Fig. 4F). Besides, the *vtg1* deficiency fish changed remarkably the pathways for inflammation and oxidative stress (Fig. 4G). These included the increase in expression of genes involved in pro-inflammation and inflammatory responses and oxidoreductase activities, and the reduction in expression of genes related to anti-inflammatory response and antioxidant activities (Fig. 4G). In general, these results reveal that, the *vtg1*-deficient larvae with less maternal DHA-PC amount exhibited impaired health with disturbed lipid metabolism, high inflammation and oxidative stress.

3.6. The Vtg1-deficient fish showed normal physiological phenotypes after early-larvae stages

In order to investigate the effects of low maternal DHA-PC content on the fish after yolk-sac depletion, we examined swimming behavior, food intake and lipid composition in the fish from 15 to 120 dpf. Of note, the swimming distances, locomotion patterns and food intake had no significant differences between the WT and *vtg1*-deficient fish at 15, 30 and 60 dpf (Figs. 5A and B, S7). The *vtg1*-deficient zebrafish had similar survival rate compared to the WT fish from 30 to 120 dpf (Fig. 5C). The *vtg1*-deficient fish still had lower total lipid content at 60 dpf, but was comparable with the WT fish at 120 dpf (Fig. 5D). Similarly, the total PC and DHA contents in the *vtg1*-deficient zebrafish at 60 dpf were still lower than the WT fish (Fig. 5E–F). However, both contents were comparable between the two strains at 120 dpf (Fig. 5E–F). Accordingly, the *vtg1*-deficient fish and WT fish had statistically comparable mRNA expression of genes related to PC and DHA syntheses from the early-phase larvae to adult fish (Fig. S6), indicating the difference of DHA-PC accumulation caused by *vtg1* depletion was not due to the modulation of PC and DHA synthetic processes. Together, these results indicate that, the negative effects of *vtg1* deficiency on larvae at early development stages might be alleviated by exogeneous food intake.

3.7. Dietary PC and DHA supplementation partly ameliorated the impaired reproduction and early stage offspring development in the vtg1-deficient female fish

In order to verify whether exogeneous PC and DHA supply can alleviate the impaired reproduction and offspring early development caused by Vtg1 deficiency, we fed mature *vtg1*-deficient female fish with diets supplemented with PC and/or DHA. The results showed no significant differences in body weight (Fig. 6A) and CF (Fig. 6B) among the WT-control (WC), *vtg1*-deficient fish (VC), VC + DHA (VD), VC + PC (VP) and VC + DHA + PC (VDP) treatments. However, all the *vtg1*-deficient treatments increased HSI (Fig. 6C) compared to WC fish. The WC fish further increased GSI than the *vtg1*-deficient fish fed on the diets supplemented with DHA and/or PC, while the VDP treatment had significantly higher GSI than the VC treatment (Fig. 6D). The diets supplemented with DHA and/or PC reduced slightly the lipid content in the liver compared to the VC treatment, and did not change lipid content in the ovary (Fig. 6E). The *vtg1*-deficient fish fed on diet supplemented with PC increased significantly the PC content in liver, but only slightly increased PC content in ovary (Fig. 6F). Apparently, the dietary DHA fortification significantly elevated the DHA contents in liver and ovary in the *vtg1*-deficient zebrafish (Fig. 6G).

Next, we characterized the female ovarian features and offspring larval development. The *vtg1*-deficient zebrafish supplemented with DHA and/or PC had higher relative follicles than the fish fed on WC diet for PG but had lower follicles for MV and FG (Fig. 6H–I). Among the *vtg1*-deficient zebrafish, the dietary fortification with DHA and/or PC did not significantly change the relative amounts of follicles at PG, PV, early-vitellogenic follicle (EV) and MV stages (Fig. 6H–I). However, the mature follicles percentages in the fish fed on the diets supplemented with VD, VP and VDP were significantly higher than the fish fed on VC, although these values were still much lower than the fish fed on WC diet (Fig. 6I). The dietary fortification with DHA and/or PC did not cause any significant differences on the spawning cycles among the *vtg1*-deficient fish (Fig. 6J), but increased the oviposition amounts per clutch (Fig. 6K) and the hatching rate (Fig. 6L) as compared to the VC treatment. Besides, the dietary PC supplementation increased dramatically swim bladder development percentages in Vtg1 deficient fish from 32.6 % to 74.6 % at 5 dpf (Fig. 6M and N), and notably decreased the total malformation rates from 11.2 % to 5.4 % at 7 dpf (Fig. 6O). Together, these data indicate that, dietary supplementation with DHA and/or PC partly alleviates the negative effects of the *vtg1*-deficiency on reproductive ability and early development.

4. Discussion

4.1. Components of Vtgs and DHA and PC transportation

Our findings showed that the *vtg1* deficient female zebrafish accumulated DHA-PC in the liver whereas decreased significantly in the plasma and ovary. These results revealed that Vtg1 plays a key transportation role for DHA-PC from the liver to ovary in zebrafish. Actually, the importance of PC and DHA in Vtg functions has been implied in some earlier studies. Norberg and Haux reported that approximately 2/3 of the lipids in the purified Vtg proteins from rainbow trout and sea trout (*Salmo trutta*) were PLs [2]. Subsequently, Fremont and Riazi indicated that, PC was the most dominant proportion in the total PLs in rainbow trout Vtgs [31]. Moreover, Lund et al. also showed the lipids in Vtgs contained nearly 80 % PC in striped bass (*Morone saxatilis*) [32]. Accordingly, results on PC dominant in zebrafish are consistent with those obtained in egg yolk [33]. Similarly, we found that PC was the major component of total PLs in zebrafish ovary (Fig. 3B). Although TG is one of the major lipids, the TG content was comparable between *vtg1* knockout and WT fish (Fig. 3B). However, as, PC content as another important lipid type was higher than TG in early larval development at 7 dpf (Fig. 4B). These data indicate that the increased HSI, total lipid

content and mRNA expressions of genes related to lipid synthesis in the liver were largely due to the high requirement of PC, rather than TG for vitellogenin generation. Furthermore, Silversand and Haux demonstrated that Vtg lipids contained considerable amount of omega-3 very long-chain unsaturated fatty acids (n-3 VLCUFA), especially the DHA and eicosapentaenoic acid (EPA) (30 % and 10 % of the total fatty acids, respectively) in Atlantic cod (*Gadus morhua*), rainbow trout, turbot (*Scophthalmus maximus*) and wolffish (*Anarhichas lupus*) [34]. Therefore, Vtg has been reported as the primary carrier for both DHA and EPA. Of note, PC was reported as the primary carrier for EPA to gonad in striped bass [32]. EPA also plays antioxidant and anti-inflammation roles as the main n-3 VLCUFA [35]. However, we did not observe a significant difference on EPA transportation between WT and vtg1-deficient zebrafish females. In addition, the total DHA percent (14.84 %) was much higher than EPA (3.07 %) in WT ovary (Fig. 3C). This suggests that the PC in Vtg1 mainly plays a vital DHA transportation role, but not EPA. The relatively stable EPA content suggest that its requirement for maturing ovary might be transported by other mechanisms apart from Vtg1. Moreover, the n-3 VLCUFA transportation is also thought to depend on both TG and PLs [36,37]. However, our present study found that PLs exert a greater effect than TG in converting DHA in maturing ovary. Consequently, we demonstrate that PC is the main vehicle for DHA transportation from the liver to ovary in female zebrafish. For the first time, we show that Vtg1 is responsible for DHA-PC transportation.

The existence of many Vtg subtypes in oviparous animals led researchers to believe that, there might be functional compensations among different subtypes [6,19]. However, the effects of Vtg1-specific deficiency on lipid mobilization dysfunction, fecundity and larval development impairment in the present study, was not compensated by other Vtg subtypes. Accordingly, the simultaneous knockout of *vtg1, 4, 5, 6* and *7* in zebrafish increased oviposition, but the *vtg3*-knockout mainly decreased fertilization rate [19]. These findings indicate that, different Vtg subtypes might exert diverse physiological functions in oviparous animals. Our study indicates that carrying DHA-PC might be a specific function of Vtg1. Moreover, it is interesting to note that liver is probably not the only tissue generating Vtg protein because the Vtg signals were also found in the white adipose tissue (WAT) in the early vitellogenesis phase in female zebrafish and rainbow trout [5]. Nevertheless, the *vtg1* expression was approximately 45 times higher in liver than in WAT at the beginning of oocyte yolk generation, and *vtg1* expression in WAT was reduced largely during late vitellogenesis phase [5]. In the present study, we did not find Vtg protein signal in the visceral adipose tissue (Fig. S1B). The absence of the Vtg protein signal in the present study may result from the much low *vtg1* gene expression in WAT during the late vitellogenesis period because the fish used in the present study had already exceeded the vitellogenesis early phase. Therefore, liver is still the main Vtg generation tissue during the whole vitellogenesis period. Of note, Vtgs transport variable amounts of nutrients from the liver to ovary for oogenesis, including lipids, amino acids, carbohydrates, vitamins, and phosphate [2], and some metal ions such as Zn^{2+} and Cd^{2+} [38], which exert different functions on the oocyte maturation. Therefore, the knockout of Vtg1 in the present study may also cause deficiency in some other nutrients. As an evidence, the dietary DHA and/or PC supplementation did not recover completely the impaired female reproduction and larval development to the WT levels, indicating that other components apart from DHA and PC transported by Vtg1 also participate in the reproductive and development processes.

4.2. Essential roles of DHA and PC carried by Vtg1 in reproduction

In the present study, we found that the *vtg1*-deficient female zebrafish with decreased DHA-PC content in ovary reduced mature follicle amounts and GSI, and then impaired female fecundity (Fig. 2D and E–I), consistency with previous studies reporting that PC and DHA are highly required for female reproduction. For example, during spawning period, the ovaries require more DHA than other organs in silver pomfret (*Pampus argenteus*) [39], and dietary DHA supplementation increased egg production in female Japanese flounder (*Paralichthys olivaceus*) [40]. Besides, PC has been shown as a crucial determinant for female reproduction by directly affecting oocyte formation in amberjack (*Seriola dumerili*) [41]. Mechanically, DHA and PC are vital structural components in cell and tissue membranes especially in fish, which is a rich source of the omega-3 VLCUFA [42]. Melzner et al. also indicated that, increased PC liposomes enhanced cholesterol biosynthesis in blood lymphocytes [43]. Since cholesterol is the precursor for steroid hormones [44], it is possible that PC regulates ovary maturation and fecundity through modulating the steroid synthesis or hormonal pathways. However, studies reporting the effects of PC and DHA loss in ovary on reproductive ability in females are missing. Therefore, our present work provides the first direct in vivo evidence indicating the DHA-PC transported by Vtg1 exerts a vital role on female reproduction in oviparous animals. Interestingly, Yilmaz et al. [19] showed that simultaneously knocked out of *vtg1, 4, 5, 6* and *7* increased oviposition in another zebrafish study. However, because Yilmaz's study did not perform a specific *vtg1* knockout, the functions of Vtg1 might be covered by the deficiency of other Vtgs.

Dietary DHA can be absorbed directly into intestinal mucosa. However, dietary PC should be first hydrolyzed into free fatty acids (FFAs) and l-acyl lysoPC via the pancreatic phospholipase A2 in intestinal lumen, subsequently the FFAs and lysoPC obtained can be absorbed directly into intestinal mucosa and re-esterified to form PC again [45]. Previous studies have shown that dietary PC or DHA supplementation increase efficiently PC or DHA content in the fish body [46,47], corroborating to our results. It should be noted that although soybean PC contains DHA, the DHA content in soybean PC is very low [48]. Our results showed that the real DHA percent in PC-supplemented diet (0.80 %) was similar as that in the control treatment (0.84 %), and was much lower than that in the DHA-supplemented diet treatment (2.45 %) (Table S1). Therefore, the DHA from soybean PC does not affect the total DHA content in the PC-supplemented diet. Of note, our study indicated that, dietary DHA and/or PC supplementation increased DHA and/or PC contents in the ovary. However, the increase in DHA and/or PC did not recover completely the adverse effects caused by the *vtg1*-deficiency. This might be contributed by unavailability of other nutrients carried by Vtg1. Moreover, the specific ovary-targeted transport using dietary DHA and/or PC feeding may be lower than that of DHA-PC contained in the Vtg1 route, causing considerable DHA and PC amounts provided by diets to fail to enter the ovary. These results further indicate that, the effects of DHA-PC carried by Vtg1 have higher bioavailability than dietary DHA and PC intake.

It is well known that, the Vtg receptor (Vtgr), which is mainly expressed in the ovary of oviparous organisms, plays a vital role during yolk formation by incorporating the Vtgs into the oocytes [49]. Many studies have reported the importance of Vtgr on female fecundity. For instance, the injection of dsRNA of Vtgr reduced eggs number laid in citrus red mites (*Panonychus citri*) [50], brown planthopper (*Nilaparvata lugens*) [51], lepidopteran insect [52], delayed the egg-laying time in Bumblebee (*Bombus lantschouensis*) [53] and affected ovary morphology in small brown plant hopper (*Laodelphax striatellus*) [54]. The findings from these studies verified that, the transportation of Vtgs into oocytes is crucial for female fecundity in various organisms. Our present study further indicates the importance of Vtg1 in transporting DHA-PC into oocytes in fish.

4.3. Essential roles of maternal DHA and PC in offspring development

Maternal nutrition has been reported to affect offspring development in animals [55–57]. For example, old female parasitic wasp (*Eupelmus vuilleti*) decreased yolk-sac nutrients, and thus reduced nutrient availability in offspring [26]. Moreover, the lipid composition in eggs derived from maternal diet played a critical role for offspring survival in red drum (*Sciaenops ocellatus*) and Pacific bluefin tuna (*Thunnus orientalis*)

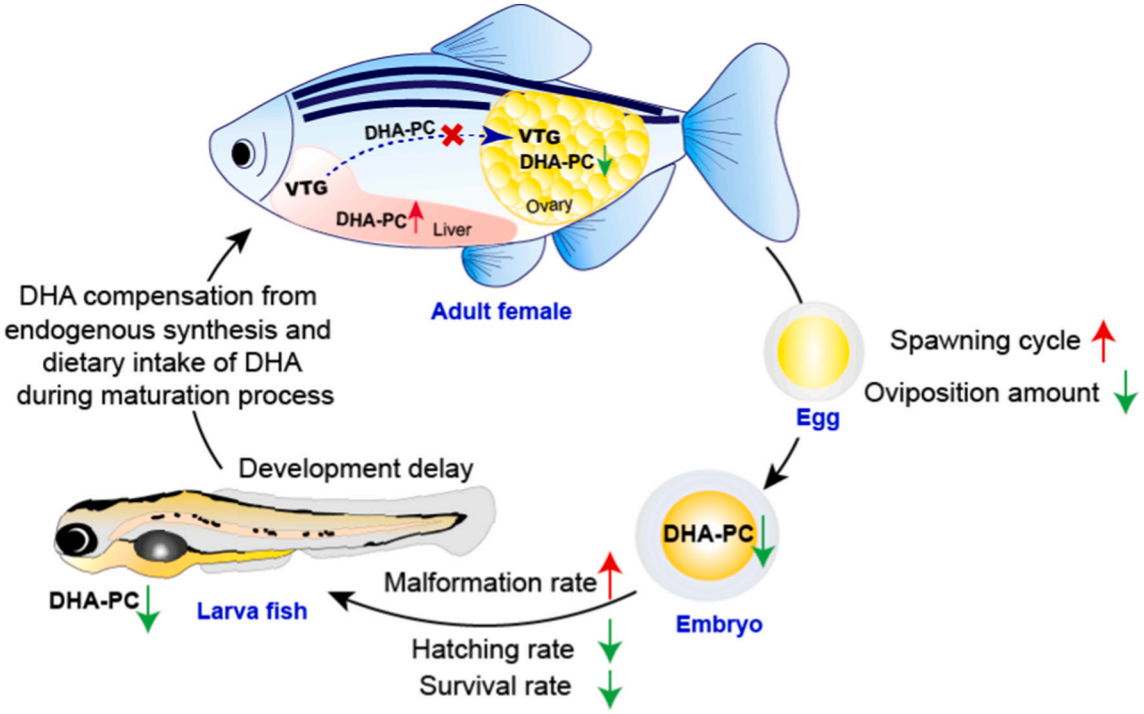


Fig. 7. A comprehensive view of *vtg1* deficiency on the disturbance of female reproductive capacity and larvae development in early phase through DHA-PC transportation from the liver to ovary. The transportation of DHA-PC from liver to ovary was inhibited in mature female *vtg1*$^{-/-}$ fish. The female *vtg1*$^{-/-}$ fish had lower oviposition amounts and longer spawning cycles than WT, and *vtg1*$^{-/-}$ embryo with lower DHA-PC decreased survival and hatching rates. The *vtg1* deficiency delayed larval development and enhanced malformation rate. The DHA-PC content was gradually compensated from larvae to adult in *vtg1*$^{-/-}$ fish.

[56,58]. In the present study, we also observed the broodstock with decreased DHA-PC content in ovary caused by Vtg1 deficiency spawned offspring with lower DHA-PC enrichment and impaired larval development (Figs. 2 and 4). Our study is the first to show that, the nutrient components transported by Vtg1 in female broodstock affect the offspring development during early stages through the maternal effects in yolk-sac animals.

It is known that, DHA and PC exert important functions on embryo and larvae development and growth in oviparous animals [35,59]. For example, dietary DHA supplementation increased growth in pikeperch (*Sander lucioperca*) larval [60] and hatching percentages in Japanese flounder larval [40]. Furthermore, DHA content correlated with egg activity and survival rates in red seabream (*Pagrus major*) [61], sea bass (*Dicentrarchus labrax*) [62] and Atlantic halibut (*Hippoglossus hippoglossus*) during larval development stages [63]. Moreover, dietary PC supplementation improved survival rate and decreased malformation in Atlantic cod larvae and early juveniles [64] and offered a better resistance to salinity stress in kuruma shrimp (*Penaeus japonicus*) larvae [65]. Previous studies also found that PC was the dominant PL in swim bladder in blue antimoral (*Antimora rostrata*) [66] and goldfish (*Carassius auratus*) [67]. Accordingly, in the present study, the Vtg1-deficient fish produced embryos and larvae with insufficient DHA-PC, which showed decreased hatching rates, survival rates, delayed swim bladder development and elevated malformations compared to the WT fish. Moreover, these adverse effects were partly alleviated by dietary DHA and/or PC supplementation in the Vtg1-deficient fish, such as increased hatching rates and swim bladder development (Fig. 6). Interestingly, the mortalities, swimming behavior and food intake were ameliorated in the juvenile and adult Vtg1-deficient fish compared to the WT, even though DHA and PC contents in Vtg1 deficiency fish were still lower than those in WT at 60 dpf. This suggests that, DHA and PC are more important in embryo and early larvae developmental stages than in later stages in fish. Similarly, a higher PC requirement was reported in embryo and early-larvae phase than in later stages in crustaceans [65] and fish [59].

It is known that oviparous animals have a limited capacity to synthesis PC de novo during early-development phases [59], meaning the yolk sac is vital for nutrient supply during this period. When the yolk-sac nutrients are completely exhausted, then the nutrients are mainly derived from exogeneous food intake. In the present study, we observed that DHA and PC contents in the body increased in the Vtg1 deficient fish from larvae to 120 dpf (Fig. 5E and F). Furthermore, PC and DHA syntheses in 60 dpf and 120 dpf were comparable between Vtg1-deficient and WT fish (Fig. S6). These results reveal that, the reduced DHA and PC contents in the Vtg1-deficient fish can be compensated through food intake and endogenous synthesis. In fact, artemia and rotifers are important live feeds for larval fish in practical aquaculture, and they are commonly supplemented with DHA to improve fish vitality [68]. These results highlight the importance of Vtgs particularly the contained DHA and PC in maternal nutrition in fish.

Some recent studies suggest that Vtgs might also play roles in promoting immune functions and antioxidant activity in developing embryos in fish apart from their nutritional functions [9]. Vtgs damage microbes' cell wall structure by binding on microbial surfaces and protect immune system from bacteria and fungi damage in honeybee [69,70]. Moreover, Vtgs have also been reported to alleviate reactive oxygen species (ROS) production because they are antimicrobial peptides [69,70]. Likewise, Zhang et al. indicated that Vtgs are the immunocompetent molecule to protect host against virus by recognizing the pathogen associated molecular patterns (PAMPs), which are microbial conserved components [71]. Furthermore, Glavinic et al. and Sun et al. also showed that Vtgs are precursors of important antimicrobial peptides required to reduce bacterial proliferation and virus infection [72,73]. Our present study also showed that the vtg1-deficient larvae with less DHA-PC contents exhibited dysfunction in immunity response and antioxidant capacity pathways (Fig. 4E and G). Our results suggest that, the non-nutritional roles of Vtgs in oviparous animals may include immunity, anti-inflammatory responses and antioxidant capacity because DHA has been reported to suppress oxidative stress and inhibit

inflammatory response in many animals [74]. These non-nutritional roles also attribute to the components transported by Vtgs such as the antimicrobial peptides, PC and DHA. Moreover, based on the findings from the present study, increasing the dietary PC and DHA contents may be a potential way to elevate female fish fecundity and larvae survival in aquaculture, especially for fish species with high mortality rates during early larval developmental phase. Besides, Vtg assessment can also be used to evaluate fish fecundity capacity for choosing the ideal female broodstock candidate in breeding programs.

5. Conclusion

The present study clarified that Vtg1 is essential for DHA-PC transportation from the liver to ovary during female vitellogenesis in fish (Fig. 7). Furthermore, the PC and DHA carried by Vtg1 are crucial for ovary maturation and female fecundity, and further affect embryo and larval development through maternal-nutrition effects. This is the first study elucidating the nutrient and physiological functions of Vtg1 in zebrafish reproduction and development. Our study also provides a reference for future studies on Vtgs functions in other oviparous animals.

CRediT authorship contribution statement

Sheng-Xiang Sun: Study design, Writing-original draft preparation, Experimental work and Data analysis. Yi-Chan Liu: Experimental work and Data analysis. Samwel M. Limbu: Writing-review and editing. Dong-Liang Li and Zhan Yin: Perform the establishment of *vtg1* knockout line. Li-Qiao Chen and Mei-Ling Zhang: Provide essential technical supports. Zhen-Yu Du: Study design, Writing review and editing, and Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

All data are contained within the manuscript and the supporting information are available for sharing from the corresponding author upon a reasonable request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbalip.2023.159289.

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