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Zebrafish as a model to screen the potential of food ingredients – fatty acids- in reproduction

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Abstract – Sperm quality is an important topic in general health, chemotherapy, and gamete preservation technology. Fatty acid (FA) composition of membranes, which is influenced by the diet, plays key roles in sperm biology and quality. Dietary supplementation with natural products can be used as a technique to screen potential agents to protect, modify, and recover sperm quality. In the present study, zebrafish (male [♂-ZF] and female [♀-ZF]) were fed a single cultivar olive oil (OO) bioencapsulated in *Artemia*. OO-treated ♂-ZF had higher (p<0.05) sperm density and motility compared to the AN. A significant difference was also observed in follicle abundance at different stages of gametogenesis, and a non-significant increase in total fecundity between OO-treated ♀-ZF and the AN, although in OO-treated ♀-ZF mature follicles had a smaller diameter. A higher fertility rate (FR) was observed in OO-treated pairs compared to the other groups. Hatching in the OO-treated fish was accelerated although no significant differences could be found in terms of hatching rate (HR) and embryo/larval survival rate (SR). These findings in FR, HR, and SR were also confirmed in

male and female replacement mating trials. Taken together, this study shows that altering the FA ratios in the diet has a clear impact on several reproductive parameters in the zebrafish adding new information about the nutritional requirement of this model species.

1. Introduction

The fatty acid (FA) composition of membrane phospholipids show large differences both in chain length and degree of unsaturation.[1,2,3] The degree of FA unsaturation determines the biophysical properties of a membrane, which in turn influences membrane-associated functions[4]. In the case of sperm cells, membrane-associated functions such as 1) the activity of membrane-bound proteins (e.g. ion channels), 2) endocytosis, 3) exocytosis,[4] 4) motility, 5) membrane resistance to osmotic shock, 6) capacitation, and 7) induced acrosomal reaction, have been identified.[5] Thus, the FA composition of cellular membranes plays key roles in sperm biology and quality.

The plasma membrane of sperm in vertebrates[6,7] contains a high level of polyunsaturated fatty acids (PUFA). Such fatty acids are highly susceptible to oxidation by reactive oxygen species (ROS) produced as the output of respiration and activity of intracellular enzymes.[8] The oxygen-induced damage of lipids or lipid peroxidation[6] reduces the fluidity and integrity of membranes[9] required for participation in the fusion events associated with fertilization.[10,11,12] Sperm (containing tightly coiled DNA) cannot transcribe electrophilic responsive genes that encode the oxidant inactivators.[9] Therefore, a number of enzymes and antioxidants present in seminal plasma and sperm cytoplasm constitute the sperm oxidative defence system required for protection from oxidative damage.[13,14,15]

Thus, the FA composition of the sperm membrane not only plays key roles in sperm biology but also, because of its susceptibility, it is considered as the sperm´s “Achilles' heel”. FA composition can be considered as a determinant factor of sperm quality, in terms of sperm

quality being an important topic concerning general health, chemotherapy, and gamete preservation technology.

The FA composition of membranes is affected by the diet and any activity of lipid-metabolizing enzymes.[16,17] Dietary FA can affect the FA composition of phospholipids from the sperm membrane and consequently its biophysical properties, as this is closely related with the functionality of sperm.[18] Thus, dietary supplementation cannot only be considered as an alternative to protect, modify, and recover sperm quality, but it can also be used as a technique to screen agents in charge of those actions.

Nowadays, the treatment for improvement of fertility[19,20,21,22] and the protection of fertility capacity[23,24] using natural products is a topic of interest. A natural product such as olive oil (OO; containing 77% monounsaturated fatty acids –MUFA-) contributes to prevention of excessive oxygen-induced damage of membrane phospholipids, increasing MUFA content of lipids[5]), with MUFAs being less easily damaged by oxidation and less likely to produce free radicals.[25]

Several studies have been published using OO dietary supplementation to improve,[26] protect,[27] and treat[5] fertility problems in mammals. The OOs used in the studies were commercial products with different FA profiles from one bottle to another. This leads to irreproducible results both at intra- (from lot to lot) and inter-laboratory levels. Such outputs cannot be considered a reliable reference to judge the reproductive potential of natural oils for the protection of reproduction capacity, or the improvement of reproduction. The above mentioned shortcoming is addressed in the current study by using a single cultivar oil that is extracted from fruits of a single olive tree.

To our knowledge, the current study is the first attempt to assess the effects of an extra virgin single cultivar OO on FA composition and reproductive success in zebrafish. Lipid metabolism (i.e. lipid digestion, absorption, and transport) in fish is similar to that in

mammals,[28,29] with zebrafish being an excellent model for lipid research[30] and vertebrate lipid metabolism.[31]

The extra virgin OO extracted from Koroneiki (Kor) has one of the highest oil contents (20-30%) and it is the most common Greek variety for oil production. Kor oil is high in 18:1*n*-9 and polyphenols. Acidity of the oil is low, and is very stable, which means it has a very good shelf life. Thus, this oil was selected as the best option to study the effect of OO on reproduction and to evaluate the use of the zebrafish as a screening agent for food ingredients or nutritive supplements on reproduction.

The current study aims to define possible relationships between the FA composition of Kor oil and (1) gonadal development, taking into account folliculogenesis and spermatogenesis, (2) fecundity, (3) sperm quality criteria such as density and motility, and (4) embryo/larva viability measured as fertilization rate (FR), hatching rate (HR), and survival rate (SR).

To establish what, if any, relationship exists between the Kor oil and improved sperm characteristics we prepared the following: 1) a determination of the FA profile of Kor oil; 2) preparation of three groups of *Artemia* nauplii: unenriched (newly hatched *Artemia* nauplii, AN), 36 hours post-hatch (AN36), and Kor-enriched *Artemia* nauplii (EAN); 3) determination of the FA profile of the three groups of *Artemia*; 4) provided zebrafish with live feed consisting of the 3 groups of *Artemia* nauplii. Endpoints were recorded and treatment groups were compared based on the recorded endpoints.

2. Materials and methods

2.1. Animals and housing facilities

Commercial rat cages of 12 L (Razirad Co, Iran) equipped with a sponge filtration unit, a 150 Watt (W) submersible heater with thermostat, a glass thermometer, a 6 W white

and a glass lid were used as fish "regular tanks" following the recommendations given by Brand *et al.*[32] and Westerfield.[33] The light was connected to a timer to provide fish with a photoperiod of 14 h L:10 h D cycle.

Municipal water was dechlorinated, charcoal filtered, conditioned with 240 mg L^{-1} rock salt and 60 mg L^{-1} sea salt, and used as system water. Physico-chemical parameters of the water were controlled and adjusted to be: temperature 28 ºC; pH: 7.5 (recommended by Brand *et al.*[32] as the best pH to rear and breed zebrafish and to maintain the biofilters,[34]) adjusted using 1M Na_2CO_3[33]; and conductivity 1043-1094 µS cm^{-1}.[35]

These regular tanks were filled leaving 1.5 cm of air space between the water surface and the tank lid to let fish gulp air[36]. The tanks were set up in an isolated room with the room temperature set 1 ºC higher than the tank water temperature.[32,37] Adult wild-type zebrafish were used as test animals. The fish either were bred in the laboratory,[38] or purchased from local suppliers. The purchased zebrafish were transported to the laboratory as described in Westerfield[33]. Newly arrived zebrafish were acclimated for 1-2 h to the water conditions by floating the fish bags in the containers and adding small amounts of the water into the bag. The stocking density per holding tank was one individual per liter[38].

The cycle was provided by turning the light on at 9:00 and off at 23:00[36]. Fish were fed 2 times per day: 1 h after turning the lights on with 2 mL of a concentrated AN suspension (1270 individuals/mL) per 8-10 fish,[33,39] and 7 h later to satiation with artificial food (flake food Vitakraft®, TetraMin®, or BioMar). Tank wastes, including uneaten food and fish feces were siphoned before first feeding and one hour after last feeding[40] on a daily basis. The tanks and their filters were washed and disinfected once a week, based on,[41] and 15% of water exchanged on a daily basis.

Zebrafish were housed in tanks for a quarantine period of 3-4 weeks.[37] During the quarantine period zebrafish were closely observed for clinical signs,[37] behavioral disorders

and/or physical changes.[42,43,44] The fresh fecal droppings of the fish were macro- and microscopically examined for any sign of the presence of intestinal parasites.[45,46] Sick fish were discarded or subjected to proper treatments. [46] The quarantine period of 3-4 weeks also allowed zebrafish to adapt to laboratory conditions.[47,48,49] Sex-dependent morphological characteristics cited by Laale,[50] Schilling,[51] Braunbeck & Lammer,[40] Paull *et al*.[52], Ruhl *et al.*[53], EOL,[54] and SOP[36] were examined to discriminate between males and females. After sex was determined, the males and females were housed in single sex tanks. The stocking density per holding tank was one fish per liter.[38]

2.2 Experimental conditions

Glass beakers of 1000 mL were placed in the regular tanks filled half-way with tap or distilled water. Six months old zebrafish,[33,35,40] selected as consistent spawners[47] were used for the experiments. The age of the fish was determined from the time of hatching of the eggs. One fish was housed in each beaker for 2 weeks before the trial for acclimation to the new environment. No aeration was provided. Fish were fed to satiation 2 times per day as indicated above. Fecal droppings were removed twice per day using a 3 mL plastic Pasteur pipette. Half of the beaker's water was changed every day.

2.3 Enrichment emulsion preparation

Koroneiki (Kor) monovarietal extra virgin olive oil was extracted and provided for the study by Prof. Dr. Seyed-Mehdi Hosseini-Mazinani (Hosseini@nigeb.ac.ir; Olive Research Group, Department of Plant Molecular Biotechnology, National Institute of Genetic Engineering and Biotechnology, Iran). Known amounts of lecithin (L-α-Phosphatidylcholine; Sigma-Aldrich, USA) used as emulsifier, Kor oil, and deionized water[55] were mixed in a ratio 1:10:100 (30 mg lecithin, 300 µL Kor, and 3 mL water) and put into a 50 mL falcon tube.

The falcon tube, with the emulsification mixture, was then placed into a water bath at 40 °C, and homogenated (Ultra-Turrax T 8, IKA®-Werke GmbH & Co. KG) for 15 min into a creamy white emulsion. The stability of the emulsion,[56] the absence of oil droplets, the size of the oil droplets (7 to 28 μm[57,58]) and the uniformity of oil droplet size in the emulsion were used to evaluate the quality. Finally, the falcon was wrapped in aluminum foil, labeled, and stored at 4 °C until use.[56] The emulsion was macro- and microscopically evaluated before use.

2.3 Artemia hatching and enrichment

Artemia franciscana, strain VC from Can Tho University, Vietnam, was used in the study. For hatching the *Artemia* cysts, a glass measuring cylinder of 100 ml was placed in a 5 L plexiglass mouse cage filled half-way with tap water and equipped with a 150 W submerged aquarium heater adjusted to 28 °C, a glass lid, a glass thermometer, and a 6 W aquarium light. The measuring cylinder was filled with 100 mL dechlorinated and charcoal filtered tap water (pH: 8.5), 2.5 g rock salt to provide a final concentration of 2.5%, kept at 28ºC and well illuminated. *Artemia* cysts were added at a density of 5g L^{-1},[41] that is 180 mg, having in mind that 1 g of cysts yields 200,000-300,000 nauplii, according to the strain and the commercial brand. The cylinder was vigorously and continuously aerated to keep cysts in suspension and maintain the oxygen levels.[33,32,41] The cysts started to hatch after 14 h and hatched completely within 24 h. To evaluate the hatching rate 6-10 samples of 250 μL were taken from the measuring cylinder and transferred into a vial of 1.5 mL. Five subsamples of 50 μL were placed on a microscope slide as 5 droplets, and examined under a dissection microscope (Zeiss). The number of AN, umbrella stage, and unhatched and empty cysts were counted. The hatching rate was calculated as the ratio of the AN number to the number of AN + umbrella stage + unhatched cysts × 100. Developmental stages of AN were determined

studying the general morphology and presence/absence of appendages and measuring the size of body and appendages.[59,60,55] This morphometric study was done using Image J (rsb.info.nih.gov/ij) and the available descriptors.[61,62,55] Newly hatched *Artemia* nauplii (AN) were collected in a 100 µm mesh, washed for 30-60 s with 2.5% salt water at 28 ºC[41], and the hatching medium collected into a beaker of 100 mL to be used as the "AN feeding suspension" that is attractive for fish,[63,64,65,66] using a ratio 1 volume AN:3 volumes of medium.[33]

2.3.1. Bioencapsulation of AN with Kor

One liter of AN suspension containing approx. 200 AN per mL was stocked in a glass measuring cylinder of 1000 mL placed in a 12 L regular tank. The tank was filled half-way with tap water and equipped with a 150 W submersible aquarium heater adjusted to 28 °C, a glass lid, a glass thermometer, and a 6 W aquarium light. Two mL Kor emulsion was added to the cyclinder with AN (2 µL Kor emulsion per mL). The pH was adjusted to 8.5 using 1 M Na_2CO_3, at 28ºC and the contents provided with continuous aeration for 12 h. Second and third doses of the emulsion were provided, after adjusting the pH to 8.5, with 12 h intervals between them. After 36 h enrichment a sample of AN was taken to assess the survival rate and gut content of the EAN using a digital camera (Zeiss) connected to a microscope. The presence of oil droplets in the EAN gut and the signs of gut content assimilation (a dark stripe was formed all along the gut) were considered as signs of success for the bioencapsulation. Three categories of EAN were considered (Fig. 1) taking into account the amount of oil droplets present in the gut. Samples containing 85% EAN in the third category (Fig. 1e-f) were considered successful and used for fish feeding, the other remaining categories were discarded. Once the EAN were evaluated, the aeration was stopped, and the bioencapsulation mixture (medium + Kor emulsion +EAN) filtered through a 100 µm mesh. The EAN was

washed gently with fresh culture medium heated to 28 °C, then transferred into a 100 mL glass beaker and diluted to obtain a concentrated suspension (1568 BSN per mL). This suspension was used to feed the fish or stored refrigerated at 4 ºC until use.

2.4. Feeding

Four treatment groups with 5 replicates each were established using **AN** (newly hatched *Artemia* nauplii), **AN36** (36 h post hatch AN), **EAN** (Kor bioencapsulated AN) and **ANEAN** (a combination of 50% EAN and 50% AN).

Each treatment included five tanks that were considered as five replicas. Each replica (tank) contained eight beakers and each beaker contained one fish, to give eight fish per replica and 40 fish per treatment. Fish were fed two times per day (1 h after light on and 7 h later) with 200 µL suspension for 1 month.

2.5. Zebrafish breeding, embryo collection, and fecundity and FR determination

A 1 L mating cage[67] with a screen of 2 mm^2 porosity that had been provided with plastic plants[32] was setup into a regular tank (of either 5 L or 12 L) equipped with a submersible heater, aerator, and thermometer (filled with system water see section 1.1). One healthy male and female zebrafish (ratio 1:1, a pair-wise crossing spawning[36]) segregated one week before mating session,[35] were transferred to the mating cage late in the afternoon (6:00 p.m. approx. 15 h before spawning) the day before spawning. Genders were housed in different chambers separated by a transparent plastic divider of the mating cage, and left there overnight. In the morning, with the beginning of the light cycle (at 9:00 a.m.), the divider was removed and the fish mated and spawned within 30-60 min.[40] After each trial, fish were returned to their original tanks and rested for 7 days before the next trial.

For the breeding trials in the current study, 16 zebrafish (8 males and 8 females) from each treatment groups were taken. Pair-wise crossings were randomly set within the same treatment groups (AN♀×AN♂, AN36♀×AN36♂, EAN♀×EAN♂, and ANEAN♀×ANEAN♂). To investigate the effect of feeding with AN groups in both sexes, male and female replacement experiments were conducted after the breeding trials. Eight male zebrafish from each treatment group were replaced by eight male fish from AN group to mate with the female fish from each treatment group (AN36♀, EAN♀, and ANEAN♀). Similar replacement experiments were done to eight female fish (AN♀) in each treatment group (AN36♂, EAN♂, and ANEAN♂). After spawning, broodstock zebrafish were removed and returned to their tanks, the embryos siphoned out from the bottom of the tank into a Petri dish, 90% of Petri water poured off and replaced with dechlorinated and autoclaved tap water containing 60 µg mL^{-1} sea salt[33,68] and 0.5 mg L^{-1} methylene blue.[41] The Petri dish was gently shaken and 90% of egg water poured off. This was repeated several times[33] and any remaining debris removed using forceps. Finally, the number of eggs per tank (fecundity) was counted. To estimate fertilization rate (FR) the eggs were examined under a stereomicroscope to determine whether they were fertilized or not.[48] Unfertilized embryos were opaque and/or with ruptured cells inside the chorion whereas fertilized embryos appear intact, transparent, and growing to the next cell division state.[69] The percentage of fertilized eggs was calculated as the ratio of fertilized eggs to the total number of produced eggs in each spawning event × 100. After determination of FR the non-fertilized eggs were discarded and fertilized ones used in the subsequent experiments.

2.6. Embryo culture and determination of hatching (HR) and survival rates (SR)

Twenty-four well microtiter plates were used as containers. Each well was filled with 3 mL egg water and 10 embryos, 4 hours post fertilization (hpf), were placed in each well

with a plastic Pasteur pipette.[70,40] Each treatment group (AN, AN36, EAN, ANEAN) was analysed using 40 replicates (10 embryos per replicate, total 400 embryos per treatment). Each plate was covered, labeled (code of the broodfish, spawning time and date, onset of incubation) and placed in a programmable light cycle incubator.[71] Embryos were incubated at 28±0.5 ºC[70] using a 14 h L:10 h D cycle. The plates were checked on a daily basis until the death of the last larva, and the most important events such as hatching (48-72 hpf), passive feeding (72-96 hpf), onset of exogenous feeding (120-144 hpf), complete yolk depletion (144-168 hpf), starved larvae (192-216 hpf), onset of larval mortality (216-240 hpf), >50% mortality (240-264 hpf), >90% mortality (264-288 hpf) and 100% mortality (288-312 hpf) were recorded. At each surveillance time point dead embryo/larvae were removed, 80% of culture medium exchanged and embryo/larvae hatching and survival rate calculated. Hatching rate was accounted as the ratio of hatched embryos to total number of cultured embryos × 100. The HR at 48 (HR-48) and 72 hpf (HR-72) were calculated for each treatment. The 48 and 72 hpf have been cited as time intervals during which the zebrafish embryo hatching take place[33]. Survival rate was calculated as the ratio of live embryo/larvae to total number of cultured embryos × 100. The SR at 24, 48, 72, 96, 120, 144, 168, 192, 216, 240, 264, 288, 312 hpf were calculated for each treatment.

2.7. Sperm analysis

Fish were fasted for 2 days,[47] anesthetized with clove oil,[72] euthanized on ice,[73] rinsed in freshwater, blotted, and weighed.[74] Testes (2-8 mg for a 6 months-old fish) were dissected, adherent fat tissue or lipid droplets removed[74], and the testes weighed in a preweighed tube. Hank's balanced salt solution (HBSS[75]) was added in a ratio 1:50 (mass:volume) (Dr. Huiping Yang, LSU AgCenter, personal communication), minced[76] with scissors and the pieces triturated by repeatedly passing through an eppendorf pipette tip more than 20 times to

disrupt the testis and to allow it to release the sperm. Large fragments of testes were removed by forceps or by a low speed centrifugation (Dr. Qiaoxiang Dong, Institute of Watershed Science and Environmental Ecology, China, personal communication) and the resulted suspension was held on ice not more than 2 h.[33,75]

Neubauer counting chambers were used to determine sperm density (Fuchs-Rosenthal Counting Chamber, HS-3720, Hausser Scientific, Germany). The slides were first sprayed with 70% ethanol, rinsed with de-ionized water, dried and covered with a coverslip (22×22 mm). Three µL of previously mixed sperm suspension (sprem+ HBSS) was put on a glass slide, 12 µL HBSS was added to the suspension, and mixed well at room temperature. This 15 µL suspension was loaded between the Neubauer and coverslip and viewed at 400x in a stereomicroscope. The central large square (1×1 mm; subdivided into 25 smaller squares) of the Neubauer chamber was considered as the counting area and photographed to count the spermatozoids (based on Hala *et al.*[77]). Density is presented as the number of spermatozoids per 0.004 µL (the volume of a sub-square in the central large square), and converted to spermatozoids per mL, and finally multipled by dilution factor (i.e. 5 in the current study).

For the determination of the number of motile spermatozoids a bright field calibrated microscope[78] (Nikon's Eclipse E600, Japan) equipped with a digital camera (Sony Cyber-shot DSC-W130 8.1MP Digital Camera with 4x Optical Zoom with Super Steady Shot, Japan) were used as the recording system. The best focal plane to use for filming was determined using an inactive sperm preparation: for this 2 µL of sperm suspension was placed onto a clean glass slide, 8 µL HBSS added to the suspension and mixed well, the 10 µL suspension drop covered with a coverslip (22×22 mm[79]), and examined under the bright field microscope at ×200[80]. The same system and procedure was used to analyse the motility of spermatozoa (2 µl) but replacing HBSS with 8µL de-ionized water[80] added to the suspension and mixed by gently pipetting twice. The drop was covered with a coverslip (22×22 mm[79]), the preparation

quickly placed on the microscope and sperm motility recorded until 100% spermatozoa were immotile. The videos were analyzed using EDIUS 6 and the percentage of motile sperms at different intervals (within 5, 10, 15, 20, 25, and 30s after activation at room temperature, denoted as spa – second post activation -) was determined.

2.8. Histology of gonads

After finishing the exposure period of 1 month, fish were fasted for at least 24 h in order to empty the gut content.[81] At least 4-6 zebrafish were taken from each treatment group,[47] euthanized in ice water for 15 minutes,[73] the fish dissected,[73] the gonads removed and placed in a 1.5 mL tube containing 10% buffered formalin solution[82] at room temperature for 48 h, and stored at 4 °C until use[83].

Fixed gonads were dehydrated in a graded series of ethanol (Emsure, Germany) solutions, cleared in cedar oil (Sigma Aldrich, USA) for 24 h at 45 °C and xylene (Emsure, Germany) for 1 h at 45 °C, infiltrated (2h at 60 °C) and embedded in paraffin (HistoPlus, Iran). Two to six, 5 μm sections from the same general area of the ovary per fish were taken using a microtome 1512 (Leitz, Germany).[84,47,83] The paraffin sections were stained with hematoxylin (4302, Merck, Germany) and eosin (165382 042, Merck, Germany), covered with coverslip and labeled. A light microscope (C82612, Olympus, Japan) equipped with a digital camera (CMEX DC. 1300, Netherland) connected to a monitor was used to take ovary photos at 100× and 400× magnifications. Ovarian follicles were assigned to their appropriate stages based on morphological characteristics.[85,86] Ovarian follicles at different gametogenic stages were counted (100 follicles from sections of each ovary) and their relative abundance (number of follicles at a known gametogenic stage/total of follicles counted×100) calculated. The diameter of ovarian follicles (μm) was determined on digital photomicrographs using

Image Focus (V. 13, Euromex Microscopen b.v., 2005-2006). The diameter of 10-12[83] follicles per developmental stage per fish was determined.

2.9. Fatty acid analysis

The sample (0.1 g, of gonad tissue or *Artemia* nauplii) was mixed with 2 mL methanol-sulphuric acid (2.5:100, v:v) and gradually heated to 80 °C in a water bath for 1 h. The sample was cooled to room temperature, 1 mL of hexane and 2 mL 0.9% NaCl added, shaken vigorously, centrifuged (4000 rpm, 5 min), the supernatant taken and transferred to a preweighed 5 mL glass vial. The hexane was evaporated at 35 ºC, flushed with nitrogen gas, and the vial weighed again. The extracted methylated fatty acids (FAME) were finally dissolved in 100 µL iso-octane and transferred to a 2 mL glass vial, flushed with nitrogen and stored at -30 °C until use.

Fatty acid methyl esters (FAMEs) were analysed by gas chromatography (GC) using an Agilent GC Model 7890A (Agilent Technologies, USA) equipped with a split/splitless injector and flame ionization detector (FID). Analytical separation was achieved on a free fatty acid phase (FFAP) fused silica capillary column (DB-225ms, 30.0 m × 0.25 mm i.d., Film thickness 0.25 µm; J&W, USA). Fatty acid methyl esters (0.25 µL, 2 mg FAMEs per mL iso-octan) were injected using nitrogen as the carrier gas at 1.0 mL min^{-1} and 43 cm s^{-1}at a split ratio 1:60. The injector was adjusted to 230 °C, FID at 230 °C, the oven was held at 160 °C for 5 min and then increased (48 °C/min) to 210 °C and held for 6 min at 210 °C.

Gas chromatography peak areas were integrated with a D-2500 integrator (Hitachi, Japan) equipped with a software (Perkin Elmer) connected to the GC. Fatty acids were identified and calibrated by comparison of retention times with those of a standard mixture of FAMEs (Nu-Chek-Prep, Elyian, MN, USA), and published data. The results were expressed

as peak area (relative) percent. The amount of FA per gram tissue was calculated using 19:0 as an internal standard.

2.10. Statistical analysis

Differences among treatments were evaluated by one-way analysis of variance (ANOVA) and multivariate analysis of variance (MANOVA) followed by Duncan's multiple range test (as a post hoc test) for all endpoints. The value 0.05 was taken as the significance level. The statistical analyses in the current study were performed using IBM SPSS (version 20; SPSS Inc., Chicago, IL, USA), and Excel 2010 (Microsoft Corporation, Redmond, WA, USA)

3. Results

3.1. Kor oil FA composition

The quantitative composition (relative abundance) of Kor FAs (Table 1) showed a predominance of MUFA (74.62 ± 0.392%), followed by SFA (16.60 ± 0.313%), and PUFA (8.73 ± 0.058%). Oleic acid, 18:1n-9 (71.92 ± 0.449) was the most abundant FA in Kor oil.

3.2. Artemia nauplii FA composition

Regarding the FA qualitative and quantitative composition (% TFA), a significant multivariate difference (MANOVA; Wilks' lambda [λ] < 0.001, $p < 0.001$, F = 302.972) was found among the four AN groups. Thus, AN and AN36 were significantly different in 7 FAs (an increase in 18:1n-7, 18:2n-6, and a decrease in 14:0, 16:0, SFA, MUFA, PUFA), AN and EAN were significantly different in 10 FAs (an increase in 18:1n-9, 18:2n-6, 20:1n-9, and a decrease in 14:0, 14:1n-5, 16:0, 16:1n-7, SFA, MUFA, PUFA), and AN and ANEAN were

significantly different from the AN in the relative amounts of 6 FAs (an increase in 18:1n-9, 18:2n-6, and a decrease in 16:0, SFA, MUFA, PUFA) (see Table 1).

3.3. FA profile of the ovaries

A significant difference (MANOVA; $\lambda < 0.001$, $p < 0.001$, $F = 246.713$) was found among the ovaries (OVA-AN, OVA-EAN, OVA-ANEAN, and OVA-AN36) regarding their FA composition. Thus, OVA-AN36, OVA-EAN, and OVA-ANEAN groups were significantly ($p < 0.05$) different from the AN group (OVA-AN) in terms of the relative abundance of 7 FAs for OVA-AN36 (an increase in 16:0, 18:1n-9, 18:2n-6, SFA, PUFA and a decrease in 14:0, 16:1n-7, 18:1n-7, 18:3n-3, 20:5n-3, MUFA), 10 FAs for OVA-EAN (an increase in 18:1n-9, 18:2n-6, 20:4n-6, 22:6n-3, PUFA, n-6 HUFA, n-3 HUFA, HUFA, HUFA+PUFA and a decrease in 14:0, 16:1n-7, 18:1n-7, 18:3n-3, 20:1n-9, 20:5n-3, MUFA), and 6 FAs for OVA-ANEAN (an increase in 18:0, 18:1n-9, 18:2n-6, PUFA and a decrease in 14:0, 16:1n-7, 18:1n-7, 18:3n-3, 20:5n-3, n-3 HUFA, HUFA) (see Table 2).

The FA ratios in the ovaries of zebrafish fed on four AN are presented in Table 3.The OVA-AN36, OVA-EAN, and OVA-ANEAN groups were significantly different from the AN (OVA-AN) regarding 12 FA ratios for OVA-AN36 (20:5n3/14:0, 16:0/16:1n-7, 16:0/18:1n-7, 16:0/20:5n-3, 22:6n-3/16:1n-7, 18:0/20:5n-3, 18:1n-7/20:5n-3, 18:1n-9/20:5n-3, 18:2n-6/18:3n-3, 18:2n-6/20:5n-3, 18:2n-6/22:6n-3, and 22:6n-3/18:3n-3), 16 FA ratios for OVA-EAN (18:2n-6/14:0, 20:4n6/14:0, 22:6n3/14:0, 16:0/16:1n-7, 16:0/18:1n-7, 18:0/16:1n-7, 18:1n-7/16:1n-7, 18:1n-9/16:1n-7, 20:4n-6/16:1n-7, 22:6n-3/16:1n-7, 18:1n-7/20:4n-6, 22:6-n/18:1n-7, 18:2n-6/18:3n-3, 20:4n-6/18:3n-3, 20:5n-3/18:3n-3, and 22:6n-3/18:3n-3), and 7 FA ratios for OVA-ANEAN (16:0/16:1n-7, 16:0/18:1n-7, 16:0/18:1n-9, 16:1n-7/18:3n-3, 18:1n-9/22:6n-3, 18:2n-6/18:3n-3, and 18:2n-6/22:6n-3) (see Table 3).

3.4. Ovarian histology and morphology

Five types of follicles were identified based on the approximate size and gross morphology: perinucleolus (PN; Fig. 2a), early cortical alveolus (eCA; Fig. 2b), mid cortical alveolus (mCA; Fig. 2c), vitellogenic (V; Fig. 2d), and mature (M; Fig. 2e).

The ovaries of EAN-treated specimens had significantly more follicles at PN stage (69.6 %) than those of fish fed AN (59.3%) and fish fed ANEAN (52.4%) and AN36 (31.7%) (Table 4, row 2), but had the same or fewer follicles at eCA, mCA, V, and M stages (Table 4, row 3-6). There was a significant difference between EAN- and AN36-treated fish regarding the abundance of follicles at all stages (PN, eCA, mCA, V, and M) (Table 4, column 6).

No significant differences between Kor-treated specimens (EAN and ANEAN) and the AN group could be found regarding follicle diameter at PN, eCA, mCA, and V stages. In the EAN-treated ovaries the follicles at M stage had a significantly smaller size compared to the other groups (Table 4, row 10). In the AN36-treated ovaries, follicles at all gametogenic stages except for M stage had a significantly higher diameters compared to the other groups (Table 4, column 6).

3.5. Fecundity

A non-significant increase in fecundity (mean ± SD; 174.7 ± 33.5) was observed in EAN-fed fish compared to those fed on ANEAN (91.0 ± 23.5), AN (138 ± 70.7), and AN36 (116 ± 14) (Table 4, row 1). In the current study, more fecund (albeit non-significant) fish had smaller oocytes.

3.6. Sperm density and motility

The highest sperm density was found in EAN- ($4.7\times10^8 \pm 6.8\times10^7$ cell mL^{-1}) and AN36-treated ($4.9\times10^8 \pm 5.0\times10^7$ cell mL^{-1}) fish. The lowest density was observed in

ANEAN- ($1.9\times10^8 \pm 7.3\times10^7$) and AN-treated ($2.2\times10^8 \pm 8.2\times10^7$) fish (Table 4, row 12). Sperm motility was estimated at room temperature (~25 °C) at 5, 10, 15, 20, 25, and 30 seconds post activation (spa). The highest sperm motility was recorded at 5 spa and at 10-30 spa in EAN-treated fish (Table 4, column 4) and in the AN group (Table 4, column 3), respectively. There was a significant (in Kor-treated groups, Table 4) or non-significant (in untreated groups AN and AN36, Table 4) decline in the percentage of motile sperm with time (i.e. from 5 to 30 spa).

3.7. Embryo and larvae viability parameters

A significant increase in FR (95.5% ± 5.9) was observed in EAN♀×EAN ♂ pairs compared to ANEAN♀×ANEAN ♂ (86.7% ± 11.5), AN♀×AN ♂ (69.2% ± 22.9), and AN36♀×AN36♂ (62.8% ± 32.8) pairs. The FR coefficient of variation in pairs fed on Kor-enriched nauplii (EAN♀×EAN ♂ [6.1] and ANEAN♀×ANEAN ♂ [13.3]) was remarkably lower than that of pairs fed non-enriched nauplii (AN♀×AN ♂ [33.0] and AN36♀×AN36♂ [52.2]) (Table 5, row 1).

To further investigate the effects of Kor enrichment in both sexes, male and female replacement trials were done using only EAN♀×EAN♂ pairs, taking into account their higher FR and lower variance coefficient than ANEAN♀×ANEAN♂ pairs. In the male replacement trial, the EAN♀×AN♂ pair had a significantly lower FR (86.2% ± 0.8) than EAN♀×EAN♂ fish (95.5% ± 5.9) with the FR (86.2% ± 0.8) not being significantly higher than that of the unenriched nauplii fed groups (AN♀×AN♂ [69.2% ± 22.9] and AN36♀×AN36 ♂ [62.8% ± 32.8]). In the female replacement trial (AN♀×EAN♂) the FR increased from 86.2% ± 0.8 in EAN♀×AN♂ to 94.3% ± 0.4 in AN♀×EAN♂. The AN♀×EAN♂ pair had a FR value similar to that obtained from EAN♀×EAN♂ fish (95.5 ±

8.1). The FR in AN♀×EAN♂ was higher (94.3% ± 0.4) than that of pairs fed unenriched AN (AN♀×AN36♂ [69.2% ± 22.9] and AN36♀×AN36♂ [62.8% ± 32.8]).

Hatching rate (HR) at 48 hpf was significantly higher in EAN♀×EAN♂ pairs (67.9%) than in ANEAN♀×ANEAN♂ (28.8%), AN♀×AN♂ (0.5%), and AN36♀×AN36♂ (43.4%), (Table 5, row 3). Such differences were not observed in the values of HR at 72 and 96 hpf (Table 5, rows 4 and 5). In the male replacement trial, EAN♀×AN♂ had no HR at 48 hpf, a lower value compared to the other groups (EAN♀×EAN♂ [67.9%], ANEAN♀×ANEAN♂ [28.8%], AN♀×AN ♂ [0.5%], and AN36♀×AN36♂ [43.4%]). No significant difference could be found between EAN♀×AN♂ and the other groups in HR obtained at 72 and 96 hpf. In the female replacement trial, the AN♀×EAN♂ had a HR at 48 hpf (44.3% ± 6.1) lower than EAN♀×EAN♂ (67.9%) and higher than ANEAN♀×ANEAN♂ (28.8%), AN♀×AN ♂ (0.5%), and AN36♀×AN36♂ (43.4%). No differences could be found in the HR obtained at 72 and 96 hpf.

There was no significant difference among Kor-treated groups, EAN♀×EAN♂ (87.4% [SR24-SR240], 43.5% [SR264], 3.3% [SR288], and 0.0% [SR312]), and ANEAN♀×ANEAN♂ (88.3% [SR24-SR240], 34.2% [SR264], 2.9 [SR288], and 0.0% [SR312]) and AN♀×AN♂ (85.3% [SR24-SR240], 50.8% [SR264], and 4.3% [SR288], 0.4% [SR312]) regarding the embryo and larvae SR at 24-312 hpf (Table 5, rows 6-18). No significant variation was observed between the offsprings of the replacement trials (male [EAN♀×AN♂; 91.6%] and female [AN♀×EAN♂; 95.9%]) and EAN-treated group (EAN♀× EAN♂ [85.3%]) in terms of embryo and larvae SR at 24-312 hpf (Table 5).

4. Discussion

In this study adult zebrafish were exposed to a bioencapsulated single cultivar OO. The results obtained here indicate that altering the FA ratios in the diet impact several

reproductive parameters in the zebrafish adding more information to the nutritional requirements of this important model species commonly used to screen biologically active food ingredients on the potential success of reproduction, a topic of high interest nowadays.

4.1. Ovarian histology and morphology

The exposure to Kor oil caused a suppression and/or delay in ovarian follicular development, indicated by the statistically significant higher presence of early-stage perinucleolar follicles (PN, 72.9% > eCA, 7.1% > mCA, 8.7% > V 5.3% > M, 7.0% [Table 4, column 4]) in EAN-treated groups compared to the AN group (PN, 61.7% > eCA, 12.4% > mCA, 12.2% > V, 7.7% > M, 5.3% [Table 4, column 3]). The highest concentration of 18:2*n*-6 found in EAN (Table 1, column 6), might explain the changes observed in folliculogenesis in the EAN-treated group and can be considered a consequence of the use of Kor oil which is rich in 18:1*n*-9, 16:0, and 18:2*n*-6. In goldfish the inhibited gonadotropin-stimulated testosterone production has been correlated to 18:2*n*-6, 18:3*n*-3 20:4*n*-6, 20:5*n*-3, and 22:6*n*-3. The role of lipids in ovarian physiology and the participation of PUFAs in regulation of ovarian steroidogenesis in teleost fish has already been cited.[87] The role of FA as precursors of some hormones has been suggested as an additional avenue by which they can affect animal physiology via endocrine hormonal pathways.[88]

In EAN-treated fish, the follicles at M stage had a significantly (ANOVA; $p < 0.05$) smaller size compared to the other treatments (Table 4, row 11). The smaller follicles at later stages are considered a disadvantage for the offspring,[84] although in the present study no significant differences could be found between EAN-treated and the other groups in terms of embryo and larval viability (HR [Table 5, rows 2-4] and SR [Table 5, rows 5-17]).

In the AN36-treated fish the follicles at all gametogenic stages except for M stage had a significantly higher diameter compared to the other treatments (Table 4, column 6). As

previously mentioned the smaller follicles at later stages are considered a disadvantage for the offspring, but the effects of the increase in the size of follicles at earlier stages in AN36-treated ovaries remains unclear. In the present study, no relationship between the size of the follicles at earlier stages in AN36-treated fish and the embryo and larvae HR and SR could be found.

4.2. Fecundity

In the present study a clear increase (non-significant) in fecundity (174.7 ± 33.5) was observed in zebrafish fed EAN compared to those fed ANEAN (91.0 ± 23.5), AN (138 ± 70.7), and AN36 (116 ± 14). Fecundity is one of the criteria used to determine fish egg quality and, in general, it is considered that a reduced fecundity could be caused either by a nutrient imbalance on the brain–pituitary–gonad system or by the restriction in the availability of a biochemical component for egg formation[89].

Nutrition is known to have a great influence in gonadal growth and fecundity in fish.[90,91] A deficiency in essential FAs (EFA) have been correlated to low egg production and quality in marine and freshwater species such as carp.[90] In the present study a higher n-6 FA content was observed in the ovaries of EAN-treated fish compared to the AN (Table 2, rows 17 and 23). Studies on Nile tilapia show that the fecundity was much higher in fish fed a diet containing higher n-6 FA.[92]

4.3. Sperm density and motility

The highest sperm density was observed in EAN- ($4.7\times10^8 \pm 6.8\times10^7$ cell mL^{-1}) and AN36-treated groups ($4.9\times10^8 \pm 5.0\times10^7$ cell mL^{-1}) (Table 4, row 12), whereas the highest and lowest FR was observed in EAN- and AN36-treated groups, respectively, (Table 5, row 1). This might indicate that in zebrafish sperm density is not a reliable criterion for quality.

EAN and AN36 are different from the AN-treated group in their FA composition, having a significantly (ANOVA; $p < 0.05$) lower SFA and higher MUFA, PUFA, and HUFA content (Table 1, rows 7, 15, 20, and 29). The AN is high in 16:0 and this is used by the *Artemia* during starvation.

The highest sperm motility at 5 spa was observed in Kor-treated specimens (80% in EAN- and 67% in ANEAN-) (Table 4, row 13), although the sperm motility decreased in both groups after that. The main differences in composition between AN36 and EAN are 18:1*n*-9 and 18:2*n*-6. At 10, 15, 20, 25, and 30 spa the motility dropped to lower values compared to the AN group (Table 4, rows 14-18). There was a significant multivariate difference (MANOVA; $p<0.008$, $\lambda<0.001$, and $F=945.4$) between the EAN and AN groups regarding FA quantitative profile. Meinelt *et al.*[93] suggested that sperm quality may be affected by FA composition. Since sperm FA composition depends on the FA content of the broodstock diet,[91,94] it might be possible that sperm motility would be affected.[89] Kor-enriched AN (EAN and ANEAN) were significantly different (ANOVA; $p<0.05$) from AN having a higher content of 18:1n-9, 20:1n-9, ∑MUFA, 18:2n-6, ∑PUFA, 20:4n-6, and ∑HUFA and a lower content of 14:0, 16:0, ∑SFA, 14:1n-5, and 16:1n-7 (Table 1). Oleic acid (18:1*n*-9) is a MUFA that is produced through desaturation of 18:0 by the microsomal FA Δ^9 desaturase.[95] The positive effects of 18:1*n*-9 on sperm motility and viability, and the enhancement of acrosome reaction has already been cited in mammals.[96] Kor-enriched AN (EAN and ANEAN) had a higher content of other MUFAs, like 14:1n-5, 16:1n-7, and 20:1n-9 compared to the AN. In general the Kor-enriched AN had a higher total MUFA (∑MUFA) than the other groups. High levels of cholesterol and MUFAs increased the cryoresistance of sperm of rainbow trout.[18]

Linoleic (18:2*n*-6) and Arachidonic (20:4n-6) acids are two *n*-6 PUFAs considered essential in fish. Both FAs are present in sperm and seminal plasma of fish such as rainbow

trout. Incubation of sperm with these two polyunsaturated FAs has been shown to increase the motility in rainbow trout in a concentration-dependent manner[97]. It has been shown that 18:2*n*-6 improves sperm motility and viability in mammals[96] whereas 20:4*n*-6 enhanced the acrosome reaction both in mammals[96] and humans.[98]

The 16:0 class of saturated fatty acids is one of the main SFAs in fish sperm. The effects of adding this SFA to sperm suspensions on motility rate and pattern in fish have been studied by Lahnsteiner *et al.*[97].

4.4. Embryo and larvae viability parameters

4.4.1. Fertility

The highest FR (95.5%) was observed in the gametes produced by pairs fed EAN (Table 1, column 6) (EAN♀×EAN♂ [Table 5, column 4, row 1]). The replacement of EAN♂ with AN♂ (EAN♀×AN♂) led to a decrease in FR (86.2%) that highlights the determinant role of sperm quality in fertility. In a previous study with zebrafish Meinelt *et al.*[93] indicated that sperm quality may be affected by FA composition and as a consequence the spawning quality is directly affected by the FA content of the broodstock diet.

In the present study EAN-treated fish were significantly different (ANOVA; $p<0.05$) from AN showing a higher content of 18:1n-9, 20:1n-9, ∑MUFA, 18:2n-6, ∑PUFA, 20:4n-6, and ∑HUFA and a lower content of 14:0, 16:0, ∑SFA, 14:1n-5, 16:1n-7, and ∑n-3/∑n-6. A positive effect of 16:0, 18:1n-9, 18:2n-6, and 20:4n-6 on sperm quality in fish and mammals has already been cited.[98,96,97]

As a warm water species, the zebrafish needs high amounts of n-6 FA, particularly for good fertilization success. The diet with the lowest proportion of n-3 per n-6 FA has led to the highest FR in other cyprinid species. The lower content of n-6 FAs in testes has been negatively correlated to FR both in zebrafish[93] and in Japanese eel.[99]

Arachidonic acid (20:4n-6) is the precursor of the series II prostaglandins (PG). 20:4n-6 stimulates testicular testosterone in goldfish testis through its conversion to PGE_2. On the contrary, 20:5n-3 or 22:6n-3 blocks the steroidogenic action of both 20:4n-6 and PGE_2, whereas both 20:4n-6 and 20:5n-3 modulate steroidogenesis in goldfish testis.[100] Thus, the timing of spermiation may be delayed and subsequently fertilization reduced by depressed steroidogenesis caused by a FA deficiency or imbalance provided to the broodstock.[89]

The success in fertilization can also be associated to the effects of 20:4n-6 on reproductive behaviour. In goldfish PGs have an important role as female pheromones, thus under the influence of the $PGF_{2\alpha}$, stimulated male sexual behaviour and synchronized male and female spawning could be observed in goldfish.[101,102] Therefore, both maturity and fertilization success might be directly affected by arachidonic acid (20:4n-6) dietary content.[93]

The decrease in FR that has been observed in the male replacement trial (EAN♀×AN♂; 86.2%) was recovered by replacing EAN♀ with AN♀ (AN♀×EAN♂ [FR=94.3%]). Thus, the determinant role of sperm quality in zebrafish fertility in the current study is made manifest by the female replacement trial.

The lower FR value of male replacement trial (EAN♀×AN♂ [FR=86.2%]) compared to pairs fed EAN (EAN♀×EAN♂ [FR=95.5%]), on one hand, and the similar FR value of female replacement trial (AN♀×EAN♂ [FR=94.3%]) to EAN-treated fish on the other hand, show that there are no significant differences between the eggs produced by EAN-treated and AN-treated females. Therefore the significant differences observed between AN pairs (AN♀×AN♂ [FR=69.2%]) and other pairs (EAN♀×EAN♂ [FR=95.5%], AN♀×EAN♂ [FR=94.3%], EAN♀×AN♂ [FR=86.2%]) concerning the FR value can be attributed to the quality of sperm.

4.4.2. Hatching and survival rates

EAN♀×EAN♂ had significantly higher HR at 48 hpf (67.9%) compared to the AN♀×AN♂ (0.5%), (Table 5, row 2). This shows an accelerated hatching in EAN-treated specimens, which is a decrease in the time needed to hatch compared to the AN. No differences could be observed between Kor oil-treated fish and other treatments concerning HR at 72 and 96 hpf (Table 5, rows 3 and 4). Therefore, although the exposure to Kor oil led to a change in the time needed to hatch, it did not have any effect in total hatching.

In the present study the ovariy FA profile of EAN-treated fish (Table 2, column 5) was significantly different to the AN (Table 2, column 4) showing a higher content of 14:1n-5, 18:1n-9, 18:2n-6, ∑PUFA, ∑n-6 PUFA/∑n-3 PUFA, 20:4n-6, ∑n-6 HUFA, and 22:6n-3 and a lower content of 14:0, 16:1n-7, 18:1n-7, ∑MUFA, 18:3n-3, and 20:5n-3. Such significant variations were also detected in the FA ratios of the ovaries (Table 3).

In previous studies, the above mentioned FAs, either have a positive correlation (18:2n-6[91]; ∑MUFA in NL[103]; 22:6n-3[90,104]; 22:6n-3 in PL, ∑n-3 in PL, ∑n-3 HUFA in PL and total lipid[103]; ∑n-3[104]; 18:2n-6[91]; 20:4n-6[105]; 20:4n-6 in NL[106]) or a negative correlation (18:1n-7; 22:6n-3 in NL; 20:4n-6; 20:4n-6/20:5n-3 in NL–Furuita et al., 2006; ∑n-6–Furuita et al., 2003a; Furuita et al., 2006) with the HR in several freshwater species. However in the present study, and despite the significant differences detected among the groups concerning ovary FA content (Tables 2 and 3), no correlation between ovary FA content and HR could be found.

Treatment groups were also compared with respect to the magnitude of the zebrafish embryo-larvae survival. No significant variation could be found among the groups in SR at 24 to 312 hpf (Table 5, rows 5-17) although a significant difference was found among them in their ovary FA content (Table 2) and the FA ratios (Table 3).

As mentioned above the FA content of ovaries in EAN-treated fish (Table 2, column 5) were significantly different from the AN (Table 2, column 4) showing a higher content of 14:1n-5, 18:1n-9, 18:2n-6, ∑PUFA, ∑n-6 PUFA/∑n-3 PUFA, 20:4n-6, ∑n-6 HUFA, and 22:6n-3 and a lower content of 14:0, 16:1n-7, 18:1n-7, ∑MUFA, 18:3n-3, and 20:5n-3. Such significant variations were also detected in the FA ratios of ovaries (Table 3).

In previous studies the above mentioned FAs, either have a positive correlation (∑MUFA in NL[103]; 22:6n-3 in PL, ∑n-3 in PL, ∑n-3 HUFA in PL and total lipid[103]; 20:4n-6 in NL[106]) or negative correlation (18:1n-7; 22:6n-3 in NL; 20:4n-6; 20:4n-6/20:5n-3 in NL[103]; ∑n-6[99,103]) with survival rate of several freshwater fish species. However, in the present study and despite the significant differences detected among the experimental groups in ovary FA content (Tables 2 and 3), no correlation between ovary FA content and HR could be found.

4.5. Conclusions

In summary, the exposure of zebrafish to Kor OO led to an increase in the frequency of follicles at an early gametogenic (perinucleolus) stage, a decrease in the diameter of matured follicles, an increase in total fecundity (non-significant), sperm density and motility, and fertility, and an accelerated hatching. The increased fertility has been attributed to the increased quality of sperm.

No significant differences could be found in terms of hatchability and embryo/larval survivability here, in agreement with earlier studies in which diet composition was cited as having a profound effect on the fish egg quality (carp[107]; rainbow trout[108]), but may not have significant effects on other parameters related to egg-quality such as hatchability and survivability at different embryo/larva developmental stages.

The physiological responses induced by a well-characterized Kor oil in the current study, suggest that FAs can be considered as nutrients that contribute to protect and improve

reproductive capacity in a model species like zebrafish (e.g. the differences in reproductive parameters between AN36 and EAN groups can be attributed to the changes in FAs such as 18:1*n*-9 and 18:2*n*-6.), and which properties can be transferred ultimately to human health as a general health criterion.

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Tables

Table 1. Fatty acid (FA) composition in Koroneiki (Kor) oil and in four groups of *Artemia* nauplii (AN): unenriched AN (newly hatched AN and 24 hours post-hatch AN36) Kor oil enriched AN (EAN), and a combination of EAN (50%) and AN36 (50%) (ANEAN).

Footnote → Data are mean ± SD. Mean values were compared by ANOVA with subsequent Duncan post hoc test. Mean values within horizontal rows superscripted by the same letter are not significantly different, $p > 0.05$. Trace = mean of FAs relative abundance less than 0.1.

Table 2. Fatty acid (FA) composition in the ovary of zebrafish those were fed with four differet groups of *Artemia* nauplii (AN): unenriched AN (newly hatched AN and 24 hours post-hatch AN36), Kor oil enriched AN (EAN), and a combination of EAN (50%) and AN(50%) (ANEAN).

Footnote → Data are mean ± SD. Mean values were compared by ANOVA with subsequent Duncan post hoc test. Values superscripted by the same letter are not significantly different, $p > 0.05$. Trace = mean of FAs relative abundance less than 0.1. OVA: ovary.

Table 3. The ratio of fatty acid (FA) composition in the ovary of zebrafish those were fed with four different groups of *Artemia* nauplii (AN): unenriched ANs (newly hatched AN and 24 hours post-hatch AN36), Kor oil enriched ANs (EAN), and a combination of EAN (50%) and AN (50%) (ANEAN).

Footnote → Data are mean ± SD. Mean values were compared by ANOVA with subsequent Duncan post hoc test. Mean values within horizontal rows superscripted by the same letter are not significantly different, $p > 0.05$.

Table. 4. Gonad (ovary and testis) and gamete (sperm) parameters in zebrafish those were fed with four groups of *Artemia* nauplii (AN).

Footnote → Data are mean ± SD. Mean values were compared by ANOVA with subsequent Duncan post hoc test. Mean values within horizontal [] and vertical () rows superscripted by the same letter are not significantly different, $p > 0.05$. spa: seconds post activation.

Table 5. Embryo-larvae viability parameters –VP- (fertilization rate FR, hatching rate HR, and survival rate SR) at different hours post fertilization (hpf) in zebrafish those were fed with four groups of *Artemia* nauplii (AN): unenriched ANs (newly hatched AN and 24 hours post-hatch AN36), Kor oil enriched AN (EAN), and a combination of EAN (50%) and AN (50%) (ANEAN).

Footnote → Data are mean ± SD. Mean values were compared by ANOVA with subsequent Duncan post hoc test. Values superscripted by the same letter are not significantly different, $p > 0.05$. M: mean, SD: standard deviation, CV: coefficient of variation

Figures

Fig. 1. The process of bioencapsulation of *Artemia franciscana*. (a-b) Unenriched *Artemia* (36 hours post-hatch [AN36]). (c-d) Incomplete bioencapsulation. (e-f) Complete bioencapsulation.

Fig. 2. Follicles at different gametogenic stages identified in the ovary. (a) Perinucleolus at PN-stage; (a,b) early cortical alveolus at eCA-stage; (d,e) mid-cortical alveolus at mCA-stage; (f-h) vitellogenic at V- stage; and mature, M- stage.

Fig. 3. The pair-wise crossings set in the current study and viability parameter of their offsprings. (a-d) Pair-wise crossings set within the same treatment groups (i.e. AN♀×AN♂, AN36♀×AN36♂, EAN♀×EAN♂, and ANEAN♀×ANEAN♂). (e) Male and (f) female replacement experiment.