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Dietary LC-PUFA deficiency early in ontogeny induces behavioural changes in pike perch (*Sander lucioperca*) larvae and fry

Ivar Lund [a,*], Erik Höglund [a], Lars O.E. Ebbesson [b], Peter V. Skov [a]

[a] Technical University of Denmark, DTU Aqua, Section for Aquaculture, The North Sea Research Centre. P.O. Box 101, DK-9850, Hirtshals, Denmark
[b] Uni Research AS, Thormøhlensgt. 49B, N-5006 Bergen, Norway

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ABSTRACT

This study examined whether dietary supply of DHA and phospholipids during early ontogeny affected the outcome of behavioural challenges in pike perch larvae and fry, and whether the history of lipid nutrition carried over in long-term effects on learning ability. Pike perch larvae were fed *Artemia* enriched with either refined olive oil high in oleic acid (A); refined olive oil supplemented with a low (B) or a high (C) level of DHA; or refined olive oil acid supplemented with fish oil with a high content of phospholipids (PL) and DHA (D). The enriched live diets were provided until 28 days post hatch (dph), at which time larval behavioural responses to visual and mechano-sensory stimuli were assessed. All dietary groups were subsequently fed an identical enriched live feed (diet D) and gradually weaned to an extruded dry feed, on which they were maintained for 112 days. At the end of this period, assessment of fry avoidance behaviour was repeated and individuals were tested for spatial learning ability in a maze. At the larval stage, individuals maintained on *Artemia* rich in DHA showed a 5–8 fold increase in swimming speed when subjected to a visually simulated predator test, a response that was not observed for larvae on diets low in DHA content. Independent of the predator simulation, larvae deficient or low in DHA exhibited significantly more time swimming along the edge of a test arena and had overall higher locomotor activities compared to larvae fed a diet with a high DHA content. Larvae on DHA rich diets showed an ability to achieve significantly higher peak acceleration rates during the escape response, which was maintained at 112 dph. Time spent locating the exit of a maze decreased with repetitious training sessions, although fish fed diets low in DHA spent longer time in the maze, caused by extended periods of inactivity or "freezing" behaviour (time lag) prior to the onset of active searching behaviour.

The consistency of behavioural responses to mechano-sensory stimuli in larvae and fry suggests long-term effects on the neuromuscular path-way involved in escape responses. A longer period of freezing in the learning test may reflect a more anxious and fragile behaviour profile in fish fed low levels of DHA. Further studies should aim at verifying whether this affects performance related traits, such as immune competence and robustness.

1. Introduction

Pike perch (*Sander lucioperca*) is considered a species with a high potential for inland freshwater aquaculture in Europe (Wang et al., 2008) and a strong candidate for the potential diversification of intensive freshwater recirculation aquaculture systems (RAS) farming in Europe (Dalsgaard et al., 2013). A major bottleneck for further expansion of pikeperch culture today is its high sensitivity to stressors (pers. comm. Martin Vestergaard, AquaPri Innovation, DK, 2013). In modern intensive aquaculture, the robustness and stress resilience are of crucial importance in terms of welfare, health, growth, quality of the end product and thus overall production costs. Studies on percid larvae suggest that dietary supplementation with phospholipids and/or specific vitamins increase the health status of farmed pike perch, by decreasing the incidence of scoliosis and lordosis and increase larval resistance to osmotic stress (Hamza et al., 2008; Henrotte et al., 2010; Kestemont et al., 1996; Lund et al., 2012). The major essential nutrient requirements for pikeperch are still unknown, and information is lacking about the link between nutritional composition early in ontogeny and the robustness of produced fish.

Pike perch eggs have a high DHA content, which could be related to its strictly carnivorous nature and/or may be an evolutionary remnant from life adapted to a marine environment. We have recently shown, that diets deficient in LC-PUFAs, particularly DHA, during first feeding (i.e. within 25 days post hatch) is accompanied by a suite of negative consequences. These effects include increased mortality and sensitivity to salinity stress in both larvae and juveniles and brain developmental disorders (Lund and Steenfeldt, 2011; Lund et al., 2012), suggesting

* Corresponding author. Tel.: +45 35883205.
E-mail address: il@aqua.dtu.dk (I. Lund).

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that DHA is crucial in cognitive function and stress responses in pike perch. This is in agreement with the previously documented role of DHA for visual acuity (Lauritzen et al., 2001; Neuringer et al., 1988) and neural development in mammals (reviewed by Dyall and Michael-Titus, 2008), in proper brain development and neuronal migration, as well as neurophysiological functioning in fish (Benítez-Santana et al., 2012; Mourente, 2003). Dietary fatty acid composition has been shown to be important for cognitive functions in mammals (including humans) due to the role of DHA in support of learning and memory formation (Benton et al., 2013; Bourre et al., 1984; Cheatham et al., 2006; Fedorova and Salem, 2006; Mohajeri and Winwood, 2012). Central effects of dietary fatty acid composition are further verified in rodent models, which have demonstrated elevated vulnerability to stress, anxiety levels, and the occurrence of depression-like conditions in animals fed diets deficient in DHA, which also results in reduced willingness to explore novel environments and behavioural latency (Bhatia et al., 2011; Fedorova and Salem, 2006; Lamptey and Walker, 1976). At present, there is only limited information and evidence for a link between dietary LC-PUFA intake, neurophysiological function and behavioural stress responses in fish (Benítez-Santana et al., 2012). As a predisposition to pathological conditions in mammals appears to be a general response to LC-PUFA malnutrition during early development (Alsop and Aluru, 2011), this might imply that the fatty acid composition of larval feed is essential in achieving robust individuals for on-growing of farmed pike perch.

Thus, the aim of the present study was to investigate if dietary fatty acid compositions in larval feed affected behavioural responses to challenges in the larval and fry stages, and if they affect learning and the endocrine stress response in the fry stage. This was carried out by studying behavioural responses to visually simulated predator attacks and fast escape responses to mechano-sensory stimuli during the larval stage. During the fry stage the fast escape response test was repeated, spatial learning ability was studied by a maze test and effects on the endocrine stress response were quantified by post stress plasma cortisol levels.

2. Materials and methods

2.1. Formulation of emulsions

Four dietary emulsions were made by the substitution of extra refined virgin olive oil (Seatons 790.1 mg oleic acid/g) with either DHA oil (Incromega DHA500TG, DHA content >500 mg DHA/g; ≤100 mg EPA/g) or a fish oil rich in phospholipids from TripleNine, Esbjerg Denmark (PL: 44.3% weight (i.e. phosphatidyl choline, PC: 16.1%; lysophosphatidylcholine, LPC: 5.4%; phosphatidylethanolamines, PE: 4.5%; APE: 6.3%; spingomyelin, SPH 3.5%, others 8.5%). The main FA in the oil constituted 16:0: 188 mg g^{-1} oil; 18:1: 109 mg g^{-1} oil; DHA: 193 mg g^{-1} oil; EPA: 135 mg g^{-1} oil. The sum of polyunsaturated FA was 400 mg/g oil. Three emulsions contained either A: 0 g, B: 50 g or C: 500 g kg^{-1} DHA oil and one emulsion D: 500 g kg^{-1} phospholipid rich fish oil (i.e. 440 g phospholipids kg^{-1}) (Table 1). In all emulsions soy lecithin was included (70 g kg^{-1}) as emulgator and E-vitamin mix was added (40 g kg^{-1}) as antioxidant (Table 1). Olive oil and DHA oil were obtained from Croda Chemicals Europe, Snaith, UK. Fish oil, soy lecithin and E vitamin mix were obtained from BioMar, Brande, Denmark.

2.2. Larval and juvenile rearing and feeding

Larvae were obtained from a commercial farm AquaPri Innovation, Egtved, Denmark at 2 day post hatching (dph). Approximately 1600 larvae were distributed into each of 12 tanks at a density of approximately 36 larvae per litre. The larval rearing tanks had a volume of 46 L, and received a water flow of 8–10 L h^{-1} from a 10 m^3 temperature controlled reservoir. Each tank had separate inlet taps with adjustable flowmeters, 500 μm drainage filters and aeration. Larvae were kept under constant dim light provided by light bulbs above the tanks. Temperature and oxygen saturation were monitored daily using a portable DO meter (OxyGuard Handy, OxyGuard, Birkerød, Denmark). The temperature was maintained at 16.6 ± 0.7 ° C during the first 28 days of experimentation. Oxygen content was kept around 7.1–7.5 mg/L in all tanks.

Larvae for each treatment were reared in triplicate tanks. Newly hatched un-enriched *Artemia* (MC 450 type, >260.000 nauplii/g, INVE-*Artemia* Systems, Belgium) were used as starter feed from dph 3 until 6 dph for all larval groups. From 7–27 dph, randomly chosen triplicate larval groups were fed EG type *Artemia* (INVE-*Artemia* Systems) enriched by one of 4 emulsions (0.6 g emulsion L^{-1} for 24 h). *Artemia* were enriched according to normal enrichment procedures at a temperature of 21–22 °C, providing vigorous aeration by airstones (by a mix of air and pure oxygen to ensure oxygen levels > 4 mg/L) at a density of 500–1000 *Artemia* /ml. *Artemia* were harvested in the morning and administered continuously for 2 periods of 6 hours (each morning and afternoon) by automatic dispensers each holding a suspension of *Artemia* in seawater. Buckets containing the remaining *Artemia* of each enrichment type were kept aerated by airstones in a refrigerator between feedings at 5 °C. The tank bottom of each larval tank was gently vacuumed on a daily basis to remove uneaten *Artemia*, debris and to examine for mortality of larvae, which were counted.

From dph 29–40 all larval groups were fed *Artemia* enriched by emulsion D (phospholipid rich fish oil) and gradually weaned to an extruded experimental feed composed of fish meal (50%); soy protein concentrate (12.5%); wheat (17.2%); fish oil (10%); rape seed oil (10%); vitamin/mineral (0.3%). Protein and lipid content was 43.6% and 28.1% respectively. The feed was initially crushed to match the size of the growing fish fry and was fed to the fry during the remaining of the study until dph 140 by 12 h band feeders. Fry were kept in their initial tanks during the entire study and tanks regularly cleaned. Temperature was kept at 19.3–20.4 ° C and oxygen above 5.1 mg/L.

Table 1
Analysed TFA Artemia content (mg g^{-1} d.w.) and FA composition (% of TFA) enriched by 4 emulsions. Formulation of emulsions (% inclusion) is shown below.

	A: OO[a]	B: OO[b] +5 DHA	C: OO[c] +50 DHA	D: OO[d] +50 PL
TFA	97.1 ± 37.6	122.1 ± 6.3	128.3 ± 75.5	79.7 ± 20.4
FA				
16:0	11.1 ± 0.0	10.1 ± 0.6	10.6 ± 0.9	11.1 ± 0.0
18:0	6.6 ± 1.2	6.0 ± 0.2	6.2 ± 0.0	6.1 ± 0.0
Total SFA	21.5 ± 2.6	19.4 ± 1.2	21.4 ± 2.1	22.5 ± 1.2
16:1 (n-7)	1.0 ± 0.3	1.1 ± 0.0	1.2 ± 0.0	1.4 ± 0.2
18:1 (n-9)	36.5 ± 0.7[c]	36.6 ± 0.4[c]	25.6 ± 0.9[a]	29.9 ± 0.8[b]
Total MUFAs	40.6 ± 1.8	43.1 ± 1.8	34.2 ± 3.5	39.1 ± 2.2
18:2 (n-6)	5.1 ± 0.2[b]	4.8 ± 0.2[b]	4.2 ± 0.2[a]	4.4 ± 0.4[ab]
18:3 (n-6)	0.3 ± 0.1	0.3 ± 0.2	0.3 ± 0.1	0.2 ± 0.1
20:3 (n-6)	0.1 ± 0.1	0.1 ± 0.0	0.1 ± 0.1	0.2 ± 0.1
20:4 (n-6) ARA	0.3 ± 0.1[a]	0.4 ± 0.1[ab]	0.7 ± 0.0[b]	0.6 ± 0.2[ab]
Total (n-6) PUFA	5.9 ± 0.5	5.8 ± 0.6	5.5 ± 0.5	5.6 ± 0.7
18:3 (n-3)	28.9 ± 2.4	27.8 ± 1.1	27.5 ± 1.5	22.9 ± 0.5
20:3 (n-3)	1.3 ± 0.4	1.1 ± 0.0	1.4 ± 0.2	1.1 ± 0.0
20:5 (n-3) EPA	0.5 ± 0.4[a]	1.4 ± 0.1[b]	3.3 ± 0.2[c]	4.4 ± 0.2[d]
22:6 (n-3) DHA	0.1 ± 0.1[a]	0.6 ± 0.1[a]	5.5 ± 0.2[c]	3.1 ± 0.2[b]
Total (n-3) PUFA	30.9 ± 3.3	30.9 ± 1.3	37.8 ± 2.0	31.6 ± 0.9
DHA/EPA	0.2 ± 0.4[a]	0.4 ± 0.1[a]	1.7 ± 0.0[b]	0.7 ± 0.1[a]
ARA/DHA	3.2 ± 7.0	0.7 ± 0.2	0.1 ± 0.0	0.2 ± 0.0
ARA/EPA	0.5 ± 0.5	0.3 ± 0.0	0.2 ± 0.0	0.1 ± 0.0
(n-3)/(n-6)	5.2 ± 0.5[a]	5.4 ± 0.2[ab]	6.9 ± 0.2[b]	5.6 ± 0.3[ab]

Values in a row followed by a different superscript are significantly different $P < 0.05$.
All emulsions included 7% soya lecithin and 4% E vitamin mix.
[a] OO: 89% OO; (OO (olive oil, Seatons refined, ≥79.1% oleic acid).
[b] OO + 5 DHA: 84% OO. 5% Incromega DHA500TG, DHA content ≥51% of total fatty acids.
[c] OO + 50 DHA: 39% OO. 50% Incromega DHA500TG, DHA content ≥51% of total fatty acids.
[d] OO + 50PL: 39% OO. 50% fish oil with phospholipid (PL) content ≥44% total lipids.