

Feeding with arachidonic acid-rich triacylglycerols from the microalga *Parietochloris incisa* improved recovery of guppies from infection with *Tetrahymena* sp.

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Abstract

Two trials were conducted to determine the effect of dietary enrichment with arachidonic acid (ARA)-rich triacylglycerols fraction, obtained from the microalga *Parietochloris incisa*, on the recovery of guppies, *Poecilia reticulata*, from infection with *Tetrahymena* sp. Commercial feed (0.7 mg ARA g⁻¹ feed) was directly enriched with the algal hexane lipid extract. Three (12.5, 25.0, 50.0 mg ARA g⁻¹ feed) and four (12.5, 25.0, 37.5, 50.0 mg ARA g⁻¹ feed) experimental diets were utilized in the first and second trials, respectively. Guppies were fed with ARA-supplemented feed for 14 days followed by infection with 1000 *Tetrahymena* sp. cells mL⁻¹. Supplemented diets significantly altered the fatty acid composition in fish body (whole body with the exception of the digestive tract and liver) and liver. The share of ARA in fatty acids of bodies and livers increased commensurately with the amount of ARA in feed. Livers accumulated the highest levels of ARA, resulting in drastic changes in the docosahexaenoic acid (DHA)/ARA and eicosapentaenoic acid (EPA)/ARA ratios.

Feeding with an algal extract that produced an ARA concentration in feed of 25 mg g⁻¹ significantly enhanced the ability of fish to recover from the disease in both experiments, as compared to the control and feed supplemented with 12.5 mg g⁻¹ of ARA. Although not significantly different, further increase in fed ARA (50 mg g⁻¹ feed) reduced the recovery as compared to the 25 mg g⁻¹ treatment.

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1. Introduction

The fatty acid composition of fish lipids is determined by the ability of fish to desaturate and elongate de

novo synthesized and dietary fatty acids (Henderson, 1996). Fish can desaturate the endogenous saturated fatty acid, 18:0, to the monounsaturated fatty acid 18:1n-9, by 18:0-CoA desaturase, but lack both $\Delta 12$ and $\Delta 15$ (n-3) desaturases which are required for the synthesis of C₁₈ and C₂₀ polyunsaturated fatty acids (PUFAs) (Henderson, 1996; Tocher, 2003). Linoleic acid (18:2n-6) and linolenic acid (18:3n-3) are thus

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essential dietary fatty acids, which fish are able to convert via a series of desaturation and elongation reactions to very long-chain (VLC) C₂₀ and C₂₂ PUFAs, e.g., the principal n-3 PUFAs, eicosapentaenoic acid (EPA, 20:5n-3) and docosahexaenoic acid (DHA, 22:6n-3), and the n-6 PUFA, arachidonic acid (ARA, 20:4n-6) (Jobling and Bendiksen, 2003; Wallis et al., 2002). Marine fish, however, require dietary supply of EPA and DHA due to their relative deficiency in either the $\Delta 5$ desaturase or one of the two enzymes of the elongase multi-enzyme complex (Tocher, 2003). Freshwater fish can produce VLC-PUFAs from dietary C₁₈ precursors (Henderson, 1996; Tocher, 2003) and their n-6 fatty acid content is therefore higher than in marine species (Ackman et al., 2002). In aquaculture, fish are supplied with diets highly enriched in n-3 PUFAs (EPA and DHA), but poor in ARA. Recently, Harel et al. (2001) suggested that such diets might suppress the capacity of fish larvae to cope with stressful events and inhibit the development of the immune system.

The importance of ARA as an essential fatty acid in fish nutrition has been overlooked in the past. The information available suggests that dietary ARA is very important in intensive aquaculture, particularly with regard to brood stock nutrition, immune function and egg and larval quality (Castell et al., 1994; Bessonart et al., 1999; Bell and Sargent, 2003). ARA is known to be the primary precursor of a group of eicosanoids, the 2-series prostaglandins and thromboxanes and the 4-series leukotrienes, which regulate many physiological processes, including homeostasis, reproduction, immune and inflammatory responses (Abbas et al., 1994; Tocher, 2003). EPA competes with ARA for the same set of enzymes leading to the production of a different group of eicosanoids, which have a different range of physiological activities than those produced from ARA. The dietary ratio of n-3 to n-6 PUFAs thus influences the pattern and properties of the eicosanoids formed (Tocher, 2003). It was therefore proposed that nutritional ARA can improve the health and quality of fish fed with EPA-and DHA-enriched diets in intensive aquaculture, particularly with regard to coping with periods of environmental stress (Bell and Sargent, 2003). Feeding sea bream (*Sparus auratus*) larvae with ARA-enriched artemia markedly improved their survival following handling stress (Koven et al., 2001, 2003, Van Anholt et al., 2004). ARA levels in tissue increased correspondingly to the ARA content in the diet (Koven et al., 2003). Growth of some *Morone* larvae positively correlated with elevated dietary ARA and DHA/ARA ratio (Harel and Place, 2003). Elevated levels of tissue ARA were generally associated with

elevated levels of cortisol (Harel et al., 2001; Koven et al., 2003). Dietary DHA and ARA affected the leukocyte relative distribution in striped bass (*Morone saxatilis*) following intraperitoneal injection with formalin-fixed *Staphylococcus aureus*. The largest increase in the proportion of lymphocytes, monocytes and neutrophils in peripheral blood occurred in larvae fed a moderate level of both DHA and ARA (Harel et al., 2001). Similarly, immune function of Atlantic salmon (*Salmo salar*) was affected in fish fed with altered n-3/n-6 ratio (Bell et al., 1996). Dietary DHA and ARA also increased larval tolerance to salinity stress (Harel et al., 2001).

Until recently, the lack of significant commercial sources of ARA has limited the study of this fatty acid as a dietary ingredient. Currently, ARA is commercially obtained from the oil of the filamentous fungus *Mortierella alpina* (Zygomycetes). This fungus was commercialized since its oil has elevated levels of ARA (about 40% of total fatty acids), minor levels of DHA and no EPA. Microalgal-derived products are utilized in aquaculture feed formulations mainly to provide DPA (docosapentaenoic acid, 22:5n-3) and DHA (the heterotrophic algae *Cryptocodinium cohnii* and *Schyzochitrium*) (Kyle et al., 1989; Barclay et al., 2005). The green microalga *Parietochloris incisa* was found to be the richest plant source of ARA, with over 90% of total ARA being deposited in triacylglycerols (TAG) (Bigogno et al., 2002). Under conditions of nitrogen starvation, ARA accounts for 60% of total fatty acids and for more than 20% of the algal dry weight (Khozin-Goldberg et al., 2002). The major molecular species of TAG, under those conditions, is the very rare triarachidonylglycerol.

Tetrahymena sp. is a free-living ciliated protozoan that under certain conditions infects fish. It is also known as the “guppy killer parasite” due to its devastating effect on guppies. Tetrahymenosis has become widespread owing to the wide trade in guppies across the world and ineffective quarantine processes, causing great losses at fish farms. Very little has been published on *Tetrahymena* sp. infection, treatment, and/or control. A combination of 0.5% sodium chloride and feeding with the immunostimulatory Chinese herb mix (C-UPIII) was shown to protect fish from infection (Ponpompisit et al., 2001), but the most effective control measure for this disease is prevention of exposure.

In the present study, the significance of a dietary supply of ARA in regulating fish immune function was tested by assessing its effect on the recovery of the ornamental freshwater fish *Poecilia reticulata* (guppy) from infection with *Tetrahymena* sp. ARA was provided

Table 1

Fatty acid composition of ARA-rich algal oil, commercial fish feed and ARA-rich algal oil-supplemented feeds

Fatty acid	Fatty acids (% of total)				
	<i>P. incisa</i> extract	Fish feed	ARA-supplemented feed (mg g ⁻¹)		
			12.5	25	50
12:0	tr	0.2	–	–	tr
14:0	0.2	2.9	2.6	2.0	1.3
16:0	7.2	19.4	17.3	15.6	12.2
16:1n-9	tr	4.7	3.8	3.0	2.2
18:0	2.2	4.0	3.6	3.5	3.0
18:1n-9	15.8	17.1	16.7	16.5	16.3
18:1n-7	4.2	2.0	2.4	3.2	3.2
18:2	10.9	21.3	18.9	17.6	15.2
18:3n-6	0.7	2.2	0.2	0.3	0.5
18:3n-3	0.3	1.8	1.8	1.3	1.1
20:0	tr	0.2	0.2	0.2	0.2
20:1	0.3	3.3	2.6	1.7	1.5
20:2n-6	0.4	0.3	0.3	0.2	0.3
20:3n-6	1.2	tr	0.3	0.5	0.7
20:4n-6 (ARA)	55.4	0.9	13.8	23.8	33.6
20:5n-3 (EPA)	0.5	6.6	5.3	3.6	3.1
22:0	tr	4.2	3.3	2.5	1.8
22:6n-3 (DHA)	–	8.3	6.6	4.1	3.7
Others	0.3	0.4	0.4	0.4	0.4
EPA/ARA		7.3	0.38	0.15	0.09
(DHA+EPA)/ARA		10	0.91	0.33	0.20

tr—traces.

as the neutral lipid extract of *P. incisa*. Changes in fish fatty acid composition were determined in whole body and liver samples.

2. Materials and methods

2.1. Algal cultivation

The microalga *P. incisa* comb. Nov was isolated from samples obtained from Mt. Tateyama in Japan (Bigogno et al., 2002). The alga was cultivated in 6 cm wide, 1000 mL glass columns in BG-11 medium (Stanier et al., 1971), which were placed in a temperature-regulated water bath at 25 °C (Bigogno et al., 2002). Cultures were aerated by bubbling with a mixture of 1.5% CO₂ in air. Illumination (175 μmol photon m⁻² s⁻¹) was provided by cool-white fluorescent lamps (20 W) external to the water bath. Nitrogen starvation was induced by resuspension of logarithmically growing cells in nitrogen-free BG-11 medium where ferric ammonium citrate was substituted with ferric citrate. Cultures were cultivated under nitrogen starvation for 14 days to achieve the highest proportion of ARA in lipids.

2.2. Preparation of fish feed supplemented with ARA-rich algal oil

Freeze-dried algal biomass was frozen in liquid nitrogen and ground to a fine powder by a mortar and pestle with extra pure sea sand (Merck, Darmstadt, Germany). The powder was sequentially extracted with several portions of n-hexane and the extract was clarified by filtration, evaporated under vacuum at 30 °C and stored under argon atmosphere at –20 °C. The β-carotene content of the extract was determined spectrophotometrically using E_{1%¹cm} absorption coefficient of 2592 at 450 nm in petroleum ether (Britton, 1995). Different amounts of the algal oil were coated onto the commercial feed (Tropical Orange, Tzemah, Israel) and the fatty acid composition and content of the modified feed were analyzed.

2.3. *Tetrahymena* sp. culture

Tetrahymena sp. was isolated from skin lesions of infected guppies from a commercial guppy farm in Northern Israel. The parasite was transferred to a *Tetrahymena* sp. culture medium (ATCC 357, Manassas, VA) in a Petri dish at 25 °C. Penicillin G and streptomycin sulfate were added to avoid growth of contaminating bacteria. After obtaining an axenic culture, the cells were transferred to 4 wells in a 24-well cell culture plate (Corning Inc., Corning, NY, USA) in ATCC 357 medium without antibiotics and were subcultured weekly.

2.4. Fish

Guppies, *P. reticulata*, used in this study were obtained from a commercial aquaculture farm in the Arava valley, Israel. Upon arrival, they were examined to determine that they were clear of *Tetrahymena* sp. and stocked in 130-L holding tanks. Tanks were supplied with biological filters and cleaned weekly by siphoning and replacing half of the water with de-chlorinated fresh water, obtained using 50 mg/L of sodium thiosulphate pentahydrate (William Blythe, Accrington, UK).

2.5. Experimental design

2.5.1. Trial 1

Two-month-old guppies (average weight 0.28 g) were stocked in four 30-L aquaria supplied with biological filters, at a density of 60 fish per aquarium. Each aquarium was fed with a commercial guppy feed, supplemented with a different concentration of the algal

lipid extract (Table 1). Feed was provided twice a day, at 2% of body weight per day, for a period of 14 days. Fish were weighed at the beginning of the experiment and at the commencement of supplemented feed application. Five fish from each treatment group were sampled for fatty acid analysis at days 8 and 14 after initiation of feeding. Anaesthetized fish (clove oil, $250 \mu\text{L L}^{-1}$) were dissected, the gastro-intestinal tract removed and livers were separated and pooled. Bodies from three fish and livers, pooled from five fish, were weighed, frozen, freeze-dried and kept at -20°C until analyzed. At the end of the 14-day period, fish were exposed to *Tetrahymena* sp. in Petri dishes (3 fish per dish) containing 40 mL of water and 1000 *Tetrahymena* sp. cells per mL, for 24 h at 25°C . Preliminary data have shown that this level of exposure produces 100% infection (data not shown). Exposed fish were pooled (each treatment separately) and ten fish from each group were randomly sampled and examined for *Tetrahymena* sp. infection (see below). Fish were then divided between 1 L beakers filled with 800 mL of clean tap water and aerated, at a density of eight fish per beaker, in triplicates for each treatment. A group of eight fish from each treatment served as a control that was similarly treated but not infected. Fish were kept in the beakers for five days at 25°C and skin scrapes were then examined for infection with *Tetrahymena* sp.

2.5.2. Trial 2

The second experiment was carried out following the protocol of the first experiment with several modifications. In this experiment an additional diet formulation, containing $17.5 \text{ mg ARA g}^{-1}$ feed, was added (Table 1). The 30-L aquaria were stocked with 80 fish (2.5 months old, average weight 0.35 g). The experimental infection and recovery were conducted in six replicates (six 1-L beakers, each stocked with 8 infected fish) and did not include the non-infected control group.

2.6. Examination of *Tetrahymena* sp. infection

Skin scrapes of about 1 cm^2 (total area) were gently collected from the lateral left side of the fish using a glass cover-slip, and examined under a light microscope for the presence of slow moving *Tetrahymena* sp., containing pigmented food vacuoles. These vacuoles indicate that *Tetrahymena* sp. have been ingesting the host's pigment cells, and therefore represent a parasitic form (Ponpornpisit et al., 2001). Infection was measured by presence vs. absence of *Tetrahymena* sp. on fish skin.

2.7. Fatty acid analysis

Samples of freeze-dried algal biomass, algal oil, fish feed and freeze-dried fish samples were transmethylated with 2% H_2SO_4 in dry methanol under argon atmosphere at 80°C for 1.5 h in the presence of 10% toluene (v/v) to facilitate solubilization of TAG. Gas chromatographic analysis of fatty acid methyl esters (FAME) was performed on a Hewlett Packard 5890 gas chromatograph with a Supelcowax 10 fused silica capillary column ($30 \text{ m} \times 0.32 \text{ mm}$) (Supelco Inc., Bellefonte, PA), using a temperature gradient from 185 to 210°C with a linear increase of $10^\circ\text{C min}^{-1}$, and helium as a carrier gas. FAME were identified by co-chromatography with authentic standards (Sigma Chemical Co., St. Louis, MO), by comparison of their equivalent chain length (Ackman, 1969) and by GS-MS. GC-MS was performed on a Hewlett Packard 6890 GC with a detector MS 5973 (Hewlett Packard, Palo Alto, CA) and an HP-INNOWAX capillary column ($30 \text{ m} \times 250 \mu\text{m} \times 0.25 \mu\text{m}$ film thickness) (Agilent, USA) using a temperature gradient from 150 to 240°C with a linear increase of 4°C min^{-1} . Each sample was analysed in duplicate.

2.8. Statistical analysis

Data of fatty acid contents and infection rates were analysed by a one-way ANOVA. Growth rates were analysed by a z-test. SigmaStat (SPSS Inc., 1992–1997) was used for all statistical analyses. Data were considered significantly different at $p < 0.05$.

3. Results

3.1. Feed preparation formulation

The hexane extract from *P. incisa* nitrogen-starved cells contained TAG as the major lipid class (data not shown) and ARA accounted for 55.4% of total fatty acids (TFA) (Table 1). The extract also contained 2.2 mg g^{-1} β -carotene. The extract was added directly to the commercial fish feed, which contained only traces of ARA (0.7 mg g^{-1} , less than 0.9% of total fatty acids). Feed enrichments with the microalgal oil were formulated to increase the proportion of ARA gradually from 0.9% in the commercial feed to 13.8%, 23.8% and 33.6%, achieving ARA contents of 12.5, 25.0 and 50 mg g^{-1} feed, respectively. During the first trial, fish were fed with regular (control) and ARA-enriched (12.5, 25.0 and 50 ARA mg g^{-1}) diets for 2 weeks. In the second trial, a diet containing 17.5 mg g^{-1} ARA was added. The β -

carotene enrichment in supplemented diets accounted for 0.06, 0.11 and 0.22 mg g⁻¹ in 12.5, 25.0 and 50 mg ARA g⁻¹ diets, respectively.

3.2. Fatty acid profiles of fish samples

Fish bodies and livers contained 27 different fatty acids, mostly saturated and monounsaturated fatty acids. In bodies of control fish sampled after 2 weeks, saturated fatty acids (SFA) and monounsaturated fatty acids (MUFA) comprised more than 30% of total fatty acids (TFA) each (Table 2). The major SFA was 16:0 (20.0 ± 2.0%) and the dominant MUFA was 18:1n-9 (25.9 ± 3.6%). C₁₈ PUFAs were represented mainly by 18:2n-6 (13.5 ± 1.0% of total fatty acids). Among VLC-PUFAs, DHA (22:6n-3) prevailed (7.2 ± 1.3%), while EPA (20:5n-3) and ARA (20:4n-6) accounted for only 1.1 ± 0.3 and 2 ± 0.7%, respectively. The fatty acid composition of livers differed from that of bodies, having higher levels of MUFA and lower levels of ARA. Thus, bodies and livers of guppies fed with unsupplemented feed were characterized by (DHA + EPA)/ARA ratios of 6.0 and 9.0, respectively.

Feeding with ARA-enriched feed for 14 days increased the ARA content (µg g⁻¹) and its proportion (% of total fatty acids) in both bodies and livers (Table 2). The proportion of ARA in fatty acids of body lipids significantly increased ($p < 0.005$) in all treatment groups compared to the control, but there were no significant differences between the different treatments. ARA content in fish dry weight significantly increased commensurately with the increase of ARA in feed ($p < 0.005$). Liver fatty acid composition was more drastically affected by the dietary manipulations. Feeding with the highest concentration of ARA (50 mg g⁻¹) produced a tenfold increase in the proportion of ARA in liver lipid (0.9% and 9.9% in control and enriched feed, respectively). The proportions of 16:0 and 18:1 in liver lipids decreased concomitantly with the increase in ARA (Table 2). However, the proportions of DHA and EPA in liver lipid changed little during the trial period, indicating that ARA was substituting less saturated and shorter fatty acids rather than VLC-PUFAs. Dietary ARA was elongated to 22:4n-6 as indicated by the gradual increase in the share of this fatty acid with the increase in the amount of ARA in the feed,

Table 2

Fatty acid composition (% of total) of body (average of two trials, $n=3$ in each trial) and liver (pooled from 5 fish, average of two trials) of guppies fed with the ARA-supplemented diets for 14 days

Fatty acid	ARA-supplemented feed (mg g ⁻¹)							
	Body				Livers			
	0	12.5	25	50	0	12.5	25	50
14:0	1.9±0.3	1.9±0.2	1.6±0.2	1.7±0.1	1.5	1.5	1.2	1.2
16:0	20.0±2.0	19.1±1.3	18.5±1.2	19.4±1.0	17.5	16.8	14.0	14.6
16:1n-7	4.0±0.5	3.9±0.7	3.3±0.5	3.7±0.7	3.5	3.1	2.7	2.8
18:0	7.6±0.7	7.0±0.4	7.5±0.4	7.3±0.5	8.7	8.0	8.3	7.4
18:1n-9	25.9±3.6	24.3±1.4	26.5±2.7	27.7±1.5	35.3	30.4	29.4	29.6
18:1n-7	2.4±0.3	2.3±0.3	2.0±1.0	2.0±1.0	2.7	2.6	2.9	3.0
18:2n-6	13.5±1.0	13.4±0.7	13.1±0.8	12.6±0.2	11.1	11.6	12.4	11.1
18:3n-6	0.8±0.4	0.9±0.5	0.8±0.4	0.7±0.1	0.4	0.4	0.5	0.5
18:3n-3	1.5±0.4	1.4±0.4	1.1±0.1	1.1±0.3	0.9	0.7	0.8	0.7
20:1	3.1±0.6	2.7±0.5	2.9±0.6	3.1±0.3	3.0	2.9	3.0	3.1
20:4n-6 (ARA)	1.5±0.8	4.4±1.0	4.9±0.9	5.4±0.9	0.9	5.0	7.8	9.9
20:5n-3 (EPA)	1.1±0.3	1.2±0.1	0.9±0.2	0.9±0.2	0.5	0.8	0.6	0.5
22:0	2.0±0.7	2.1±0.6	2.1±0.8	1.6±0.1	0.7	0.9	0.9	1.0
22:4n-6	0.5±0.3	1.6±0.8	1.5±0.7	1.2±0.1	0.4	1.4	2.1	3.1
22:5n-3	1.9±0.5	1.9±0.3	1.7±0.5	1.6±0.2	1.8	1.5	1.5	1.3
22:6n-3 (DHA)	7.2±1.3	7.7±1.2	7.5±1.2	6.4±0.9	7.5	8.0	7.9	6.1
SFA	32.5±0.9	31.1±1.5	30.5±1.0	30.4±0.6	28.7	27.6	24.8	24.6
MUFA	35.7±3.8	33.3±1.9	34.9±2.8	36.6±1.2	44.7	38.1	38.0	38.6
PUFA	31.7±4.5	35.5±2.3	34.4±2.5	33.4±1.4	26.6	33.2	37.3	36.6
(EPA+DHA)/ARA	6.0±1.7	2.0±1.4	1.7±1.4	1.3±1.1	9.0	1.8	1.1	0.7
EPA/ARA	0.8±0.3	0.3±0.1	0.2±0.2	0.2±0.2	0.6	0.2	0.1	0.1
DHA/ARA	5.2±1.4	1.8±1.2	1.5±1.3	1.2±0.9	8.4	1.6	0.6	0.6
ARA (µg g ⁻¹ DW)	2.4±0.8	6.0±2.3	8.2±1.9	10.4±2.6	3.1	15.3	22.5	26.3

Table 3
Average weight gain of fish fed with the ARA-supplemented diets for 14 days

ARA (mg g ⁻¹ feed)	Average weight gain (g)	
	Trial 1	Trial 2
0.7	0.02	0.05
12.5	0.02	0.08
17.5	ND	0.07
25	0.03	0.13
50	0.03	0.11

ND—not determined.

especially in liver lipids. The change in ARA proportion in liver lipids was accompanied by drastic changes in the ARA content in liver dry weight and by changes in the ratio of the n-3 VLC-PUFAs, DHA and EPA, to ARA. Similar but less dramatic changes were observed in fish sampled 8 days post feeding initiation (data not shown).

3.3. Effect of ARA enriched diet on fish growth and recovery from infection

Growth rates in the different treatment groups did not significantly change in the two experiments (Table 3). In the second trial (but not in the first) a weight gain was observed, accompanying the increased ARA levels. *Tetrahymena* sp. infection rate in fish fed with a diet enriched with 25 mg g⁻¹ ARA was significantly lower than in the groups fed with control or 12.5 mg g⁻¹ ARA-enriched diet, in both experiments (Fig. 1). Although not significantly different, a further increase in dietary ARA (50 mg g⁻¹) resulted in somewhat elevated infection rates.

4. Discussion

The majority of research on ARA in aquaculture feeds has been conducted either with pure ARA (Bessonart et al., 1999; Bell et al., 1994, 1995) or ARA-rich oil derived from the cultured filamentous fungus *M. alpina* (Koven et al., 2001, 2003; Harel et al., 2001, 2002). So far, algal-derived ARA has not been utilized in aquaculture. *P. incisa* is a unique oleaginous microalga, producing TAG that is extremely rich in ARA. Under N-starvation, the share of ARA reaches more than 55% of total fatty acids (Table 1). The ARA-rich TAG fraction was added to commercial fish feed, increasing its ARA content and altering its n-3/n-6 ratio (Table 1). The presence of β -carotene in the hexane extract may have served as an endogenous antioxidant, preventing the harmful effects of lipid peroxidation and deterioration of ARA in the feed. This β -carotene is

derived from the extraplastidial oil bodies that *P. incisa* forms under starvation simultaneously with the accumulation of ARA-rich TAG (Shrestha et al., 2004).

Guppies, being fresh water species, contain ARA in their lipids (Table 2). We found a significant impact of dietary ARA intake on ARA levels in the body and especially in liver tissues. ARA is one of the major fatty acid components of fish phospholipids, specifically phosphatidylinositol (Bell and Tocher, 1989), and dietary ARA significantly alters the fatty acid composition of liver phospholipids (Bell et al., 1994). Indeed, increasing ARA content in the feed drastically altered the proportion of ARA and its elongation product 22:4n-6, particularly in liver. These data indicate that ARA-rich TAG from *P. incisa* was efficiently digested and metabolized by the fish. The elevated ARA did not significantly affect the proportions of DHA and EPA,

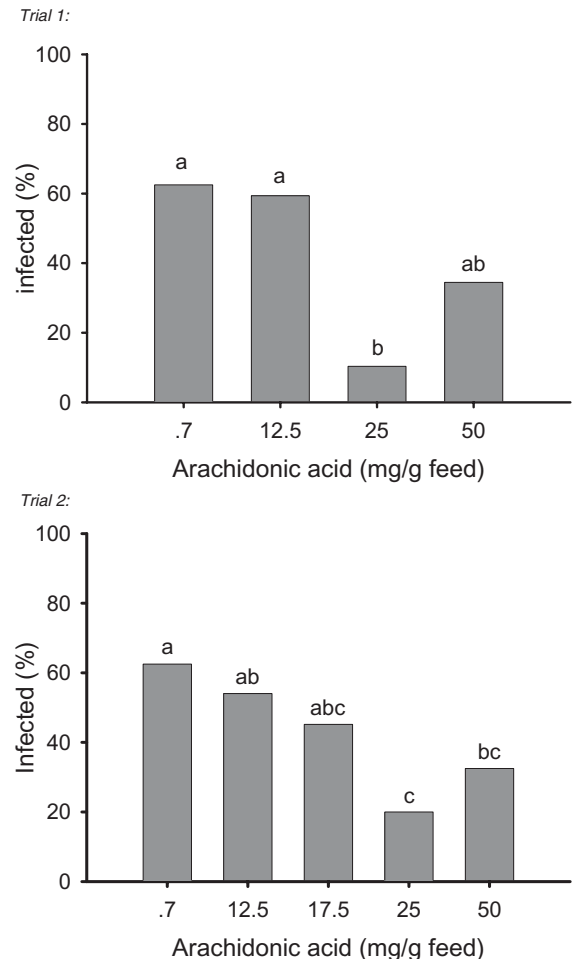


Fig. 1. Recovery from *Tetrahymena* sp. infection in guppies fed with the ARA-supplemented diets in the two trials. a, b and c denote significant differences in different treatments ($p < 0.05$).

suggesting that ARA does not compete with n-3 VLC-PUFA but rather substitutes shorter and less desaturated fatty acids within lipids.

Dietary ARA positively affected recovery of guppies from infection with *Tetrahymena* sp. An ARA content of 25 mg g⁻¹ produced the best results in terms of percentage of healthy fish observed after infection with the disease. A further increase in the ARA content to 50 mg g⁻¹ did not improve recovery and may even have reduced it. In fish, as in other invertebrates, ARA is the main fatty acid precursor of several highly active eicosanoids, which have important roles in inflammation and in the regulation of immunity (Bell and Sargent, 2003; Tocher, 2003). The level of dietary ARA determined the levels of prostaglandin PGE₂ and 6-ketoPGF₁ measured in homogenates of various organs of juvenile turbot fed with ARA (Bell et al., 1994). In brains, the concentration of PGE₂ was significantly elevated in fish fed the highest dietary ARA (Bell et al., 1994, 1995). Taking into account that the EPA/ARA ratio in cellular membranes is determined by the dietary intake, dietary ARA enhances the biosynthesis of eicosanoids with high immunoregulatory activity. It is possible that guppies fed with high levels of ARA may have exhibited an elevated synthesis of eicosanoids that induced an immune response against the infection. This suggestion is supported by the dramatic influence of ARA supplementation on EPA/ARA ratio in fish lipids, indicating that in the treatment group, ARA might be preferentially converted to high potency eicosanoids.

Dietary manipulations of ARA in the nutrition of fish larvae were shown to positively affect survival, growth and resistance to acute handling stress (Bessonart et al., 1999; Koven et al., 2001, 2003). Resistance to stress was achieved only when ARA was added prior to the stress event, while subsequent feeding with ARA did not affect stress-related mortality (Koven et al., 2001). The effect of dietary ARA on survival of fish larvae following exposure to chronic salinity stress was concentration-related, with maximal survival at intermediate ARA supplementation (Koven et al., 2003). Similar results were obtained in the present study. Although not significantly different, the highest dietary ARA resulted in reduced recovery. Koven et al. (2003) found that elevated dietary levels of ARA were associated with elevated cortisol levels and suggested that the reduced survival was caused by immunosuppression induced by the high cortisol. The chronic stress which was likely induced by *Tetrahymena* sp. infection, together with the high dietary ARA, may have similarly induced an enhanced secretion of cortisol, reducing

circulatory lymphocytes (Barton and Iwama, 1991) and subsequently reducing the fish's ability to recover from the infection.

Acquired protective immunity against the ciliated protozoan parasite *Ichthyophthirius multifiliis*, which produces external infection (skin and gills), was thoroughly studied in channel catfish (*Ictalurus punctatus*) and specific antibody production was shown to be protective and to induce recovery from infection (Wang et al., 2002; Maki and Dickerson, 2003). There is no information in the literature regarding protection and recovery from infection with *Tetrahymena* sp., but it is possible that similarly to *I. multifiliis*, an immobilizing antibody would be protective. ARA was effective in facilitating a change in circulating white blood cells in response to antigenic exposure (Harel et al., 2001). An altered n-3/n-6 PUFA ratio was shown to be important in facilitating an optimal immune response (Bell et al., 1996).

There are two factors that may have played a role in the enhanced recovery. One is the *Tetrahymena* sp. infection per se, and the other is the chronic stress induced by the parasite itself and possibly also by the conditions to which the fish were exposed during infection (Petri dishes) and recovery (1-L beakers). ARA may have positively affected the fish's immune response, but beyond a certain level, its negative effect on the chronic stress response may have reduced the fish's ability to recover from infection. It is also possible that at the higher levels, ARA negatively affects the immune system due to its concentration per se, the concomitant reduction in n-3/n-6 PUFA ratio or the immunosuppression caused by excessive synthesis of ARA-derived eicosanoids (Bell et al., 1995).

We did not observe any visible adverse effects in the fish, even at the higher level of dietary ARA, which produced a 10-fold increase in ARA liver content. However, high levels of malpigmentation were reported in Atlantic halibut and turbot larvae when fed an ARA-enriched diet (Estevez et al., 1999).

Carotenoid-facilitated immunological benefits were demonstrated in fish (Amar et al., 2004; Tachibana et al., 1997). The vitamin A precursor β -carotene was a component of the algal neutral lipid extract and was therefore present in experimental diets. At the effective concentration of ARA (25 mg g⁻¹ feed), the β -carotene content amounted to 0.11 mg g⁻¹ feed. Amar et al. (2004) reported that feeding rainbow trout, *Oncorhynchus mykiss*, with feed containing similar concentrations of β -carotene for a period of 9 weeks enhanced several components of the innate immune system, including serum alternative complement activity,

serum lysozyme activity and phagocytic rate of head kidney phagocytes. We cannot exclude the immunostimulative contribution of dietary β -carotene in the present study.

In conclusion, the supplementation of dietary ARA increased the recovery rate of guppies infected with *Tetrahymena* sp. We speculate that the enhanced level of ARA modulated the fish's immune response, improving recovery. Due to the small size of the guppy, we are restricted in the number of immunological assays that can be carried out, but examination of cortisol and eicosanoid levels and distribution of WBC following infection may shed light on the effect of ARA on the guppies immune response.

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