

## Short-term dietary supplementation with the microalga *Parietochloris incisa* enhances stress resistance in guppies *Poecilia reticulata*

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### Abstract

Two trials were conducted to determine the effects of dietary enrichments with the microalga *Parietochloris incisa*, rich in arachidonic acid (ARA), on stress resistance in guppies *Poecilia reticulata*. The microalga was added to commercial diets as a neutral lipid (NL) extract and its fractions or as broken cells. Experimental diets were applied for a period of 14 days. In trial 1, commercial diets were supplemented with NL (containing 25 mg ARA and 0.11 mg  $\beta$ -carotene  $g^{-1}$  feed), its triacylglycerol (TAG) fraction (containing 25 mg ARA  $g^{-1}$  feed and no  $\beta$ -carotene) and the  $\beta$ -carotene fraction (containing 0.11 mg carotenoid  $g^{-1}$  feed and minute amounts of ARA). Neutral lipid-fed fish demonstrated the highest resistance ( $P < 0.05$ ) to osmotic stress (32-ppt NaCl), followed by fish fed with diets supplemented with TAG and  $\beta$ -carotene alone, which were more resistant than control ( $P < 0.05$ ). In trial 2, fish fed diets supplemented with higher levels of broken alga (26.1 mg ARA  $g^{-1}$  feed) were more resistant ( $P < 0.05$ ) to stress as compared with fish fed lower ARA (16.3 mg  $g^{-1}$ ) or an unsupplemented control diet. We suggest a dietary supplementation with broken *P. incisa* cells to enhance stress resistance in guppies before a stressful event.

**Keywords:** arachidonic acid,  $\beta$ -carotene, guppy, microalgae, *Parietochloris incisa*, stress

### Introduction

Mortalities due to stress events routinely occur in aquaculture and lead to substantial economic losses.

There are no adequate practical solutions to common stresses experienced by fish during the culture period (such as handling, water quality fluctuations, etc.). In the ornamental fish production, exposure to stresses does not end in the production period at the aquaculture farm (unlike food fish). Their marketing involves shipment of densely packed fish, stressful conditions that often last for about 2 days. At their new location, they are often exposed to new conditions and are relocated several times until reaching their final destination, often a home aquarium. The new environment poses environmental challenges and also exposure to different infectious diseases. The existing practice of decision-making regarding the fish health status is based on visual observation that is unreliable; thus, fish with low stress resistance are detected only following postshipment morbidity and mortality. A short-duration salinity stress test was developed by Lim, Dhert, Chew, Dermaux, Nelis and Sorgeloos (2002) to quantitatively evaluate the guppy's resistance before a stressful condition, such as shipment. This stress test was suggested to be more stable than exposure to other parameters such as temperature, pH and ammonia (Lim, Dhert & Sorgeloos 2003).

Development of effective means to improve stress and disease resistance in ornamental fish, such as through immunostimulation, is of great importance. Diets that lead to more successful adaptation and higher survival may be a valuable resource to the ornamental fish culturist.

Microalgae are an important live feed source for aquaculture due to their high nutritional values

attributed to the contents of protein, vitamins, carotenoids and highly unsaturated C20 and C22 fatty acids (HUFA). Microalgae-based diets have been shown to have positive effects on the immune system of developing fish larvae (Reitan, Rainuzzo, Oie & Olsen 1997).

The green microalga *Parietochloris incisa* is the richest plant source for the HUFA arachidonic acid (ARA) (Bigogno, Khozin-Goldberg, Boussiba, Vonshak & Cohen 2002; Khozin-Goldberg, Bigogno, Shrestha & Cohen 2002). This microalga was also found to deposit ARA-rich triacylglycerols (TAG) along with  $\beta$ -carotene in extraplastidial lipid globules (Solovchenko, Khozin-Goldberg, Didi-Cohen, Cohen & Merzlyak 2008), both of which are enriched in the neutral lipid (NL) extract of the microalga. Thus far, research on dietary supplementation with ARA in aquaculture has examined the use of commercial ARA (Bell, Tocher & Sargent 1994; Bessonart, Izquierdo, Salhi, Hernandez-Cruz, Gonzalez & Fernandez-Palacios 1999) or ARA-rich lipid fractions derived from the cultured filamentous fungus *Mortierella alpina* (Harel, Gavasso, Leshin, Gubernatis & Place 2001; Koven, Barr, Lutzky, Ben-Atia, Weiss, Harel, Behrens & Tandler 2001; Koven, van Anholt, Lutzky, Ben Atia, Nixon, Ron & Tandler 2003). In our previous work, feeding ornamental fish, guppies *Poecilia reticulata*, with the NL from *P. incisa* significantly reduced infection with the protozoan parasite *Tetrahymina* sp. (Khozin-Goldberg, Cohen, Pimenta-Leibowitz, Nechev & Zilberg 2006).

Dietary supplementation with HUFA can alter the stress response in fish and many other physiological processes. Because of the well-documented importance of n-3 HUFA in fish nutrition for optimal growth, development and reproduction (Sargent, Henderson & Tocher 1989; Lall 2000), fish feed in aquaculture is enriched in eicosapentaenoic acid (EPA, 20:5n-3) and docosahexaenoic acid (DHA, 22:6n-3), but is poor in ARA. Recently, it was proposed that such ARA-poor diets might suppress the capacity of fish to cope with stressful events and inhibit the development of the immune system (Harel *et al.* 2001; Bell & Sargent 2003). ARA is the primary substrate in eicosanoid biosynthesis: the 2-series prostaglandins (PGs) and thromboxanes and the 4-series leukotrienes, which regulate many physiological processes, including homeostasis, reproduction, immune and inflammatory responses (Abbas, Lichtman & Pober 1994; Tocher 2003). Eicosapentaenoic acid competes with ARA for the same set of enzymes, leading to the production of highly active eicosanoids with a different range of physiological activities.

Thus, the dietary ratio of n-3:n-6 HUFA influences the pattern and properties of the eicosanoids formed (Bell & Sargent 2003).

Several studies conducted with marine fish species demonstrated that dietary ARA supplementation may improve growth and could be effective in moderating the stress response, thus improving survival following exposure to handling and transport (Bessonart *et al.* 1999; Harel *et al.* 2001; Koven *et al.* 2001; Van Anholt, Koven, Lutzky & Wendelaar Bonga 2004). Studies on ARA-mediated stress response in freshwater fish are limited. Because freshwater fish can produce HUFA from dietary C18 PUFA, it is generally accepted that their requirements for dietary supplementation with HUFA are lower than in marine fish. In the freshwater white bass *Morone chrysops*, HUFA requirements are similar to that of marine fish and optimal dietary DHA and ARA ratios positively affected growth and tissue fatty acid composition during larval development and metamorphosis (Harel, Lund, Gavasso, Herbert & Place 2000). Arachidonic acid was suggested to be essential in the maturation and spawning of freshwater ornamental fish (Tamaru, Ako & Paguirigan 1997) and was suggested to play an integral part in the reproductive mechanism of swordtail, *Xiphophorus helleri* (Ling, Kuah, Tengku Muhammad, Kolkovski & Shu-Chien 2006). In a study conducted at our laboratory, feeding guppy fry with *P. incisa*-enriched *Artemia* increased their survival to handling stress (Nath, Khozin-Goldberg, Cohen, Boussiba & Zilberg 2008).

Dietary carotenoids have been reported to positively affect growth (Torrissen 1984; Karino & Haijima 2004), broodstock performance (Verakunpiriya, Watanabe, Mushiake, Kawano, Kobayashi, Hasegawa, Kiron, Satoh & Watanabe 1997; Karino & Haijima 2004) and disease resistance in fish (Latscha 1990; Tachibana, Yagi, Hara, Mishima & Tsuchimoto 1997; Kolluru, Grether, South, Dunlop, Cardinali, Liu & Carapiet 2006). Dietary intake of  $\beta$ -carotene from the microalga *Dunaliella salina* and astaxanthin from the yeast *Phaffia rhodozyma* enhanced factors of the innate immune system in rainbow trout *Oncorhynchus mykiss*, including serum alternative complement activity and phagocytic activity (Amar, Kiron, Satoh & Watanabe 2004). In this study, lysozyme was elevated only after  $\beta$ -carotene supplementation. Microalgae-based diets affected the immunocompetence of guppies in a field experiment where the load of the ectoparasite *Gyrodactylus turnbulli* was significantly lower in fish raised on a diet containing an intermediate level of carotenoids as compared with the

trace- and high-carotenoid-fed groups (Kolluru *et al.* 2006). Dietary supplementation of  $\beta$ -carotene-rich *D. salina* reduced lipid peroxidation in larvae of *Solea senegalensis* (Canavate, Prieto, Zerolo, Sole, Sarasquete & Fernandez-Diaz 2007).

In the present work, we investigated the effect of short-term (14 days) dietary supplementation of guppies with extracts or broken biomass of the ARA-rich green microalga *P. incisa* on their resistance to stress. Exposure to a high osmotic stress test was used (after Lim *et al.* 2002), standardized to determine fish's stress tolerance (Lim *et al.* 2003). The positive effect of  $\beta$ -carotene from the algal extract was also revealed.

## Materials and methods

### Algal cultivation

The microalga *P. incisa* comb. Nov. (Trebouxiophyceae, Chlorophyta) (Watanabe, Hirabashi, Boussiba, Cohen, Vonshak & Richmond 1996) was cultivated on BG 11 medium (Stanier, Kunisawa & Cohen-Bazir 1971) in 6 cm wide, 1000 mL glass columns that 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% CO<sub>2</sub> in air. Illumination (175  $\mu\text{mol photon m}^{-2} \text{s}^{-1}$ ) 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 a nitrogen-free BG 11 medium where sodium nitrate was omitted and 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 and biomass content of ARA.

### Preparation of supplemented feeds

#### Neutral lipid extract-supplemented feed

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 to obtain the NL extract containing mainly TAG and  $\beta$ -carotene. The extract was clarified by filtration, evaporated under vacuum at 30 °C and stored under an argon atmosphere at –20 °C. The extract was coated onto the commercial guppy feed (Tropical Orange, Tzemah, Israel) to achieve 24.9 mg ARA g<sup>-1</sup> feed. The fatty acid compo-

sition and content of the modified feed were analysed by gas chromatography (GC) as described below.

#### TAG- and $\beta$ -carotene-supplemented feed

A separation of NL extract into two major components was performed using column chromatography (19.5 × 2 cm; Silica gel 60, particle size 0.040–0.063 mm, Merck, Darmstadt, Germany).  $\beta$ -Carotene and TAG were resolved using sequential elution with *n*-hexane and an *n*-hexane:ethanol mixture (98.5:1.5, v/v) respectively. Fractions were evaporated and stored under an argon atmosphere at –20 °C. The purity of the fractions was examined by thin-layer chromatography (TLC) in a solvent system consisting of petroleum ether:diethyl ether:acetic acid (80:20:1, v/v/v). Different amounts of the TAG and  $\beta$ -carotene fractions were added to commercial feeds, to produce 25.7 ARA mg g<sup>-1</sup> feed and 0.11 carotenoid mg g<sup>-1</sup> feed in TAG- and  $\beta$ -carotene-enriched diets respectively. The content of  $\beta$ -carotene in enriched diets was adjusted to the levels obtained in feeds supplemented with NL (0.11 mg g<sup>-1</sup>). The  $\beta$ -carotene content in extracts and feeds was determined spectrophotometrically using an absorption coefficient of 2592 at 450 nm in petroleum ether (Britton 1995). Feed samples were extracted with acetone and  $\beta$ -carotene was isolated from evaporated extracts by *n*-hexane elution on Bond-Elute silica cartridges (Varian, Palo Alto, CA, USA). TAG-enriched diets did not contain the increased amounts of  $\beta$ -carotene.

#### Broken-algae-supplemented feed

To prepare broken cell algal powder, a concentrated algal cell suspension was prepared in double-distilled water (pH adjusted to 9 with 1 M NaOH) and heated at 80 °C for 1 h under an argon atmosphere. Cells were broken by passing three times through a grinding bead mill (Dyno-Mill, WAB, Switzerland), freeze-dried and stored at –20 °C. Dried broken algal powder was mixed with commercial fish feed powder and distilled water and feed was repelleted using a HL5 extruder (Loser, Germany), to achieve 16.3 and 26.1 ARA mg g<sup>-1</sup> feed. The fatty acid composition and content of the modified feed were analysed by GC as described below.

### Fish

Male guppies, *P. reticulata* (average weight 0.5 g; 2.3–2.8 cm), were obtained from a commercial aquaculture farm in the Arava valley, Israel. Upon arrival, fish

were stocked in 130 L holding tanks and kept there for at least 2 weeks to acclimate before commencement of the experiment. Tanks were supplied with biological filters and cleaned every other day by siphoning and replacing 10% of the water with dechlorinated fresh water, treated with 50 mg L<sup>-1</sup> of sodium thiosulphate pentahydrate (Willam Blythe, Accrington, UK). Commercial guppy feed was applied at 2% body weight day<sup>-1</sup>. Water quality parameters, including dissolved oxygen (> 80%), temperature (26 °C), ammonia (0–0.2 mg L<sup>-1</sup>) and nitrite (0 mg L<sup>-1</sup>), were monitored one to two times a week. Oxygen levels were measured using a YSI 52-dissolved oxygen meter (YSI, Yellow Springs, Oh, USA). Ammonia and nitrite were measured using visocolor kits (Macherey-Nagel, Duren, Germany). Experimental procedures were in accord with the ethical care and use of animals in experimentation, authorized by our institutional committee.

## Experimental design

### Trial 1

Guppies were stocked in 30 L aquaria, 50 fish-aquarium<sup>-1</sup> and preacclimated for at least 1 week. Fish were fed commercial fish feed supplemented with NL extract, TAG and  $\beta$ -carotene-rich fractions, containing, 24.9, 25.7 and 0.6 mg ARA g<sup>-1</sup> feed respectively. Unsupplemented feed (0.2 mg ARA g<sup>-1</sup>) was used as control. The experiment was conducted in duplicate. Six fish from each treatment group were sampled for fatty acid analysis at day 15 after initiation of feeding. Anaesthetized fish (clove oil, 250  $\mu$ L L<sup>-1</sup>) were dissected, the gastrointestinal tract (GIT) was removed and livers were separated and pooled. Bodies of six fish and pooled livers (pooled from six fish) were weighed, frozen, freeze-dried and kept at -20 °C until analysed for fatty acid composition and content. Experimental diets were applied for 14 days, followed by a salinity stress test.

### Trial 2

Guppies were stocked in 30 L aquaria, 50 fish-aquarium<sup>-1</sup>, and preacclimated for at least 1 week. Fish were fed commercial fish feed supplemented with broken-whole algal cells containing 16.3 and 26.1 ARA mg g<sup>-1</sup> feed or unsupplemented feed for control (0.2 ARA mg g<sup>-1</sup>). The experiment was conducted in duplicate. Six fish from each treatment group were sampled for fatty acid analysis at day 15 after initiation of feeding. Anaesthetized fish (clove

oil, 250  $\mu$ L L<sup>-1</sup>) were dissected, the GIT was removed and livers were separated and pooled. Bodies of six fish and pooled livers (pooled from six fish) were weighed, frozen, freeze-dried and kept at -20 °C until analysed for fatty acid composition and content. Experimental diets were applied for 14 days, followed by a salinity stress test.

## Fatty acid analysis

Fatty acid composition and content were determined in freeze-dried algal biomass, fish feed, fish tissue samples and lipid extracts. Samples were trans-methylated with 2% H<sub>2</sub>SO<sub>4</sub> in dry methanol, under an argon atmosphere at 80 °C for 1.5 h in the presence of 10% toluene (v/v) to facilitate solubilization of TAG. The reaction was terminated by adding distilled water. Fatty-acid methyl esters (FAME) were extracted with *n*-hexane. GC analysis of FAME was performed on a Hewlett Packard 5890 gas chromatograph with a Supelcowax 10 fused silica capillary column (30 m  $\times$  0.32 mm) (Supelco, Bellefonte, PA, USA), using a temperature gradient from 185 to 210 °C with a linear increase of 10 °C min<sup>-1</sup> and helium as a carrier gas. Fatty-acid methyl esters were identified by co-chromatography with authentic standards (Sigma Chemical, St. Louis, MO, USA) and FAME of fish oil (Larodan Fine Chemicals, Malmö, Sweden), by comparison of their equivalent chain length (Ackman 1969). The data shown represent mean values with a range < 5% error for major peaks (over 10% of fatty acids) and 15% error for minor peaks.

## Stress test

A high osmotic stress test was performed by a protocol modified from Lim *et al.* (2002). Fish were subjected to 32 ppt NaCl solution, made up of pre-aerated tap water and NaCl, in 1 L glass beakers filled with 500 mL of saline solution (10 fish beaker<sup>-1</sup>). Fish from each treatment aquarium were tested in four (Trial 1) or three (Trial 2) separate beakers. Cumulative mortality was reported at 3-min intervals over 120–135-min periods. Stress resistance was expressed as the stress index, calculated as the average sum of cumulative mortality value in replicate beakers, as described by Lim *et al.* (2002).

## Statistical analysis

Mortality of guppies in salinity stress test was expressed as the stress index (Lim *et al.* 2002) and as cumulative mortality. The stress index was analysed by

ANOVA and cumulative mortality was analysed by the proportional hazard (Cox) regression test for survival data (STATISTICA AX PROGRAM). Fatty acid body contents were analysed by a one-way ANOVA. No statistical analysis was conducted for liver fatty acid composition, as due to the small sample quantity, livers of six fish had to be pooled and analysed as a single sample. STATISTICA (version 7.0) was used for all statistical analyses. Data were considered to be significantly different at  $P < 0.05$ .

## Results

### Feed formulations

*Parietochloris incisa* was added to the diets either as the NL extract, its fractions (Trial 1) or as broken cells (Trial 2). A protocol for algal cell breakage allowing efficient recovery of ARA-rich TAG was developed. To deactivate lipases and prevent ARA oxidation, we explored a thermal treatment of the cell suspension to 80 °C at pH 9 under an argon atmosphere and dim light. Under these conditions, TAG and FFA accounted for 98% and 2% in NL extract of broken cells respectively. Arachidonic acid amounted to 61% of the total fatty acids (TFA) (Table 1), in comparison with 58% in unbroken cells (not shown), indicating that ARA was not deteriorated during the breakage.

In trial 1, dietary ARA was adjusted to about 25.0 mg g<sup>-1</sup> feed by supplementing commercial feed

**Table 1** Fatty acid composition of the NL extract, TAG fraction and broken algae, used in the supplemented-feed preparation

Fatty acid	Fatty acid composition (% of TFA)		
	NL	TAG	Broken algae
16:0	8.7	8.0	8.8
16:1n-7	0.1	0.4	0.4
18:0	2.6	2.6	2.6
18:1n-9	12.2	11.6	9.2
18:2n-6	7.5	8.9	9.2
18:3n-6	0.6	0.6	0.7
18:3n-3	0.2	0.2	0.5
20:1	0.2	0.3	0.2
20:4n-6 (ARA)	62.1	61.2	60.5
20:5n-3 (EPA)	0.6	0.8	1.1
22:0	0.1	0.4	0.4
Other*	5.1	5.0	6.4

\*Other fatty acids include 14:0, 16:1n-9, 18:1n-7, 20:0, 20:2n-6 and 20:3n-6.  
ARA, arachidonic acid; EPA, eicosapentaenoic acid; NL, neutral lipid; TAG, triacylglycerols; TFA, total fatty acid.

**Table 2** Fatty acid composition and content and β-carotene content of commercial guppy feed and feeds supplemented with the NL extract, TAG or β-carotene fractions, used in trial 1

Fatty acid	Fatty acid composition (% of TFA)			
	Control	NL	TAG	β-Carotene
14:0	2.5	1.8	1.8	2.7
16:0	19.8	14.7	15.2	19.4
16:1n-7	4.0	2.5	2.5	4.2
18:0	3.7	3.3	3.4	3.9
18:1n-9	22.1	16.1	17.1	20.4
18:2n-6	23.1	17.6	17.2	23.2
18:3n-6	0.1	0.3	0.3	0.2
18:3n-3	2.1	1.4	1.2	2.0
20:1	4.3	2.7	2.8	4.2
20:4n-6 (ARA)	0.3	24.7	24.0	1.0
20:5n-3 (EPA)	4.8	3.3	3.2	4.6
22:0	4.3	3.0	2.6	3.9
22:6n-3 (DHA)	6.8	4.6	4.6	6.7
EPA+DHA/ARA	39.0	0.3	0.3	11.3
EPA/ARA	16.1	0.1	0.1	4.4
DHA/ARA	22.9	0.2	0.2	6.9
Other*	2.1	4.0	4.1	4.1
ARA (mg g <sup>-1</sup> )	0.2	24.9	25.7	0.6
EPA (mg g <sup>-1</sup> )	3.1	3.3	3.4	3.0
DHA (mg g <sup>-1</sup> )	4.4	4.6	4.9	4.3
TFA (mg g <sup>-1</sup> )	65.0	100.9	107.1	64.5
β-Carotene (mg g <sup>-1</sup> )	0.06	0.11	NQ†	0.11

\*Other fatty acids include 16:1n-9, 18:1n-7, 20:0, 20:2n-6 and 20:3n-6.

†NQ, not quantified since TAG fraction used for enrichment was found to be free of β-carotene.

ARA, arachidonic acid; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; NL, neutral lipid; TAG, triacylglycerols; TFA, total fatty acid.

with either NL or its TAG fraction. In feed supplemented with the β-carotene fraction (also obtained from the NL extract), ARA was only slightly elevated in comparison with the unsupplemented control feed (0.2 and 0.6 mg g<sup>-1</sup> feed, respectively, and TFA levels were not affected; Table 2); the β-carotene content was 0.11 mg g<sup>-1</sup> feed in the NL- and β-carotene supplemented diets and 0.06 mg g<sup>-1</sup> feed in the control diet (Table 2). The TAG-supplemented feed did not contain elevated levels of carotenoid, because the TAG fraction did not contain β-carotene (not shown).

In trial 2, diets were supplemented with broken algae and consisted of unsupplemented control (0.2 ARA mg g<sup>-1</sup>), low ARA (16.3 mg g<sup>-1</sup>) and high ARA (26.1 mg g<sup>-1</sup>) diets (Table 3). The TFA contents of supplemented feeds increased mainly due to the increase in the ARA content.

**Table 3** Fatty acid composition and content of commercial guppy feed (control) and broken-alga supplemented diets, used in trial 2

Fatty acid	Fatty acid composition (% of TFA)		
	Control (unsupplemented)	Supplemented-low	Supplemented-high
14:0	2.5	1.9	1.5
16:0	19.8	16.5	15.5
16:1n-7	4.0	2.8	2.4
18:0	3.7	3.5	3.4
18:1n-9	22.1	19.2	18.7
18:2n-6	23.1	19.1	18.1
18:3n-6	0.1	0.4	0.4
18:3n-3	2.1	1.5	1.4
20:1	4.3	2.7	2.2
20:4n-6 (ARA)	0.3	21.3	27.0
20:5n-3 (EPA)	4.8	3.0	2.4
22:0	4.3	2.5	2.1
22:6n-3 (DHA)	6.8	4.0	3.3
(EPA+DHA)/ARA	39.0	0.3	0.2
EPA/ARA	16.1	0.1	0.1
DHA/ARA	22.9	0.2	0.1
Other*	2.1	1.6	1.6
ARA (mg g <sup>-1</sup> )	0.2	16.3	26.1
EPA (mg g <sup>-1</sup> )	3.1	2.3	2.3
DHA (mg g <sup>-1</sup> )	4.4	3.1	3.2
TFA (mg g <sup>-1</sup> )	65.0	76.6	96.5
β-Carotene (mg g <sup>-1</sup> )	0.06	0.072	0.102

\*Other fatty acids include 16:1n-9, 18:1n-7, 20:0, 20:2n-6 and 20:3n-6.

ARA, arachidonic acid; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; TFA, total fatty acid.

(Correction added on 28 July 2009, after first online publication: Parts of the table heading were removed to make the data clearer.)

### Fatty acid profiles of fish samples

Fatty acid analysis of fish tissues (whole body excluding the GIT and liver) showed that ARA-supplemented diets significantly altered the FA composition in both experiments. In trial 1, the ARA proportion of TFA and DW content in bodies and livers increased in fish fed NL and TAG supplemented diets, with no significant difference in ARA accumulation between the two treatments for bodies ( $P < 0.05$ ; Table 4), but higher accumulation of ARA (as % of TFA) was measured in the livers of NL-enriched fish. The increase in the proportion of ARA was accompanied by a drastic decrease in the ratios of DHA and EPA to ARA (Table 4). The proportions of EPA and DHA in the liver and body lipid changed little during the experimental period (except for some decrease in TAG-supplementation), indicating that ARA was substituting more saturated and shorter fatty acids, rather than HUFA.

Supplementation with β-carotene did not alter the FA composition of fish tissues.

Similar results concerning ARA accumulation in fish tissues were obtained in trial 2 utilizing broken algal cells. Liver FA composition in fish that were dietary supplemented with broken algal cells was more drastically affected by the dietary manipulations as compared with the fish body FA composition (Table 5). Feeding with the high ARA diet resulted in an eight-fold increase in the proportion of ARA in the liver (0.7% and 5.6% in control and high ARA diets respectively). Arachidonic acid proportion of TFA and content in fish bodies significantly increased, commensurate with the increase in dietary ARA. Dietary ARA was elongated to 22:4n-6, as indicated by the increase in its share with the increase in the amount of ARA in the diets, both in the body and in liver lipids (Table 5).

### Effect of *P. incisa* supplementation on the resistance of guppies to high osmotic stress

In all treatments, fish behaved normally and showed high acceptance of the enriched diets. No mortality was reported during the feeding periods nor any other apparent adverse effects such as behavioral alteration, changes in the body coloration or growth rate. Moreover, fish did not show any symptoms of distress. Feeding with supplemented feeds at 25.0 mg ARA g<sup>-1</sup> from NL, TAG fraction of NL (Trial 1) or broken alga (Trial 2), significantly ( $P < 0.05$ ) enhanced guppies' resistance to high salinity stress, as compared with the control (Figs 1 and 2). Feeding with ARA at 16.3 mg g<sup>-1</sup> feed from broken alga (Trial 2) did not affect the stress resistance of guppies (Fig. 2). In addition, feeding with β-carotene-enriched feed at 0.11 mg g<sup>-1</sup>, same as the carotenoid amount in the NL-enriched feed, enhanced ( $P < 0.05$ ) guppies' resistance to high salinity stress as compared with the control (Fig. 1). There was no significant difference between the effects of β-carotene- and TAG-enriched diets.

### Discussion

Arachidonic acid amounted to about 60% of TFA in the extracts and biomass of *P. incisa* used for feed enrichments in the present work. Aiming to avoid usage of solvents for lipid extraction, conditions that allowed the production of ARA-rich feed supplements

**Table 4** Fatty acid composition and ARA content of body and liver (pooled from six fish) in guppies fed for 14 days with diets supplemented with NL extract, TAG and  $\beta$ -carotene fractions from *Parietochloris incisa* (trial 1)

Fatty acid	Fatty acid composition (% of TFA)							
	Body				Liver			
	Control	NL	TAG	$\beta$ -carotene	Control	NL	TAG	$\beta$ -Carotene
14:0	1.8 ± 0.3	1.6 ± 0.2	1.8 ± 0.1	1.9 ± 0.2	1.4	1.0	1.3	1.5
16:0*	19.1 ± 2.0 <sup>a</sup>	18.2 ± 1.3 <sup>a</sup>	18.4 ± 1.5 <sup>a</sup>	22.1 ± 3.9 <sup>b</sup>	14.7	12.0	14.4	14.4
16:1n-7	5.0 ± 0.6	4.7 ± 0.6	4.5 ± 0.6	4.6 ± 0.2	4.9	3.9	4.4	4.5
18:0*	8.4 ± 1.5 <sup>a</sup>	8.1 ± 1.1 <sup>a</sup>	8.8 ± 2.9 <sup>a</sup>	11.2 ± 3.8 <sup>b</sup>	7.6	7.2	6.8	7.7
18:1n-9	29.5 ± 1.4	29.8 ± 2.7	29.7 ± 4.0	27.7 ± 4.3	38.2	34.9	39.9	37.2
18:2n-6	10.1 ± 0.8	9.4 ± 0.8	9.5 ± 0.7	8.6 ± 1.0	10.8	10.5	10.1	9.8
18:3n-6	0.5 ± 0.1	0.5 ± 0.1	0.5 ± 0.1	0.6 ± 0.2	0.6	0.5	0.6	0.7
18:3n-3	1.3 ± 0.4	1.5 ± 0.5	1.2 ± 0.2	1.3 ± 0.3	0.9	0.9	0.9	1.0
20:1	2.4 ± 0.4	2.5 ± 0.3	3.1 ± 1.8	2.9 ± 0.8	2.9	3.4	3.0	3.0
20:4n-6 (ARA)*	1.3 ± 0.4 <sup>a</sup>	3.9 ± 1.2 <sup>b</sup>	3.5 ± 1.1 <sup>b</sup>	1.1 ± 0.3 <sup>a</sup>	1.5	6.9	4.4	1.9
20:5n-3 (EPA)	1.3 ± 0.3	1.2 ± 0.2	1.1 ± 0.2	1.3 ± 0.2	0.7	0.7	0.6	1.0
22:0	1.1 ± 0.3	1.0 ± 0.1	1.0 ± 0.1	0.9 ± 0.1	0.6	0.5	0.6	0.6
22:4n-6	0.6 ± 0.2	0.9 ± 0.2	0.8 ± 0.2	0.4 ± 0.1	0.5	2.1	1.3	0.5
22:5n-3	2.4 ± 0.3	2.1 ± 0.3	2.1 ± 0.3	2.0 ± 0.3	1.9	1.7	1.6	1.9
22:6n-3 (DHA)	12.5 ± 1.0	12.1 ± 1.8	11.7 ± 2.0	10.9 ± 1.8	11.0	10.7	8.0	12.1
EPA+DHA/ARA*	10.7 ± 2.9 <sup>a</sup>	3.4 ± 1.0 <sup>b</sup>	3.6 ± 1.1 <sup>b</sup>	11.2 ± 2.9 <sup>a</sup>	7.7	1.7	1.9	6.7
EPA/ARA	9.7 ± 2.6 <sup>a</sup>	3.1 ± 0.9 <sup>b</sup>	0.3 ± 0.1	1.2 ± 0.3	0.4	0.1	0.1	0.5
DHA/ARA*	1.0 ± 0.4	0.3 ± 0.1	3.3 ± 1.0 <sup>b</sup>	10.0 ± 2.7 <sup>a</sup>	7.3	1.6	1.8	6.2
Other†	2.7	2.5	2.3	2.5	2.0	3.1	2.0	2.1
ARA (mg g <sup>-1</sup> DW)*	0.5 ± 0.2 <sup>a</sup>	1.2 ± 0.3 <sup>b</sup>	1.4 ± 0.4 <sup>b</sup>	0.5 ± 0.3 <sup>a</sup>	0.1	2.9	4.2	0.3

Values represent average ± SD.

\*Statistical analysis of variance (ANOVA) was conducted. Significance was considered at  $P < 0.05$ . a, b and c, different letters denote significant differences in different treatments.

†Other fatty acids include 16:1n-9, 18:1n-7 and 20:0.

ARA, arachidonic acid; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; NL, neutral lipid; TAG, triacylglycerols; TFA, total fatty acid. (Correction added on 28 July 2009, after first online publication: Parts of the table heading were removed to make the data clearer.)

**Table 5** HUFA composition and ARA content of body and liver (pooled from six fish) of guppies fed with broken-alga supplemented diets for 14 days (trial 2)

Fatty acid	Fatty acid composition (% of TFA)					
	Body			Liver		
	Control (unsupplemented)	Supplemented low	Supplemented high	Control	Low	High
20:4n-6 (ARA)*	1.0 ± 0.1 <sup>a</sup>	3.2 ± 0.7 <sup>b</sup>	4.2 ± 0.9 <sup>c</sup>	0.7	4.1	5.6
20:5n-3 (EPA)	1.6 ± 0.1	1.4 ± 0.2	1.4 ± 0.1	0.7	0.6	0.8
22:0	1.6 ± 0.3	1.4 ± 0.2	1.5 ± 0.1	0.9	0.8	1.2
22:4n-6*	0.4 ± 0.0 <sup>a</sup>	0.8 ± 0.2 <sup>b</sup>	1.5 ± 0.7 <sup>c</sup>	0.5	1.2	1.8
22:5n-3*	3.2 ± 0.5 <sup>a</sup>	2.5 ± 0.1 <sup>b</sup>	1.1 ± 0.3 <sup>c</sup>	2.0	1.6	2.3
22:6n-3 (DHA)	13.3 ± 0.8	12.4 ± 1.7	12.5 ± 0.9	9.3	8.8	10.1
(EPA+DHA)/ARA*	14.9 ± 1.2 <sup>a</sup>	4.5 ± 1.1 <sup>b</sup>	3.4 ± 0.9 <sup>b</sup>	14.2	2.3	1.9
EPA/ARA*	1.6 ± 0.2 <sup>a</sup>	0.5 ± 0.1 <sup>b</sup>	0.3 ± 0.1 <sup>b</sup>	0.9	0.1	0.1
DHA/ARA*	13.4 ± 1.1 <sup>a</sup>	4.0 ± 1.0 <sup>b</sup>	3.1 ± 0.8 <sup>b</sup>	13.2	2.2	1.8
ARA (mg g <sup>-1</sup> DW)*	0.4 ± 0.1 <sup>a</sup>	1.1 ± 0.2 <sup>b</sup>	1.6 ± 0.5 <sup>c</sup>	0.5	3.5	4.4

Values represent average ± SD.

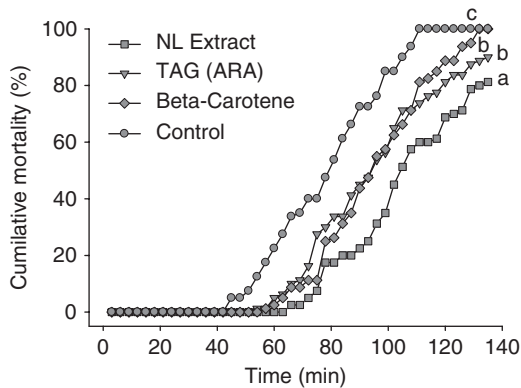
\*Statistical analysis of variance (ANOVA) was conducted. Significance was considered at  $P < 0.05$ . a, b and c, different letters denote significant differences in different treatments.

ARA, arachidonic acid; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; TFA, total fatty acid.

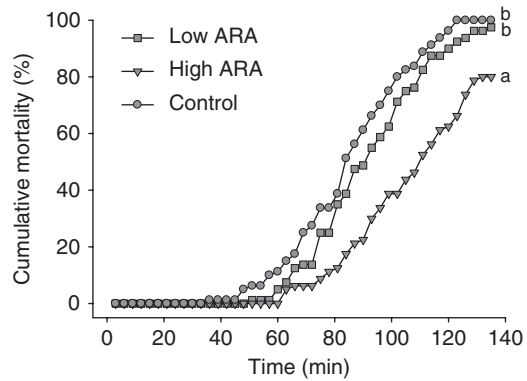
(Correction added on 28 July 2009, after first online publication: Parts of the table heading were amended to make the data clearer.)

from broken *P. incisa* cells were developed. The micro-alga possesses a very strong cell wall and high endogenous lipolytic activities, thus complicating cell breakage and recovery of intact TAG (Shrestha, Cohen, Khalilov, Khozin-Goldberg & Cohen 2004). Degradation of TAG was prevented by incubation of cell suspensions at pH 9 and at 80 °C before cell breakage. Similar, thermal treatment procedures are used to preserve the high ARA and lipid content in the biomass of *M. alpina* during harvesting (Ratledge, Streekstra, Cohen & Fichtali 2005).

Supplemented diets (except for the  $\beta$ -carotene enrichment) attained significantly elevated ARA levels and altered n-3/n-6 HUFA ratios in comparison with the control feed (Tables 2 and 3). The TFA content in the diet was increased, mainly due to the increase in the ARA content. Selection of the dietary ARA levels to be tested was based on our previous results on the effect of supplementation with the NL extract from *P. incisa* (in the range of 12.5–50 ARA mg g<sup>-1</sup> feed) on guppies' recovery from infection with the protozoan parasite *Tetrahymena* sp. (Khozin-Goldberg et al. 2006). In two separate trials, dietary ARA supplementation at 25 mg g<sup>-1</sup> significantly increased fish recovery from infection, as compared with the control, higher and lower dietary ARA levels. Similarly, supplementation with 25 mg ARA g<sup>-1</sup> feed, obtained from the NL extract, TAG or broken cells, significantly increased guppies' resistance to high osmotic stress (Figs 1 and 2). The diet with lower levels of broken alga (16 mg ARA g<sup>-1</sup>) was unable to enhance stress



**Figure 1** Trial 1: Effect of feeding guppies with 25 ARA mg g<sup>-1</sup> supplemented diets as a neutral lipid (NL) extract or triacylglycerols (TAG) and a diet supplemented with  $\beta$ -carotene from *Parietochloris incisa*, on resistance to high-salinity stress. a, b and c, different letters denote significant differences between different treatments ( $P < 0.05$ ).



**Figure 2** Trial 2: Effect of feeding guppies with *Parietochloris incisa* supplemented diets as broken algal cells, on resistance to high-salinity stress. Diets included unsupplemented control, low ARA (16.3 mg g<sup>-1</sup>) and supplemented high ARA (26.1 mg g<sup>-1</sup>). a and b, different letters denote significant differences between different treatments ( $P < 0.05$ ).

resistance, and yet it contained high levels of ARA and an elevated  $\beta$ -carotene level. We suggest that a certain threshold apparently exists for both active components to induce stress tolerance. Similarly, in our previous study, a low level of dietary enrichment with *P. incisa*, which provided ARA levels of 12.5 and 17.5 mg ARA g<sup>-1</sup> (close to the low supplemented diet in the present study, containing 16.3 mg ARA g<sup>-1</sup>), did not affect resistance to *Tetrahymena* infection (Khozin-Goldberg et al. 2006).

The effective ARA levels were higher than those reported in several studies demonstrating the beneficial effect of ARA on growth, survival and stress resistance in marine fish larvae (Bessonart et al. 1999; Harel et al. 2001; Koven et al. 2001, 2003; Willey, Bengtson & Harel 2003).

Guppies fed a commercial unsupplemented diet, containing linoleic acid as the major n-6 PUFA (Tables 2 and 3), were able to convert it to ARA as evident by the measurable ARA in the body and livers in both experiments (Tables 4 and 5). Generally, freshwater fish species have higher levels of ARA in comparison with marine fish species because they are able to convert the essential linoleic acid to ARA. Nevertheless, substantial differences are reported within freshwater as well as marine fish spp. (Gutierrez & da Silva 1993; Ackman, McLeod, Rakshit & Misra 2002). One can speculate that fish response to dietary ARA may depend on the basal ARA levels in fish lipids, as well as on the dose and duration of feeding.

Arachidonic acid is one of the major FA components of fish membrane phospholipids, specifically phosphatidylinositol (Bell & Tocher 1989). Following exposure to specific stimuli, such as inflammation and stress, ARA is released from membrane phospholipids and converted to eicosanoids, which play important roles in the regulation of stress, immunity, signalling and gene expression (Rowley, Knight, Lloyd-Evans, Holland & Vickers 1995; Bell & Sargent 2003). Alterations in cortisol levels in fish exposed to various stresses are generally associated with dietary manipulations of ARA (Harel *et al.* 2001; Koven *et al.* 2003). Arachidonic acid-derived PGs have been shown to alter the sensitivity of the stressor-activated HPI axis and ACTH-dependent cortisol release (Lands 1991; Sumida 1995; Ganga, Tort, Acerete, Montero & Izquierdo 2006).

Marine fish depend largely on cortisol to maintain their osmotic balance, because it increases the development of chloride cells and enhances the activity of the Na<sup>+</sup>/K<sup>+</sup>ATPase (Dange 1986; Sampathkumar, Munro, Lee & Lam 1993; Ayson, Kaneko, Hasegawa & Hirano 1995; McCormick 2001). Nevertheless, in freshwater tilapia, cortisol increased chloride cell numbers as well as Na<sup>+</sup>/K<sup>+</sup>ATPase abundance in the tubular system of the chloride cell (Dang, Balm, Flik, Bonga & Lock 2000). Enhanced tolerance of freshwater guppies fed *P. incisa* to an acute increase in salinity probably involves various coordinated physiological responses aiming to compensate for the increased salinity. These responses may include the function of osmoregulatory organs, the maintenance of plasma osmolality, ion uptake and secretion as well steroidogenesis.

Resistance to a same stress was enhanced through nutritional prophylaxis that stimulated the fish immune system, by supplementation of high doses of vitamin C (Lim *et al.* 2002). Similarly, in our study, the enhanced resistance was induced by the administration of  $\beta$ -carotene from *P. incisa*, the vitamin A precursor, immunostimulant and antioxidant. Supplementation with the  $\beta$ -carotene-rich fraction, containing minute amounts of ARA (Table 3), significantly increased guppies' resistance to high osmotic stress. As resistance in fish fed with the NL extract was higher than that in fish fed with  $\beta$ -carotene-or ARA-rich TAG alone, thus our results suggested an additive effect of  $\beta$ -carotene-and ARA-rich TAG in the NL extract supplementation. Remarkably,  $\beta$ -carotene was applied at substantially lower doses than the ARA-rich TAG, signifying its high efficiency. Our results are in line with the results of several studies

showing the beneficial effect of dietary carotenoids on fish non-specific immune responses (Amar, Kiron, Satoh & Watanabe 2001; Rodríguez, Cuesta, Esteban & Meseguer 2004) and disease resistance (Tachibana *et al.* 1997; Kolluru *et al.* 2006).  $\beta$ -Carotene in *P. incisa* is mainly derived from the extraplastidial oil bodies that alga forms under N-starvation conditions and is deposited concurrently with the buildup of ARA-rich TAG, however, at substantially lower levels (Solovchenko *et al.* 2008). Moreover, this endogenous antioxidant serves as a beneficial component as it can prevent the harmful effects of lipid peroxidation and deterioration of ARA in the feed.

In conclusion, the high osmotic stress allowed an easy monitoring of stress resistance of guppies under laboratory conditions and a reproducible evaluation of the effect of immunostimulants. The elevated dietary levels of both ARA and  $\beta$ -carotene modulate guppies' defence mechanism, possibly through their effect on the innate immune system and/or on the stress response, enhancing the resistance to acute stress.

Diets supplemented with broken algal cells at 25 mg ARA g<sup>-1</sup> provided an effect similar to that of the corresponding ARA concentrations in TAG- and NL-extract-supplemented diets. Besides ARA-rich TAG and  $\beta$ -carotene, whose effect was investigated in the present study, algal cells may contain additional components, e.g. polysaccharides, proteins, nucleotides, etc., with potential immuno- and stress-modulating activities. To conclude, we propose the use of broken *P. incisa* as a dietary supplement in guppies for short-term feeding before the onset of an acute stress event.

## Acknowledgments

We thank Mr Ran Epstein, the owner of Colors fish farm, Hazeva, Israel, and Mr Evyatar Ginat, the Owner of a commercial fish farm in Ein Yahav, Israel, for providing guppies for this study. This research was supported by the Regional Research and Development grant, the Israeli Ministry of Science and Technology and the Prof Dan Koshland Fund.

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