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Fatty acid food source affects expression of genes involved in the stress response in tilapia (*Oreochromis niloticus*)

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Fatty acid source in tilapia (*Oreochromis niloticus*) feeding can affect stress resistance and thus fish welfare. We aimed to test the hypothesis that diets based on different sources of fatty acids in tilapia reared in a Recirculating Aquaculture System (RAS), affect fish welfare as measured by different stress and immunity indicators. A total of twenty four male tilapia individuals were fed Fish Oil (FO), SunFlower Oil (SFO), Linseed Oil (LO) or High-Oleic SunFlower Oil (HO-SFO) as a fatty acid source. Water quality, cortisol and liver transcript expression (genes PRLRa, KLR, HSP70, IGFI, IGFII, GHR) were measured. No differences in growth performance were shown when changing fatty acid source. Water quality was improved in the tanks where fish were fed on plant oil [Electrical Conductivity (EC_{25}) was lower ($p < 0.023$)]; concentration of chloride (Cl^-, $p < 0.001$), calcium (Ca^{2+}, $p < 0.0001$) and sodium (Na^+, $p < 0.012$) ions were lower when HO-SFO oil was used, suggesting better nutrient absorption. Also, KLR, HSP70 and PRLRa genes were significantly inhibited and IGFI, IGFII and GHR significantly over-expressed in individuals fed vegetal oils when compared to FO, indicating an osmoregulatory stress in fish fed the latter. We conclude that growth is not affected by substitution of fish oil by vegetal oils, and that the latter lowers stress, improving fish welfare.

Key words: feed ingredients, fatty acid source, nutrigenomics, gene expression, stress response, tilapia.

INTRODUCTION

Polyunsaturated Fatty Acids (PUFAs) are nutritionally essential for normal growth, development and reproduction in all vertebrates including fish and humans (Haave & Innis, 1991). Fish and marine mammals are the main source of this dietary lipid and provide essential polyunsaturated fatty acids, especially Highly Unsaturated Fatty Acids (HUFA: EPA; 20:5n-3 and DHA; 22:6n-3) (Sargent *et al*., 2002) to the feed of many farm-raised species, from pigs and poultry to farmed fish. However, the relatively high cost of fish meal and fish oil, and the growing pressure on wild fisheries requires alternative oils that are nutritionally suitable and sustainable (Tacon, 2004; FAO, 2009). Plant oils hold considerable promise as cost-effective partial replacements for fish oil. Studies performed in Atlantic salmon fed lipid rich diets containing high-oleic sunflower oil (Torstensen *et al.*, 2000), rapeseed oil (Bell *et al*., 2001) and linseed oil (Bell *et al*., 2003; Menoyo *et al*., 2005) were found to have no detrimental effects on growth.

In recent years, several groups considered the effects of different plant oil sources on fish welfare, but mostly in carnivorous species (e.g., sea bream, Gómez-Requeni *et al*., 2004). However, little information is available on the effect that different vegetal oil sources may have on omnivorous species. In tilapia (*Oreochromis niloticus*), stress due to high density (Hrubec *et al*., 2000) or confinement (Vijayan *et al*.,

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1997), clearly increases plasma cortisol concentration, but few studies have considered how plant oil source may affect those levels.

Variation in dietary fatty acid profiles caused by the inclusion of vegetal oil sources may alter fish metabolism, which can affect stress resistance and thus fish welfare. One neglected aspect of fish welfare is osmoregulation, an important homeostatic process in fish, which represents more than half of maintenance energy requirements (Bijvelds *et al*., 1997). It is known that stress increases salt excretion during fish transport (Wurts, 1999). However chronic stress has been less studied and Recirculating Aquaculture Systems (RAS) provide an excellent environment to measure possible effects of plant oil substitution on fish stress in terms of changes in water quality.

Stress in fish has been measured indirectly through the classic stress hormone cortisol or by glucose plasma levels (Ziková *et al*., 2010), the immunomodulatory action through superoxide dismutase (SOD) (Campa-Córdova *et al.,* 2002) and catalase (CAT) enzyme activities, by means of thiobarbituric reactive substances (TBARs) level, or serum markers of oxidative stress such as malondialdehyde and glutathione (Tricot *et al*., 2010). Here, we quantify stress levels in tilapia fed with different fatty acid sources using plasma cortisol levels as well as the expression of heat shock protein 70 (HSP70) known to play a critical role in salinity tolerance of fish when exposed to osmotic stress (Deane *et al*., 2002) and prolactin which plays an essential role in freshwater adaptation by preventing changes in ion concentrations (Manzon, 2002). The KRL gene expression was also measured to describe immune system health, and growth levels were described by IGFI, IGFII and GH expression. The main objective of this study was to test the hypothesis that diets based on different sources of fatty acids in tilapia reared in a RAS system, affect fish welfare and growth as measured by different stress and immunity indicators.

MATERIALS AND METHODS

Installations

The work was carried out using RAS units at the Field Station of the Agricultural College of the Polytechnic University of Madrid (Madrid, Spain). Twenty-four white fiber glass tanks (110 l capacity; 0.38 m height, 0.64 m diameter) were used, with one filter (EHEIM Classic; Mod. 2217, 6 l capacity, 20 W, 1000 l hr^{-1} flow rate) per two tanks (n = 12 filters). Although these types of aquarium filters are not the most suitable for high output production in aquaculture, they were specifically used to control waste production per two tanks and to increase the replicate number for water quality samples (three per treatment). Each tank was oxygenated using individual air pumps (Million Air-MA 200, 4 W) with an inlet of 20 cm from the bottom of the tank. Each tank was also individually heated (Atman N2867, 50-60 Hz, 20-34°C, 150 W) to 26°C at the beginning of the experiment. The flow rate of water per tank was measured to be 160-180 l hr^{-1} at different times during the trial. Tanks were also covered with a 1 cm thick plastic lid (75 cm × 75 cm) to prevent fish from jumping out of the tank and to decrease evaporative losses. Photoperiod was controlled automatically to be 12 L:12 D.

Animals

We used 24 male tilapia (n = 1 fish per tank), with an average initial weight (±sd) of 70 ± 2 g, that had been purchased as larvae from a breeding population of genetically male tilapia (GMT®) from Valenciana de Acuicultura (Puçol, Valencia). All experimental fish originated from one tank from a larger recirculation unit and had been handled and fed the identical manner (fed twice a day) for eight weeks previous to the start of the trial. All experimental fish were weighed individually at the beginning and end of the trial and feeding rate (g of feed per fish) was adjusted weekly. Using the data on feeding rate and live weight, we calculated the average initial weight, average final weight, weight gain, Specific Growth Rate (day^{-1}), Feed Conversion Rate (FCR = g of feed/g weight gain), and feed intake per fish. The experimental protocol was approved by the Animal Experimentation Ethics Committee of the Polytechnic University of Madrid.

Diets

Individuals were fed four isoenergetic and isoproteic extruded diets (pellet diameter 3.0 mm), with 350 g kg^{-1} crude protein, 79 g kg^{-1} ash, 150 g kg^{-1} crude fat, 48 g kg^{-1} crude fiber, vitamin and mineral premix 8 g kg^{-1} (proprietary composition, Skretting ARC, Stavanger, Norway), 15.9 MJ kg^{-1} gross energy and 6.8 g kg^{-1} phosphorus. They were formulated to meet or exceed all the essential nutrient requirements of tilapias (NRC, 1993; Jauncey, 1998) and kindly produced by Skretting ARC (Stavanger, Norway). Each feed contained either Fish Oil (FO), SunFlower Oil (SFO),

Linseed Oil (LO) or High-Oleic SunFlower Oil (HO-SFO). Six fish were fed three times a day using a programmable feeder (EHEIM 3581) at 9:00, 13:00 and 17:00. Feed was provided at 10% of live weight. We assumed that all feed provided was consumed by the fish (no pellets were observed to accumulate on the surface or bottom of the tanks).

Water quality

Eight weeks before placing the fish in the tanks, a fishless nitrogen cycle was commenced. Tanks were filled with city water (day -56), loaded filters turned on and residual chlorine gas removed by aeration for 24 hrs. Starting on day -56, tank water was inoculated weekly with Clear-FLO® 1100 1x containing *Nitrosomonas* and *Nitrobacter* (Alken Murrary Corporation) according to manufacturer's instructions. After ammonia and nitrite levels peaked and subsided to near zero levels, the experimental fish were added. Tanks were topped-up once every two weeks with city water (approx 10 l) due to water loss from evaporation. After the fish were introduced, sodium bicarbonate was added daily (25 g kg^{-1} by weight of the feed provided) to each tank (except controls) to maintain appropriate pH levels (~7.5) and stimulate bacterial growth in the biofilters. In addition to the 24 experimental tanks with fish, four control tanks were kept under similar conditions but without fish or feed to monitor water quality changes in the absence of biomass.

We measured dissolved oxygen and temperature (Orion model 810, Orion Research Inc.) and pH (Crison, mod. 507) three times a week throughout the trial. In addition, one water sample (250 ml) was taken from each tank at the beginning and end of the trial, to measure electrical conductivity (EC), bicarbonate (HCO_3^-), nitrate (NO_3^-), ammonium (NH_4^+), phosphates ($H_2PO_4^-$), sulphates (SO_4^{2-}), chloride (Cl^-), sodium (Na^+), potassium (K^+), calcium (Ca^{2+}) and magnesium (Mg^{2+}). The EC and bicarbonates were measured with a 716-Titrino DMS, sample exchanger 730, automatic arm 759 and conductivimeter 712. For the rest of the measurements we used ion chromatography (Compact IC 761 and IC Sample Processor 766 linked to a computer). Before analysis, samples were passed through a 0.45 μm pore filter.

Plasma cortisol

Blood samples were obtained from all 24 fish at the end of the trial to measure plasma cortisol levels. Fish were firstly anaesthetized (tricaine methanesulphonate, Orgamol Inc.; 1g per 10 l), weighed and sampled from the caudal vein (0.2 to 0.5 ml). Blood plasma was maintained in heparin (Chiesi España, S.A.) until analysis. To determine cortisol levels, samples were centrifuged at 1500 × *g* (3000 rpm) for 4 min and plasma kept at –80˚C until competitive radio-immuno analysis following Yalow & Berson (1971) using I-125 (DSL Cortisol RIA kit, DSL-2000, Diagnostic Systems Laboratories, Inc.).

RNA extraction, cDNA synthesis and quantitative real-time PCR

The total RNA was extracted from liver sample of twelve male tilapia (three for each dietary treatment) using commercial spin-columns (RNeasy® Midi Kit, QIAGEN, Izasa, Spain) according to the manufacturer's specifications. RNA concentration was determined by conventional agarose electrophoresis and through absorbance measurements (ratio 260/280 ≥ 2). Two ml of total liver RNA were used to produce a retrotranscription reaction using iScript™ cDNA Synthesis Kit (Bio-Rad, Madrid, Spain). Real time PCR was performed in iCycler IQ Real-Time PCR Detection System (Bio-Rad, Madrid, Spain) and a master mix was prepared using Dynamo™ HS SYBR® Green qPCR Kit (Finnzymes, Madrid, Spain). Each amplification reaction mixture (20 μl) contained 2 μl of cDNA; 0.5 mM of each primer and 10 μl master mix. Primers were designed using Primer3 online programme (http://frodo.wi.mit.edu/primer3) with the default parameters standing in the program and using known sequences of *Oreochromis niloticus* and *O. mossambicus* found in nucleotide database. Six genes were chosen: 1) stress genes – Heat Shock Protein 70 (HSP70 – GenBank Accession No JF957367; 5′-AAAAGGTGTCCAACGCTGTC-3′ and 5′-AGATGCCGTCTTCAATGGTC-3′) and Prolactin Receptor a (PRLRa – GenBank Accession No XM_003451495; 5′-CTGGCCCTTTCTTCGAGTGT-3′ and 5′-CGCTTTCCTCATCCTCCAAC-3′); 2) immunosuppression – C-type lectin natural killer cell-like protein (KLR – GenBank Accession No AY_495714.1; 5′-TACTGTATGTCCACGAAACG-3′ and 5′-CGCCTCAGAGCAAAAGAGAA-3′); 3) growth – Insulin-like growth factor 1 (IGFI – GenBank Accession No XM_00344 8059; 5′-GTCTGTGGAGAGCGAGGCTTT-3′ and 5′-GCGAGAAATCTTGGGAGTCTT-3′), insulin-like growth factor 2 (IGFII – GenBank Accession No XM_003440474; 5′-GAAAC

CCAGCAAAGATACGG-3′ and 5-GTTCTCCCC-CACACAGAGTC-3′) and growth hormone receptor type 1 (GHR – GenBank Accession No XM_00344 6082; 5′- TGCTGAGTGGTAGAGGAATGG-3′ and 5′-TGACTGACAGGTTGGGACTG-3′). Elongation factor 1-alpha (EEF1a – GenBank Accession No JQ392736) was used as reference gene and amplified with the primer pair (5′- CCCCTCCAGGACGTT-TACAAA-3′ and 5′-CACACGGCCCACAGGTA-CA-3′). As two different genes are found to encode different prolactin receptors, primers were designed to be specific to PRLRa known for its role in fresh water osmoregulation.

After the selection of the most adequate annealing temperature, standard curves and non-template controls were produced in triplicate for each gene to test intra-assay variation (which was set at < 0.5 Ct), together with the sample assays. Forty five steps of PCR were performed, each one consisting of heating 95˚C for 25 sec, 10 sec at annealing temperature and 15 sec at 72˚C, ending with a melting program ranging from 68˚C to 95˚C with a heating rate of 0.1˚C per 10 sec and continuous fluorescence measurement.

Data Analysis

The data on growth and plasma cortisol were analyzed using the PROC MIXED procedure of the SAS statistical package (SAS, 2009) with oil type as the fixed effect, biological filter as a random effect and initial weight as the covariate. In addition, the Repeated Measures procedure was used to analyze water quality data collected five times over the experimental period. Means were compared using the LSD test; significance level was set at $p < 0.05$.

For the qRT-PCR data, significant differences were considered at $p < 0.1$. Relative quantification of the target gene transcript was performed through the use of the reference gene (EEF1a) and analysed following the Pfaffl method with the Relative Expression Software (REST©) (Pfaffl *et al*., 2002). This algorithm computes an expression ratio, based on real-time PCR efficiency and the crossing point deviation of the unknown sample *versus* a control group: $R = (\text{Etarget gene})^{\Delta\text{CTTarget gene (control-sample)}} / (\text{ERef gene})^{\Delta\text{CTRef gene (control-sample)}}$, where E is PCR efficiency of reference gene transcript determined by standard curve using serial dilution of cDNA. Statistical differences in gene expression between control and sample were evaluated in group means by randomisation test.

RESULTS AND DISCUSSION

Growth of tilapia was similar when fed either LO, SFO, HO-SFO or FO, which agrees with other studies in tilapia (Karapanagiotidis *et al*., 2007) and in carnivorous fish (Bell *et al*., 2003; Bransden *et al*., 2003; Menoyo *et al*., 2005). Although fish growth was moderate (our system was not designed for high output commercial growth), there was no mortality, and final weight (range 119.4-146 g) and SGR (range 0.93-1.18% day) were within reasonable ranges, while FCR ranged between 2.03-2.49.

Although growth results were similar, some differences in water quality were observed (Table 1), which may have been an indirect cause of stress (see cortisol and expression of PRLRa, KLR and HSP70). It is important to note that ions were allowed to build up during the trial and no water was added or exchanged outside the double-tank unit. Thus, ion accumulation was only influenced by fish growth and feed type. Ion concentrations in water from control tanks (no fish or feed) were similar to treatment tanks at the beginning of the experiment and did not change significantly throughout the study (data not shown). Among the treatment tanks, water pH was similar but electrical conductivity (EC_{25}) was slightly lower in the tanks with fish fed HO-SFO, suggesting better nutrient absorption by those fish. In line with that result, the concentration of Cl^-, Ca^{2+} and Na^+ were also significantly lower in HO-SFO. When comparing plant oil treatments against FO, the latter had higher EC_{25}, Cl^- and Na^+, suggesting lower water quality and the possibility for a higher osmotic stress.

Plasma cortisol levels were quite low in all treatments (Table 2), but significantly higher in SFO ($p < 0.01$), indicating higher stress levels. Those results seem to contradict Montero *et al.* (2003) who reported much higher plasma cortisol in sea-bream fed linseed oil. These authors concluded that substituting more than 60% of fish oil with only one plant oil (instead of a blend), affects immunity and stress resistance.

We estimated immune suppression by considering KRL gene expression, encoding for a natural killer protein which allows NK cells to perform apoptosis of the target cells (Watts *et al*., 2001; Sato *et al*., 2003; Kikuno *et al*., 2004; Dong *et al.,* 2007). Phagocyte activity is well-documented in fish, and natural cytotoxic cells have shown to be present with analogous functions as in mammalian natural killer cells (Watts *et al*., 2001). KLR expression in liver was significantly

TABLE 1. Effects of vegetal oil in feed pellets with linseed oil (LO), sunflower oil (SFO), fish oil (FO) and high-oleic sunflower oil (HO-SFO) on water quality parameters in a small-scale experimental recirculation system with Nile tilapia (*Oreochromis niloticus*). PROC MIXED procedure of the SAS statistical package (SAS, 2002) was used with plant oil type as the fixed effect, biological filter as a random effect and initial weight as the covariate

	LO	SFO	FO	HO-SFO	SEM[1]	p_{trt}	p_{date}	p_{t*d}
pH	8.3	8.3	8.1	8.1	0.1	0.218	< 0.0001	0.490
EC_{25}	0.8[b]	0.8[b]	0.8[b]	0.7[a]	0.03	0.023	< 0.0001	0.008
HCO_3^-	139.7	162.8	121.9	147.6	11.9	0.308	< 0.0001	0.290
CO_2	1.9	1.8	2.6	2.5	0.7	0.429	0.0017	0.676
NH_4^+	0.0	0.0	0.0	0.0	0.00	0.512	0.0054	0.617
NO_3^-	204.4	175.3	230.8	177.6	15.7	0.058	< 0.0001	0.012
$H_2PO_4^-$	10.6[a]	10.2[a]	9.5[b]	10.1[ab]	0.7	0.031	< 0.0001	0.513
SO_4^{2-}	15.9	14.6	14.3	12.9	0.9	0.171	< 0.0001	0.090
Cl^-	65.5[b]	69.9[ab]	73[a]	56.1[c]	2.6	0.001	< 0.0001	0.025
Na^+	140.[a]	141.9[a]	149.7[a]	129.4[b]	5.6	0.012	< 0.0001	0.067
K^+	17.9	18.2	17.6	16.7	0.8	0.517	< 0.0001	0.205
Ca^{2+}	23.3[a]	23.1[a]	19.9[b]	19.8[b]	0.8	0.000	< 0.0001	0.894
Mg^{2+}	4.5	4.2	4.4	4.7	0.3	0.196	< 0.0001	0.183

All concentrations in mg l^{-1} except pH, and EC (electrical conductivity in mS cm^{-1})
[a,b,c] Mean values with different superscript letters are significantly different ($p < 0.05$)
p_{trt}: significance level of treatments; p_{date}: significance level of date; p_{t*d}: significance level of the interaction treatment × date

TABLE 2. Effect of plant oil source in feed pellets on plasma cortisol levels in tilapia: Linseed oil (LO), Sunflower oil (SFO), Fish oil (FO) and High-Oleic Sunflower oil (HO-SFO). PROC MIXED procedure of the SAS statistical package (SAS, 2002) was used

	LO	SFO	FO	HO-SFO	SEM	*p*
Cortisol (ng ml^{-1})	14.2[a]	19.8[b]	15.1[ab]	10.4[a]	1.65	0.007

[a,b] Different letters indicate statistical significant difference ($p < 0.01$)

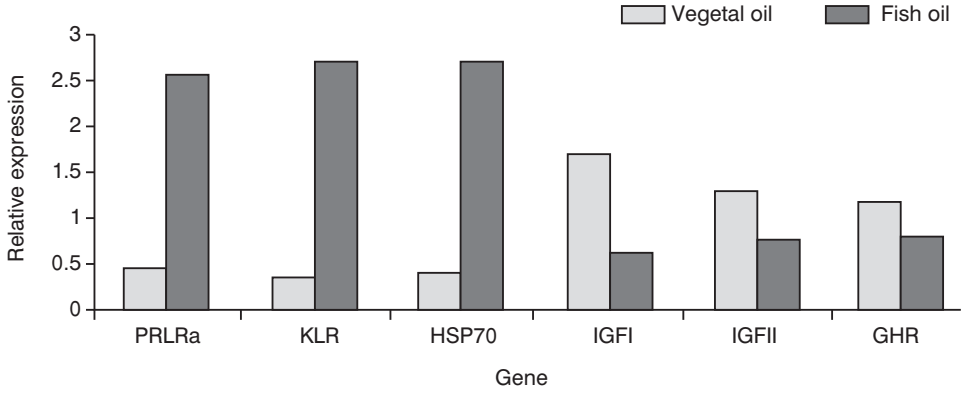

FIG. 1. Significant effects of fish oil diet *versus* vegetal oil diet on the hepatic expression of PRLRa (Prolactin receptor a), KRL (C-type lectin natural killer cell-like protein), HSP70 (Heat shock protein 70), IGFI (Insulin-like growth factor I mRNA), IGFII (Insulin-like growth factor II mRNA), GHR (Growth hormone receptor type 1 mRNA) in Nile tilapia (*Oreochromis niloticus)*. Results were calculated with REST 2008 software using EEF1a as reference gene and expressed relative to values for individuals fed fish oil diet. For *p*-values, see Table 3.

TABLE 3. Changes in hepatic gene expression of tilapia comparing fish based diets *versus* each vegetal oil based diets. Relative quantification of the target gene transcript was performed through the use of the reference gene EEF1a and analysed following the Pfaffl method with REST© (Pfaffl *et al*., 2002). No significant differences were found when comparing vegetal oils among them

	LO			SFO			HO-SFO		
	Relative Expression	Expression change[1]	*p* value	Relative Expression	Expression change[1]	*p* value	Relative Expression	Expression change[1]	*p* value
PRLRa	0.5	D	0.06	0.35	D	0.048	0.34	D	0.037
KRL	0.37	D	0.03	0.27	D	0.005	0.50	D	0.11
HSP70	0.35	D	0.03	0.25	D	0.014	0.55	D	0.082
IGFI	1.89	I	0.006	1.63	I	0.017	1.60	I	0.007
IGFII	1.81	I	0.009	1.29	NC	0.456	0.94	NC	0.74
GHR	1.29	I	0.05	1.16	NC	0.307	1.01	I	0.09

[1] NC: no significant change in expression; I: expression increases; D: expression decreases

down-regulated (Fig. 1 and Table 3) in all vegetal oil diets compared to fish oil, which indicates better stress resistance.

Other proteins known to be induced by multiple stressors including osmotic stress are heat shock proteins, specifically Heat Shock Protein 70 (HSP70), and prolactin (which has been measured indirectly through the expression of the prolactin receptor a (PRLRa) were down-regulated ($p < 0.1$) in liver using vegetal oil (Fig. 1 and Table 3). HSP-mediated adaptation processes are regarded as fundamental protective mechanisms that decrease cellular sensitivity to damaging events and can be expressed by multiple stressors, including osmotic stress (Sørensen *et al*., 2003). Although HSP70 has been mainly used as a marker in salinity tolerance (Deane *et al*., 2002; Deane & Woo, 2004), this gene has been recently suggested as a potential biomarker of fish health (Tine *et al.,* 2010). Significant differences found between vegetal and fish fat sources indicate increased stress when using fish oil and confirm its potential as a health marker.

In euryhaline teleosts, such as tilapia, PRL plays an essential role in fresh water adaptation and is essential for their survival by either preventing ion loss or increasing ion retention (especially Na^{2+} and Cl^{-}) and by decreasing water uptake (Manzon, 2002). Prolactin has been found to stimulate the cellular immune system in the rainbow trout (*Oncorhynchus mykiss*) (Yada *et al*., 1999), and recent studies support a critical function of this hormone for teleost osmoregulation (Sakamoto & McCormick, 2006). The two-fold significant increase in PRLRa expression in the fish oil fed individuals indicated an osmoregulatory stress, probably produced by a state of unbalanced tissue oxidation. Also the reduced GH and IGFI signalling in the FO fed animals would indicate a response to combat oxidative stress, as has been described in sea beam (Saera-Vila *et al*., 2009). Both IGFI and IGFII in fish have been associated with growth as well as with metabolism, development, reproduction and osmoregulation in seawater (McCormick & Bradshaw, 2006). There is also evidence that in bony fish, both the IGFI gene and IGFII gene are controlled by GH (Vong *et al*., 2003) in all organs. So the fact that the three genes (IGFI, IGFII and GH - measured here through its receptor) are up-regulated in fish fed FO may indicate an osmoregulatory stress due to the existence of an unappropriate cellular redox, as stated by Haddad (2002).

Finally, it has been documented that, under some conditions, cortisol promotes ion uptake and interacts with prolactin during acclimation to fresh water (McCormick, 2001). Results obtained here (gene expression, cortisol level and ions concentration) indicated that individuals fed with vegetal oil seem to have a better balanced cellular redox state and thus a better health status. As growth performance did not vary among diets, it is advisable to use plant oils in the diet to rear tilapia in commercial conditions under a RAS management system. This will contribute to an efficient use of resources, improve water conditions in circulating systems and lower stress level. Moreover, as HSP70 has been suggested as biomarker for fish health, the rest of the genes measured here can be used as biomarkers of stress resistance.

In conclusion, our results corroborate recent findings in many fish species that fish oil can be substitut-

ed with plant oils without altering growth, and can improve stress resistance resulting in a probable positive effect on fish welfare.

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