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Title

Effects of dietary limonene and thymol on the growth and nutritional physiology of Nile tilapia (*Oreochromis niloticus*)

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Abstract

Phytogenic compounds such as limonene and thymol have been shown to have growth-promoting properties in farmed animals but studies in fish are scarce. Two Experiments (Experiments I and II) were carried out to investigate the individual effects of dietary limonene and thymol on the growth and nutritional physiology of juvenile Nile tilapia (*Oreochromis niloticus*). In Experiment I, the fish were fed on a commercial diet coated with limonene at 0 (control), 200, 400, and 600 mg kg^{-1} (ppm), while in Experiment II thymol was supplemented in the diet at 0 (control), 250 and 500 ppm. Our results showed a significant increase in fish weight and weight gain with diets supplemented with 400 and 600 ppm limonene compared to the control. Moreover, the expression of insulin growth factor I (*igf-I*), mucin-like protein (*muc*), oligo-peptide transporter I (*pept1*), lipoprotein lipase (*lpl*), alkaline phosphatase (*alp*) and catalase (*cat*) was up-regulated by dietary limonene. Our results confirm that dietary limonene can enhance the growth of Nile tilapia juveniles through the activation of key genes involved in somatotropic axis-mediated growth, nutrient digestion and antioxidant enzyme defence. Dietary thymol did not seem to influence growth or regulate the same pathways activated by limonene in Nile tilapia juveniles at inclusion levels up to 500 ppm. Overall, the present results suggest that potential growth-promoting effects are dependent upon the phytogenetic itself and its inclusion level.

Keywords

Essential oil; gene regulation; growth promoters; Nile tilapia; phytochemical; phytogenics

1. Introduction

Limonene and thymol are major phytogenic compounds of the essential oils (EOs) from citrus fruits and thyme herbs, respectively (Gad, 2012; Sun, 2007). Currently phytogenics are being widely investigated as naturally derived growth-promoters for use in animal production (Hashemi et al., 2010; Hernandez et al., 2004; Peric et al., 2009). Phytogenics are reported to be safer and healthier than antibiotics and hormone growth-promoters, compounds that have been discouraged from use in animal feeds by the World Health Organisation since 2006 (Brenes and Roura, 2010; Windisch et al., 2008). Thus, antibiotic growth-promoters have the risk of creating resistance to bacteria pathogenic to animals and humans, while hormones might deposit in animal tissue and negatively affect human health (Jeong et al., 2010; Mathivanan and Edwin, 2012; Sicuro et al., 2010; Syahidah et al., 2015).

Some studies have shown that dietary supply of limonene and thymol have demonstrated growth-promoting effects on growth of poultry and some fish species (Acar et al., 2015; Ahmadifar et al., 2011, 2014; Dalkilic et al., 2015; Ngugi et al., 2017; Pérez-Sánchez et al., 2015; Shad et al., 2016; Zidan et al., 2016). The effects of these compounds are largely dose-dependent and appear to be species-specific. Among fish species, weight gain of Mozambique tilapia (*Oreochromis mossambicus*) was significantly improved with a diet formulated with 1000 ppm of an EO containing 83.0 % limonene but not with 3000 and 5000 ppm (Acar et al., 2015). The growth of Ningu (*Labeo victorianus*) was improved with diets supplemented with 1000, 2000, 5000 and 8000 ppm of an EO containing 81.4 % limonene (Ngugi et al., 2017). However, in both studies it remained uncertain whether the enhanced fish growth was a result of synergistic effects between limonene and the compounds present at lower proportions in the EO. With regards to thymol, a dietary

combination of thymol and carvacrol at 1000, 2000 and 3000 ppm enhanced the growth of rainbow trout (*Oncorhynchus mykiss*) and European sturgeon (*Huso huso*) (Ahmadifar et al., 2011, 2014). Conversely, 500 ppm of dietary thymol did not stimulate growth of channel catfish (*Ictalurus punctatus*) (Zheng et al., 2009). Giannenas et al. (2012) did not find significant increase in weight of rainbow trout fed a diet with 1000 ppm of a phytogenic product with 6000 ppm thymol. Overall, the studies above highlight the importance of identifying the right phytogenic compounds and doses that result in growth improvement in different fish species. Presently, there is scarce information on the impact of limonene or thymol on the growth of Nile tilapia (*Oreochromis niloticus)*.

Nutritional physiology mechanisms underlying the growth-enhancing effects of phytogenic compounds are also not fully understood. Although it is known that diets can influence appetite, nutrient digestion, absorption and transport, lipid metabolism, antioxidant enzyme activity and somatotropic axis-mediated growth among other processes (Qiang et al., 2012; Rust, 2003), there are few reports in the literature on the pathways regulated by phytogenic compounds. In addition, different phytogenic compounds seem to have different effects on the physiology of animals (Lee et al., 2003a, b, 2004; Lillehoj et al., 2011), thus potentially activating different pathways to enhance growth. Presently, most of the studies that have investigated the mode of action of phytogenic compounds in fish, as well as terrestrial monogastric animals such as pigs and poultry, have been carried out using diets containing combinations of several phytogenic compounds (Awaad et al., 2014; Hafeez et al., 2016; Hashemipour et al., 2013; Hashemipour et al., 2016; Jiang et al., 2015; Li et al., 2012; Matysiak et al., 2012; Pérez-Sánchez et al., 2015; Zheng et al., 2009). From these studies, it is difficult to elucidate the specific pathways activated by individual

compounds. However, other studies have investigated the effects of phytogenic compounds supplemented individually in the diet (Galeotti et al., 2012; Yilmaz et al., 2014), although there is still limited understanding on pathways that different phytogenic compounds influence in nutritional physiology, particularly for fish.

Some nutritional physiology processes through which phytogenics exert their alluded to above growth-promoting effects include feed intake, digestion, absorption and lipid metabolism among other functions (Hashemi and Davoodi, 2010; NRC, 2011). The aim of the present study was to investigate the individual effects of dietary limonene or thymol on the growth and nutritional physiology of Nile tilapia. Specifically, the study investigated the expression of genes regulating food intake, nutrient digestion and transport, lipid metabolism, antioxidant enzyme status and somatotropic axis-mediated growth in Nile tilapia fed on diets with either limonene or thymol along with control diets.

2. Materials and Methods

2.1. Ethics statement

All experiments were subjected to ethical reviewed and approved by the University of Stirling through the Animal and Welfare Ethical Review Body. The project was conducted under the UK Home Office in accordance with the amended Animals Scientific Procedures Act implementing EU Directive 2010/63.

2.2. Experimental design

Two feeding experiments (Experiment I and II) were carried out at the Institute of Aquaculture, University of Stirling, UK. In each experiment, the pathways in the nutritional physiology of Nile tilapia potentially influenced by limonene or thymol were investigated by analysing the expression of genes regulating food intake, nutrient digestion and transport, lipid metabolism, antioxidant enzyme status and somatotropic axis-mediated growth. Both feeding experiments were run for 63 days.

2.3. Diets and fish feeding

A commercial fish diet (INICIO Plus, BioMar Ltd., Stirling, UK) (see proximate composition in Table 1) was used as a standard diet to which limonene or thymol was supplemented at increasing concentrations. Limonene (97 % purity) and thymol (99.5 % purity) were obtained from Sigma Aldrich Ltd., England, UK. In Experiment I, the diets were supplemented with 0 (control), 200 (L1), 400 (L2) and 600 (L3) mg kg^{-1} (ppm) of limonene. In Experiment II, the diets were supplemented with 0 (control), 250 (T1), and 500 (T2) ppm of thymol. In order to achieve the above concentrations in the experimental diets, the phytogenic compounds were first dissolved in 100 ml of absolute ethanol and subsequently sprayed evenly onto 1 kg of feed. The same volume of ethanol was added to the control diet to ensure similar conditions with phytogenic compound-supplemented diets. The diets were air-dried for one day before being used to feed the fish.

2.4. Experimental fish and husbandry conditions

Nile tilapia juveniles from the same spawning batch were obtained from the Tropical Aquarium, Institute of Aquaculture, University of Stirling, UK. The fish were size graded

during which they were anaesthetised with a low dose of benzocaine of 0.05 g L^{-1} for 3 - 5 min and kept in aerated water to minimise stress. The initial weight of the fish was 1.5 ± 0.0 g for Experiment I (limonene) and 1.5 ± 0.1 g for Experiment II (thymol) (mean ± standard error). Both experiments were conducted in 60 L plastic tanks in a recirculating aquaculture system. All treatments were assessed in three replicate tanks allocated using a complete randomised design. In Experiment I (limonene supplementation), each tank was stocked with 37 fish, whereas 25 fish per tank were stocked in Experiment II (thymol supplementation). In both experiments, fish were fed to apparent satiation by hand between 9:00 - 10:00 am and 4:00 - 5:00 pm. The amount of feed eaten was recorded daily.

The water quality in the experimental system was assessed weekly and maintained within the conditions for growth of Nile tilapia. An oxygen meter (OaKton DO 6+, Eutech Instruments Pte Ltd., Landsmeer, The Netherlands) was used to determine dissolved oxygen levels and water temperature. The pH and ammonia-nitrogen levels were measured using a fresh water test kit from Tropic Marin Company Ltd. (Wartenberg, Germany) following the instructions from the manufacturer. The water temperature ranged from 26.0 to 27.0 ^{o}C, pH was 6.8 ± 0.5, dissolved oxygen 6.8 ± 0.6 mg L^{-1} and ammonia-nitrogen 0.8 ± 1.0 mg L^{-1} (mean ± standard deviation). A light regime of 12L:12D was maintained.

2.5. Sampling and measurements

Growth of fish, previously anesthetised with 0.05 g L^{-1} benzocaine for 3-5 min, was monitored every two or three weeks by measuring weight (0.1 g accuracy) and total length (0.1 cm accuracy). The number of fish in each tank was also recorded. At the end of the experiments, fish were humanely killed with an overdose of benzocaine. Samples of liver,

fore intestine, and brain were collected from three fish per replicate (N = 9 per treatment) and placed in 1.5 mL tubes containing RNAlater (Sigma aldrich, Poole, UK) to preserve RNA integrity. The samples were kept at 4 ºC overnight and transferred to a -70 ºC freezer until RNA extraction.

2.6. Fish performance computations

At the end of each experiment (I and II), final average fish weight (FW) was assessed as FW (g) = total fish biomass at end of the experiment (g) / number of fish at the end of the Experiment; growth rate (GR) was calculated as GR (g d^{-1}) = (final average fish weight (g) - initial average fish weight (g)) / duration of Experiment (d); percentage (%) weight gain (% WG) was computed as % WG = (final average fish weight (g) - initial average fish weight (g)) / initial average fish weight (g) × 100; condition factor (CF) was expressed as CF = (final average fish weight / final average total $length^3$) × 100; percentage fish survival (% survival) was calculated as % survival = (number of fish at end of the Experiment / initial number of fish) × 100; feed intake as a percentage of body weight per day (% FI) was computed as % FI = (100 × (average feed intake per fish / ((initial average body weight ± final average body weight)/2))) / duration of the experiment (d); feed conversion ratio (FCR) was expressed as FCR = average feed intake per fish / average weight gain; protein efficiency ratio (PER) was assessed as PER = weight gain / protein intake (Jobling, 1994; Workagegn et al., 2014).

2.7. RNA extraction and complementary DNA (cDNA) synthesis

Total RNA from the tissue samples of brain, liver and fore intestine was extracted by homogenising the samples in TRI Reagent (Sigma Aldrich, Dorset, UK). The quantity and quality of the RNA was assessed by spectrophotometry using an ND-1000 Nanodrop (Nanodrop 1000, Thermo Scientific, Glasgow, UK) at absorbance wavelength ratios of 260/280 and 260/230. The integrity of the RNA was verified by agarose gel electrophoresis using 200 ng RNA in 1 % agarose gel and 0.5 x TAE buffer containing 0.3 µl ethidium bromide (10 mg mL^{-1}). Complementary DNA (cDNA) was synthesised from 2 µg of each RNA sample (N = 9 per treatment) using a high capacity reverse transcription kit without RNase inhibitor from AB Applied Biosystems (Warrington, UK) following the procedure described by the manufacturer.

2.8. Quantitative real-time polymerase chain reaction

The expression of candidate genes was analysed by quantitative real-time polymerase chain reaction (qPCR) in either liver, fore intestine or brain depending on the organ in which they play major biological functions. Table 2 shows the nucleotide sequence of the primers for each of the analysed genes. Efficiency of the primers was assessed using the standard curve dilution method, melting curves and cycle threshold (Ct) values (Larionov *et al*., 2005). The relative expression of each gene was determined (N = 9) using a Biometra TOptical Thermocycler (Analytik Jena, Goettingen, Germany) in 96-well plates. Each sample was analysed in duplicates of 20 µl reaction volumes comprising 5 µl of 20-fold diluted cDNA, 3 µl nuclease-free water, 1 µl (10 pmol) each for the forward and reverse primer of the candidate gene and 10 µl of Luminaris color higreen qPCR Mix (Thermo Scientific, Hemel Hempstead, UK). The same reaction volume was used for the

reference genes but 6 µl of nuclease-free water and 2 µl of 20-fold diluted cDNA of each sample were used. In each qPCR, a calibrator sample (20-fold dilution of pool cDNA from all the samples) and a negative control (non template control-NTC) with no cDNA were included. This was to compare the gene expression among the different samples and control any genomic DNA contamination, respectively. Each qPCR cycle consisted of pre-heating samples at 50 °C for 2 min followed by 35 cycles: initial denaturing at 95 °C for 10 min, denaturation at 95 °C for 15 s, annealing at 60 °C for 30 s, and an extension at 72 °C for 30 s.

The relative expression for each gene was determined based on the PCR efficiency and the Ct 20 value of every sample compared with that of the control, and expressed in relation to the reference genes (*β-actin* and *ef-1α*) following the mathematical model of Pfaffl (Pfaffl, 2001). Data were normalised using the geometric mean expression of the references genes (*β-actin* and *ef-1α*). Heat maps enabling observation of gene expression patterns but not based on statistical differences were generated representing relative expression patterns of the analysed marker genes. The data were plotted using Java Tree View and clustered according to Euclidean distance. Expression level of each gene was natural log transformed and normalised against two reference genes (*β-actin* and *ef-1α*).

2.9. Statistical analysis

SPSS version 19 (Chicago, USA) was used to perform statistical analyses (Landau and Everitt, 2004). For each treatment, data on performance indicators and gene expression are presented as means ± standard error. Normality of distribution of the data was assessed

using Kolmogorov-Smirnov test. Data not normally distributed were subjected to square-root (FW), natural logarithm (qPCR data) and arcsin square-root (GR, % WG, CF, % fish survival, % FI, FCR and PER) transformation. Differences among treatments were analysed by one-way ANOVA followed by Tukey's test. If heterogeneity of variances existed Welch's test was used with Game-Howell's test to establish differences among treatments. Significant differences were considered at P value < 0.05. The interaction between the duration of the growth experiment (days) and supplementation level of limonene or thymol in the diet was analysed using two-way ANOVA.

3. Results

3.1. Performance of Nile tilapia fed on limonene supplemented diets (Experiment I)

Growth performance of Nile tilapia fed on diets supplemented with limonene for 63 days (Experiment I) is presented in Table 3. A significant increase in FW, GR and % WG was found in fish fed on diets supplemented with 400 (L2) and 600 (L3) ppm limonene compared to the control. Differences in FW were detected after 42 (P = 0.008) and 63 (P = 0.012) days of the experiment. Condition factor and fish survival were not significantly different among treatments.

The influence of limonene dietary supplementation level (0, 200, 400, 600 ppm) and cumulative fish rearing days (1, 14, 28, 42 and 63 d) on fish weight showed a significant interaction F (12, 1771) = 2.17 (P < 0.05; Table 4). An increase in cumulative fish rearing days and concentration of limonene in the diet increased the weight of the fish. An increase in the number of days (time) of the experiment had a bigger contribution (P < 0.0001) than the concentration of limonene in the diet (P < 0.005) in influencing the somatic growth of

the fish (fish weight). No significant differences among treatments were found in % FI, FCR and PER.

3.2. Performance of Nile tilapia fed on thymol supplemented diets (Experiment II)

No significant differences were found in survival, mean weight, GR, % WG, CF, % FI, FCR and PER (Table 5) with thymol supplemented diets at the end of the experimental period. Fish weights measured during days 1, 14, 28, 42 and 63 of the experiment did not show significant differences among treatments. A two-way ANOVA on the impact of diets containing thymol (0, 250 and 500 ppm) and the duration of the growth experiment (1, 14, 28, 42 and 63 d) on the fish weight found no significant interaction of thymol inclusion level and growth duration, $F(8, 927) = 0.71$, $P > 0.05$ (Table 6).

3.3. Relative expression of candidate genes

Fig. 1 and 2 show heat maps representing relative expression patterns (not based on statistical significance) of the marker genes involved in the pathways of somatotropic axis growth-mediation (a), lipid and energy metabolism (b), and antioxidant enzyme defence (c) in the liver, nutrient digestion, absorption and transport (d) in the fore intestine and food intake regulation (e) in the brain of Nile tilapia fed on limonene (Fig. 1) and thymol (Fig. 2) supplemented diets. Fish fed on limonene supplemented diets had the highest number of genes activated with higher relative expression patterns (red). Fish fed on thymol supplemented diets (Fig. 2) exhibited more stable (black to maroon) or low (green) gene expression patterns.

3.3.1. Expression of genes involved in somatotropic axis in liver

Insulin growth factor I (*igf-I*) expression was significantly ($P < 0.01$) up-regulated in the liver of fish fed diets supplemented with limonene compared to control fish (Fig. 3A). Conversely, there was no significant difference in the expression of growth hormone (*gh*) and growth hormone receptor 1 (*ghr-I*) in the livers of fish fed on limonene supplemented diets (L1, L2, L3) and the control diet. The mRNA levels of *gh*, *ghr-I* and *igf-I* were not significantly different between treatments among livers of fish-fed the thymol supplemented diets compared to the control (Fig. 3B).

3.3.2. Expression of genes involved in lipid metabolism in liver

Significantly higher expressions of lipoprotein lipase (*lpl*) ($P = 0.002$) and alkaline phosphatase (*alp*) ($P = 0.038$) were found in the liver of fish fed on the limonene supplemented diets compared to the control (Fig. 4A). For *lpl*, gene expression differences were found between the L1, L2, L3 and the control, while only fish fed diet L2 had significantly higher levels of *alp* mRNA compared to control fish (Fig. 4A). No significant effect of dietary limonene was found in the expression of fatty acid synthase (*fas*), sterol regulatory element binding transcription factor 1 (*srebf1*) and peroxisome proliferator-activated receptor alpha (*pparα*) in the fish liver. Similarly, no significant differences in the expression of *alp*, *fas*, *srebf1* and *pparα* were found in fish from Experiment 2 (Fig. 4B).

3.3.3. Expression of genes regulating nutrient digestion, absorption and transport in fore intestine

The mRNA levels of mucin-like protein (*muc*) and oligo peptide transporter 1(*pept1*) were significantly higher ($P = 0.026$ and $P = 0.015$, respectively) in the fore intestine of

fish fed on diets L2 (*pept1*) and L3 (*muc* and *pept1*) compared to the control (Fig. 5A). No significant differences in the expression of intestinal *alp* (P = 0.575), aminopeptidase (*ap;* P = 0.858), phospholipase A2 (*pla2;* P = 0.266), glucose transporter 2 (*glut2*; P = 0.657), pancreatic alpha-amylase (*p-amy;* P = 0.951) and chymotrypsinogen A-like *(ctra;* P = 0.322) were observed with increasing concentrations of limonene in the diet (Fig. 5A). In the Experiment 2 (thymol supplementation), the average expression levels of *alp*, *pla2*, *p-amy*, *pept1*, *muc* and *ctra* in the fore intestine of the fish were statistically similar among treatments (Fig. 5B).

3.3.4. Expression of antioxidant enzymes in liver

Catalase (*cat*) was significantly up-regulated (P < 0.01) in the liver of fish fed diets supplemented with 200 and 400 ppm limonene compared to the control (Fig. 6A). The relative expression of glutathione peroxidase (*gpx*) in the liver of fish fed on diets L1, L2 and L3 did not differ significantly from the control (P = 0.716). Similarly, superoxide dismutase 2 (*sod2*) gene was not significantly regulated in the fore intestine by the experimental treatments. None of the analysed antioxidant enzymes (*gpx*, *sod2* and *cat*) was significantly up-regulated in the liver by thymol compared with the control (Fig. 6B).

3.3.5. Expression of genes regulating food intake in the brain

There was no significant difference in the expression of pro-neuropeptide Y-like *(npy;* P = 0.918) and leptin receptor variant X1 (*lepr;* P = 0.329) in the brain of Nile tilapia with increasing dietary inclusion of limonene (Fig. 7A). The fish fed on thymol supplemented diets also had statistically similar expression levels of *npy* (P = 0.227) and *lepr* (P = 0.111) compared with the control (Fig. 7B).

4. Discussion

This study investigated the effects of increasing concentrations of limonene and thymol on the performance of Nile tilapia, as well as the pathways regulating somatic growth and nutritional physiology in the fish. The expression of key genes participating in selected pathways of somatotropic axis-mediated growth, food intake, nutrient digestion and transport, lipid metabolism and antioxidant enzyme defence in Nile tilapia were investigated in major tissues where they exert their action, namely brain, fore intestine and liver. Specifically, the brain is a key site for stimulating food intake through the action of appetite regulating peptides (Kiris et al., 2007; Pierce et al., 2012). Moreover, the fore intestine is a pivotal site for digestion, absorption and transport of nutrients, as well as elimination of microbes (Bakke et al., 2010; Fascina et al., 2012; Kim and Ho, 2010). On the other hand, liver carries out many metabolic processes including lipid metabolism, antioxidant enzyme status and somatotropic axis peptide activities (Picha *et al*., 2008). The present study found that specific genes involved in the somatotropic axis growth (*igf-I*), nutrient digestion, absorption and transport (*muc* and *pept1*), lipid metabolism (*lpl* and *alp*) and antioxidant enzyme activity (*cat*) were regulated by dietary limonene, with less clear effects observed with dietary thymol. Such different response of fish to both phytogenics compounds expanded as well to growth performance. While the somatic growth of the fish from Experiment I showed that dietary limonene at 400 and 600 ppm has growth-promoting effects in Nile tilapia, dietary thymol up to 500 ppm did not enhance somatic growth.

In the somatotropic axis, insulin growth factor I (*igf-I*) plays a core role in regulating and controlling somatic growth (Dyer et al., 2004; Fox et al., 2010; Qiang et al., 2012).

Generally, with fish of the same species, age and rearing conditions under optimal feeding regimes, higher fish weights correlate with high mRNA expression of *igf-I* (Picha et al., 2008; Reindl and Sheridan, 2012). Consistently, the results from this study showed that fish fed on diets supplemented with 400 and 600 ppm limonene exhibited increased mRNA levels of *igf-I*, final weights, growth rates and percentage weight gain (% WG) compared to the control, implying that *igf-I* accounted for improved growth of the fish. Previous observations on enhanced levels of *igf-I* by dietary limonene (Miller et al., 2013) or a combination of cinnamaldehyde and thymol (Li et al., 2012) have been reported in mammals. In fish, dietary supplementation with curcumin, an active ingredient of turmeric (*Curcuma longa*), resulted in incresased expression of *igf-I* in Mozambique tilapia (*O. mossambicus*) (Midhun et al., 2016). Interestingly, our results showed that the final weight of the fish was influenced by an interaction between increasing number of days of the experiment and concentration of limonene. This suggests that the effectiveness of limonene to enhance somatic growth at concentrations of 400 and 600 ppm was time dependent and probably *igf-I* up-regulation followed the same trend given that it is a growth indicator. In agreement, Steiner (2009) reported that the effects of dietary phytogenics on improving growth of animals do occur after a period of time as shown with studies that monitored cumulative performance.

One of the possible mechanisms by which dietary limonene stimulates growth is through increasing energy availability from feed ingredients. Indeed dietary limonene increased expression of genes encoding two key enzymes, namely lipoprotein lipase (Lpl) and alkaline phosphatase (Alp). Lpl is a key enzyme involved in the breakdown of plasma lipids releasing fatty acids that are subsequently transported from the blood stream to

tissues for energy production (Georgiadi and Kersten, 2012). Increased expression of *lpl* suggests that limonene enhanced energy availability from dietary lipids and spared protein for somatic growth (Li et al., 2012). Additionally, Alp, also up-regulated by dietary limonene, plays a role in maintaining an energy or lipid balance by participating in carbohydrate metabolism, which also can to some extend contribute to the protein sparing effect (Hernandez-Mosqueira et al., 2015; Olagunju et al., 2000).

Enhanced digestion, absorption and transport of nutrients is vital for efficient utilisation of dietary components that ultimately contributes to enhanced growth. In this study mucin-like protein (*muc*) was up-regulated by limonene supplementation (600 ppm) and, provided the role that Muc plays in secretion of mucus required for efficent nutrient transportation in the intestine into the blood (Kamali et al., 2014; Neuhaus et al*.,* 2007; Pérez-Sánchez et al., 2015), it can be hypothesised that limonene increased mucus secretion improving nutrient absorption and accounting in part for the observed growth enhancement. Such mucus stimulatory effect by limonene has been previously reported in human and rat (Moraes et al., 2009; Rozza et al., 2011) but, to the best of our knowledge, not in fish. The present study suggests that enhanced protein absorption is a major mechanisms resulting from increased mucus secretion as indicated by up-regulation of oligo-peptide transporter 1 (*pept1*) in fish fed 600 ppm limonene diets. Pept1 is a nutrient transporter that uses mucus as a medium for active transport of di/tri peptides from enterocytes into the blood stream (Verri et al., 2011). In agreement, dietary di/tri peptides have been often reported to enhance growth of fish in a more efficient manner compared to individual amino acids (Tengjaroenkul et al., 2000; Verri et al., 2011). Other accessory genes to *muc* and *pept1* involved in nutrient digestion that were assessed herein, namely *p-amy* (pancreatic alpha-

amylase), *ctra* (chymotrypsin A-like) and *pla2* (phospholipase A2), showed no regulation by dietary limonene or thymol.

All metabolic processes including those investigated herein can potentially result into production of reactive oxygen intermediates (ROIs), whose levels in the organism are prevented by antioxidant defences including both enzymatic and non-enzymatic systems (Abele and Puntarulo, 2004; Patnaik et al., 2013). While no effect of thymol could be detected, limonene supplemented up to 400 ppm may have enhanced the antioxidant capacity as evidenced by up-regulation of catalase (*cat*), a key antioxidant enzyme in fish (Vasylkiv et al., 2011). The herein observed role of dietary limonene on *cat* regulation appears not to be common among other growth-promoting phytogenetic compounds such as thymol and carvacrol when tested on Channel catfish (Zheng et al., 2009), suggesting that different action modes occur within phytogenetic compounds, animals and fish species. For instance, Tiihonen et al. (2010) reported that a dietary combination of phytogenic compounds thymol and cinamaldehyde stimulated food intake and appetite of broiler chicken. However, neither limonene nor thymol had such an effect on Nile tilapia juveniles according to the lack of differences observed in feed intake and expression of neuropeptide Y-like (*npy*) and leptin receptor variant XI (*lepr*), key players in appetite regulation in the brain (Copeland et al., 2011; Prokop et al., 2012; Volkoff, 2006; Zhou et al., 2013).

In conclusion, this study showed that dietary limonene enhanced the growth of Nile tilapia, with its mode of action involving activation of key genes within the somatotropic axis-mediated growth (*igf-I*), nutrient absorption and transport (*muc* and *pept1*), lipid assimilation (*lpl* and *alp*) and antioxidant enzyme defence (*cat*). The growth enhancing

effect of limonene was not associated with an increased appetite and feed intake of the fish. On the contrary, dietary thymol supplemented up to 500 ppm did not improve the growth of Nile tilapia or regulate the selected genes within key pathways accounting for the growth-promoting effects of phytogenic compounds previously reported in the literature.

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Figures

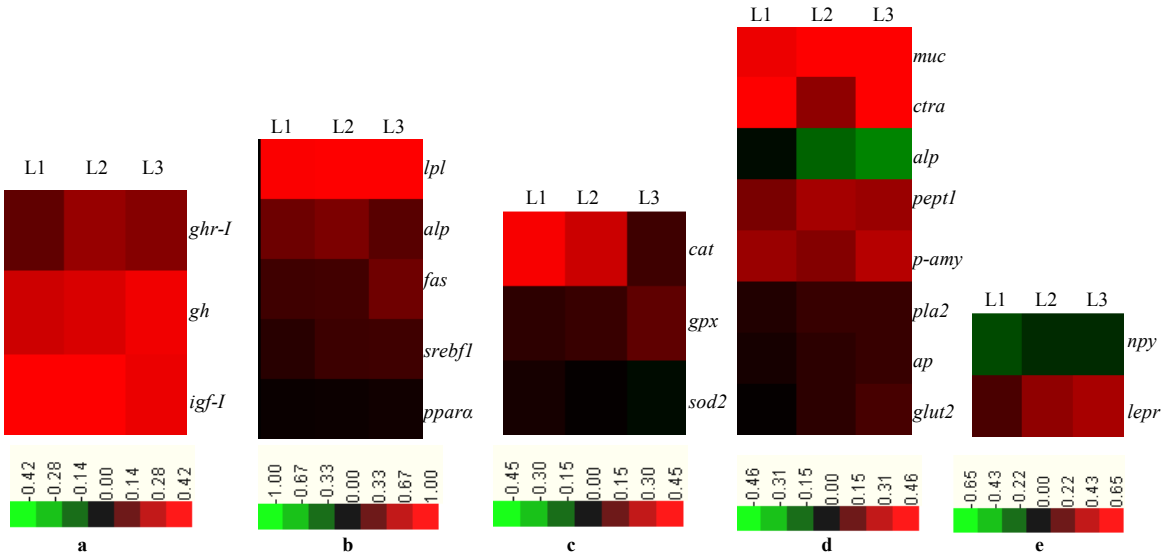

Fig. 1. Heat map indicating expression patterns of twenty selected genes analysed using qPCR data from Nile tilapia fed limonene supplemented diets. The columns represent mean data values of three dietary treatments L1, L2 and L3 (200, 400 and 600 ppm limonene, respectively). The rows indicate each of the analysed genes in the pathways of somatotropic axis growth-mediation (a), lipid and energy metabolism (b), and antioxidant enzyme defence (c) in the liver, nutrient digestion, absorption and transport (d) in the fore intestine and appetite regulation (e) in the brain of Nile tilapia. Expression level of each gene was natural log transformed and normalised against two reference genes. The colour bars at the bottom represent the mean relative expression levels as low (green), neutral (black) or high (red). The black colour represents genes that had similar relative expression level with the control. *lpl*, lipoprotein lipase; *gpx*, glutathione peroxidase; *pparα*, peroxisome proliferator activated receptor alpha; *cat*, catalase; *fas*, fatty acid synthase; *gh*, growth hormone; *ghr-I*, growth hormone receptor I; *srebf1*, sterol regulatory element-binding transcription factor 1; *pla2*, phospholipase A2; *igf-I*, insulin growth factor I; *alp*,

alkaline phosphatase; *muc*, mucin-like protein; *ctra*, chymotrypsin A-like; *sod2*, superoxide dismutase 2; *ap*, aminopeptidase N-like; *glut2*, glucose transporter 2; *pept1*, oligo-peptide transporter 1; *p-amy*, pancreatic alpha-amylase; *npy*, pro-neuropeptide Y-like; *lepr*, leptin receptor variant XI.

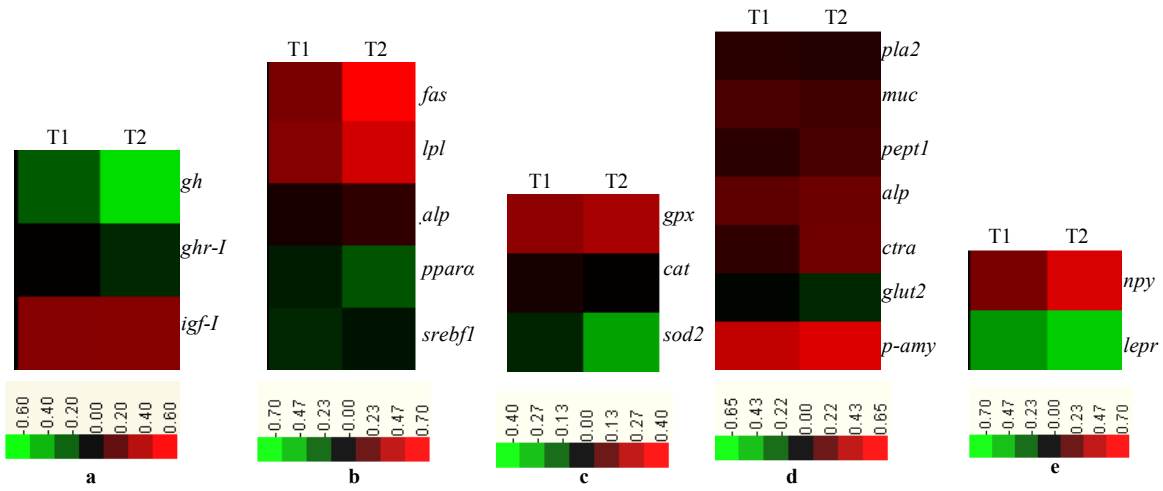

Fig. 2. Heat map indicating expression patterns of twenty selected genes analysed using qPCR data from Nile tilapia fed thymol supplemented diets. The columns represent mean data values of two dietary treatments T1 and T2 (250 and 500 ppm thymol respectively). The rows indicate each of the analysed genes in the pathways of somatotropic axis growth-mediation (a), lipid and energy metabolism (b), and antioxidant enzyme defence (c) in the liver, nutrient digestion, absorption and transport (d) in the fore intestine and appetite regulation (e) in the brain of Nile tilapia. Expression level of each gene was natural log transformed and normalised against two reference genes. The colour bars at the bottom represent the mean relative expression levels as low (green), neutral (black) or high (red). The black colour represents genes that had similar relative expression level with the control. *lpl*, lipoprotein lipase; *gpx*, glutathione peroxidase; *pparα*, peroxisome proliferator activated receptor alpha; *cat*, catalase; *fas*, fatty acid synthase; *gh*, growth hormone; *ghr-I*, growth hormone receptor I; *srebf1*, sterol regulatory element-binding transcription factor 1; *pla2*, phospholipase A2; *igf-I*, insulin growth factor I; *alp*, alkaline phosphatase; *muc*, mucin-like protein; *ctra*, chymotrypsin A-like; *sod2*, superoxide dismutase 2; *ap*, aminopeptidase N-like; *glut2*, glucose transporter 2; *pept1*, oligo-peptide transporter 1; *p-*

amy, pancreatic alpha-amylase; *npy*, pro-neuropeptide Y-like; *lepr*, leptin receptor variant XI.

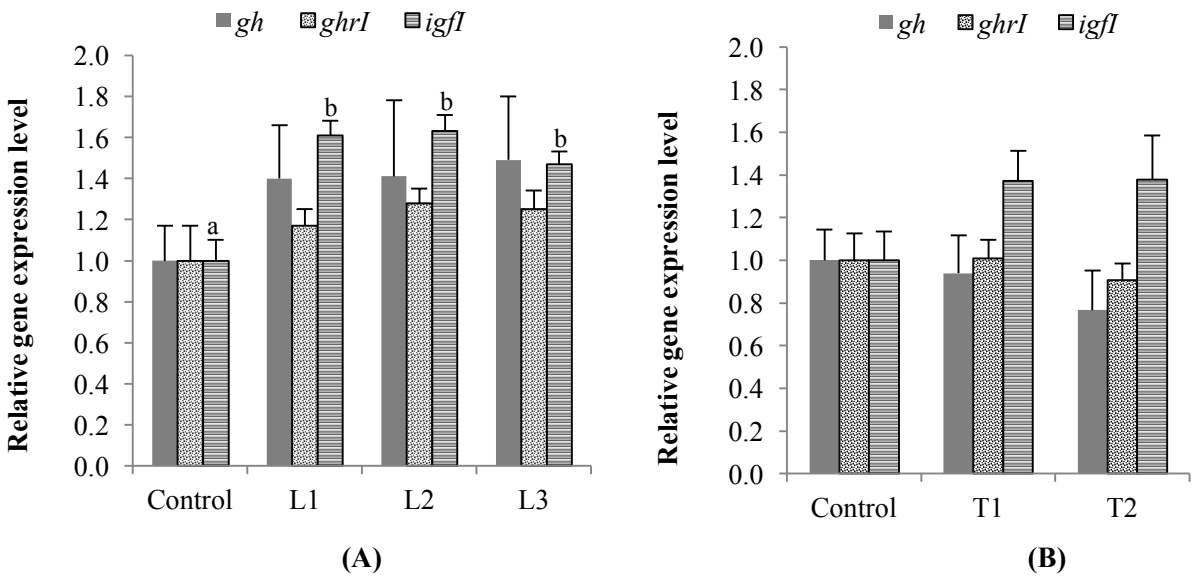

Fig. 3. Expression of growth hormone (*gh*), growth hormone receptor I (*ghr-I*) and insulin growth factor I (*igf-I*) in the liver of Nile tilapia fed on diets with 0 (Control), 200 (L1), 400 (L2) and 600 (L3) ppm limonene (A), and 0 (Control), 250 (T1), and 500 (T2) ppm thymol (B). All values are means of treatments ± standard error (N = 9). Different superscript letters denote significant differences among treatments.

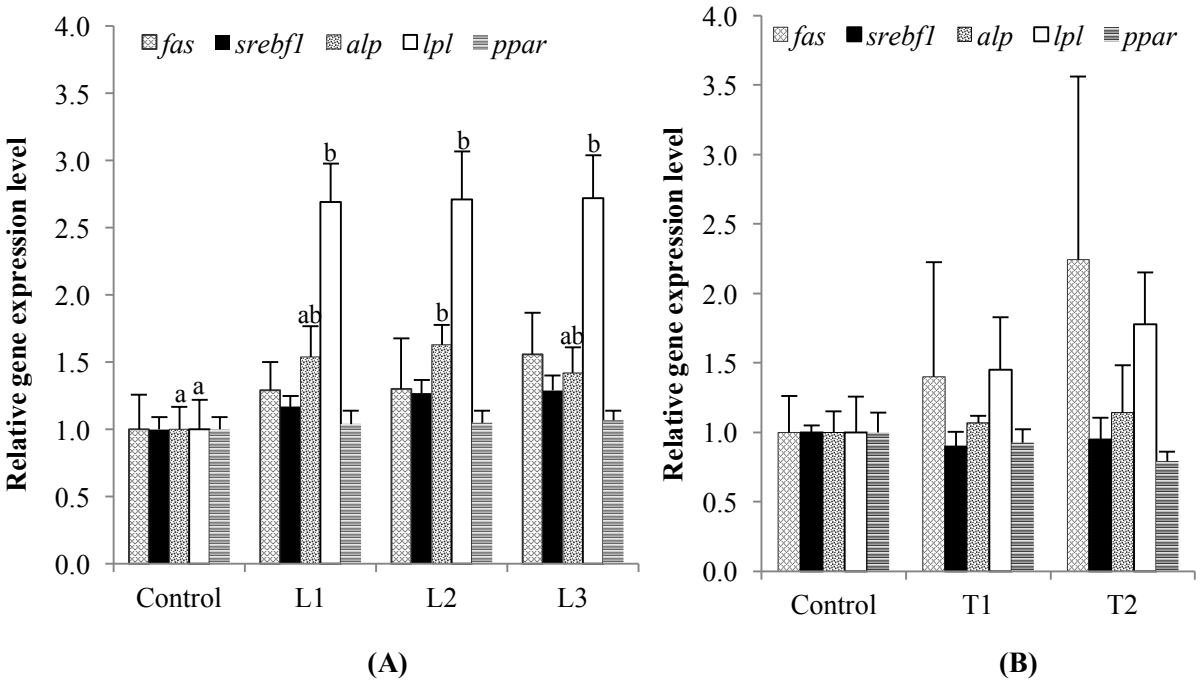

Fig. 4. Expression of fatty acid synthase (*fas*), sterol regulatory element binding transcription factor 1 (*srebf1*), alkaline phosphatase (*alp*), lipoprotein lipase (*lpl*), and peroxisome proliferator-activated receptor (*ppar*) alpha in the liver of Nile tilapia fed on diets with 0 (control), 200 (L1), 400 (L2) and 600 (L3) ppm limonene (A) and 0 (Control), 250 (T1), and 500 (T2) ppm thymol (B). All values are means of treatments ± standard error (N=9). Different superscript letters denote significant differences among treatments.

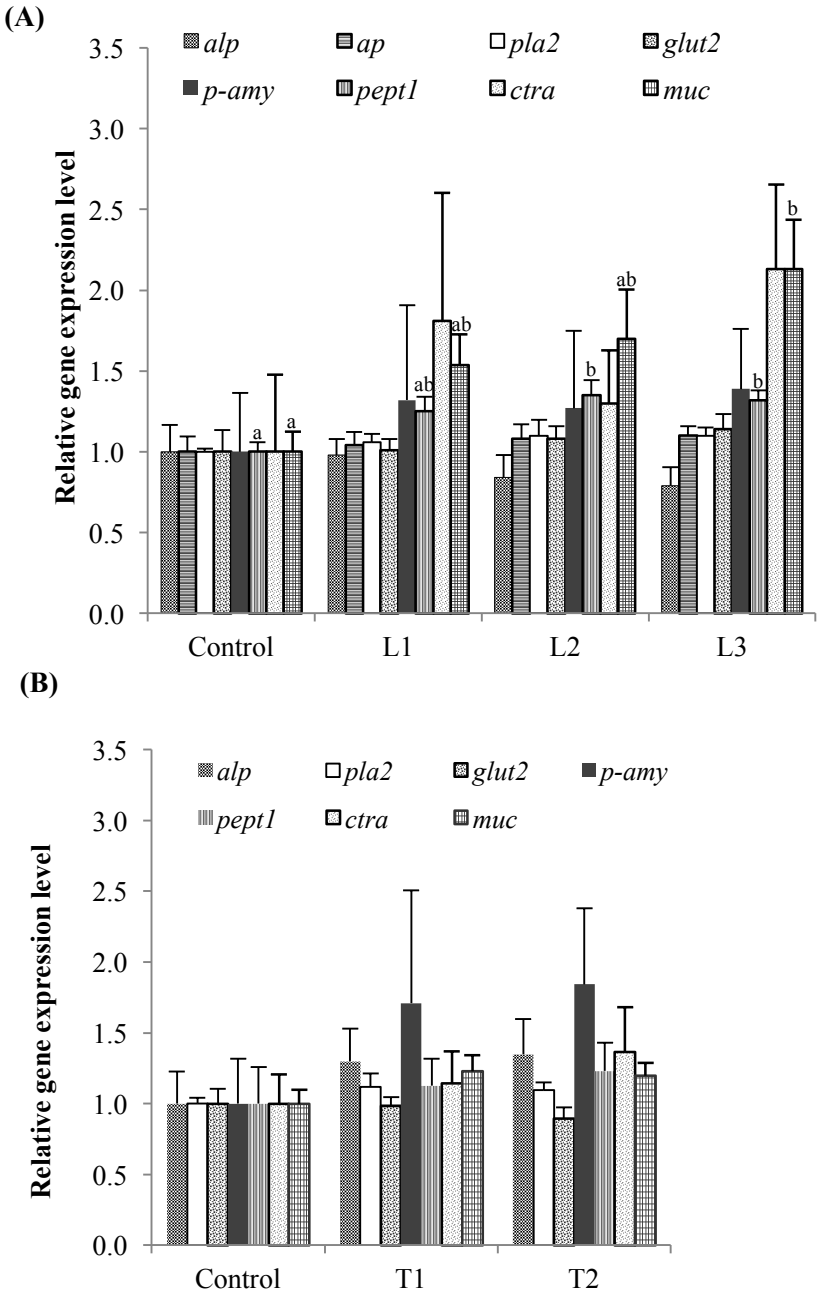

Fig. 5. Expression of alkaline phosphatase (*alp*), amino peptidase (*ap*), phospholipase A2 (*pla2*), glucose transporter 2 (*glut2*), pancreatic alpha-amylase (*p-amy*) oligo-peptide transporter 1 (*pept1*), mucin-like protein (*muc*), and chymotrypsin A-like (*ctra*) genes in the fore intestine of Nile tilapia fed on diets with 0 (Control), 200 (L1), 400 (L2) and 600 (L3) ppm limonene (A) and 0 (control), 250 (T1), and 500 (T2) ppm thymol (B). All values are means of treatments ± standard error (N=9). Different superscript letters denote significant differences among treatments.

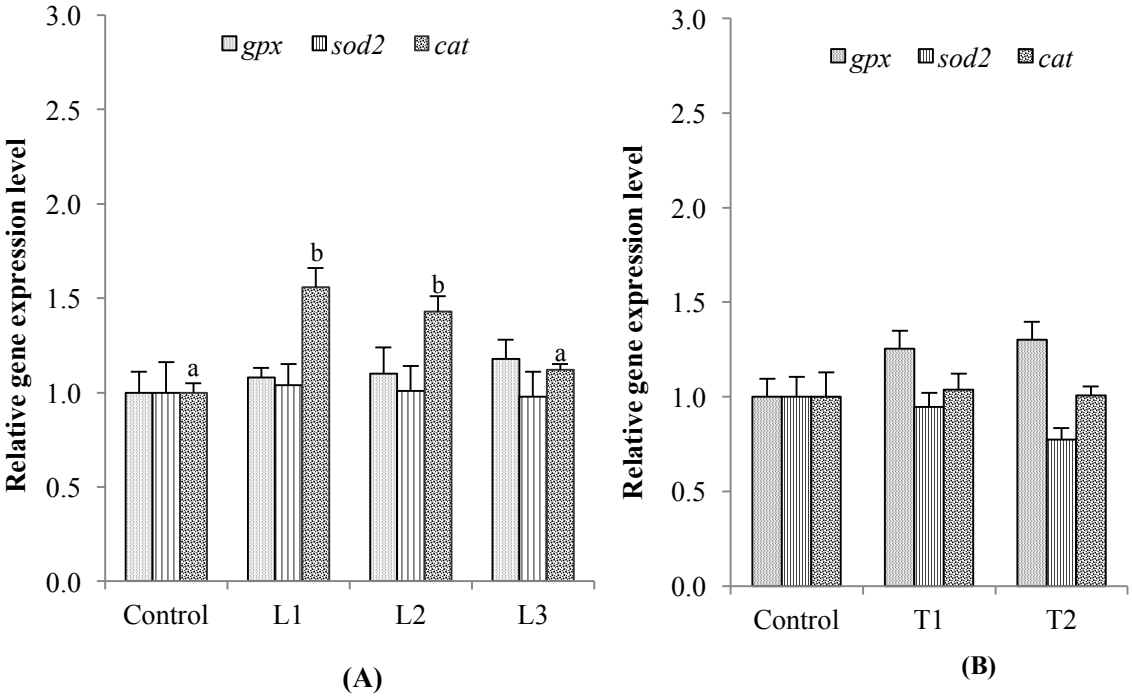

Fig. 6. Expression of antioxidant enzymes glutathione peroxidase (*gpx*) and catalase (*cat*) in the liver, and superoxide dismutase 2 (*sod2*) in the fore intestine of Nile tilapia fed on diets with 0 (Control), 200 (L1), 400 (L2) and 600 (L3) ppm limonene (A), and 0 (Control), 250 (T1), and 500 (T2) ppm thymol (B). All values are means of treatments ± standard error (N=9). Different superscript letters denote significant differences among treatments.

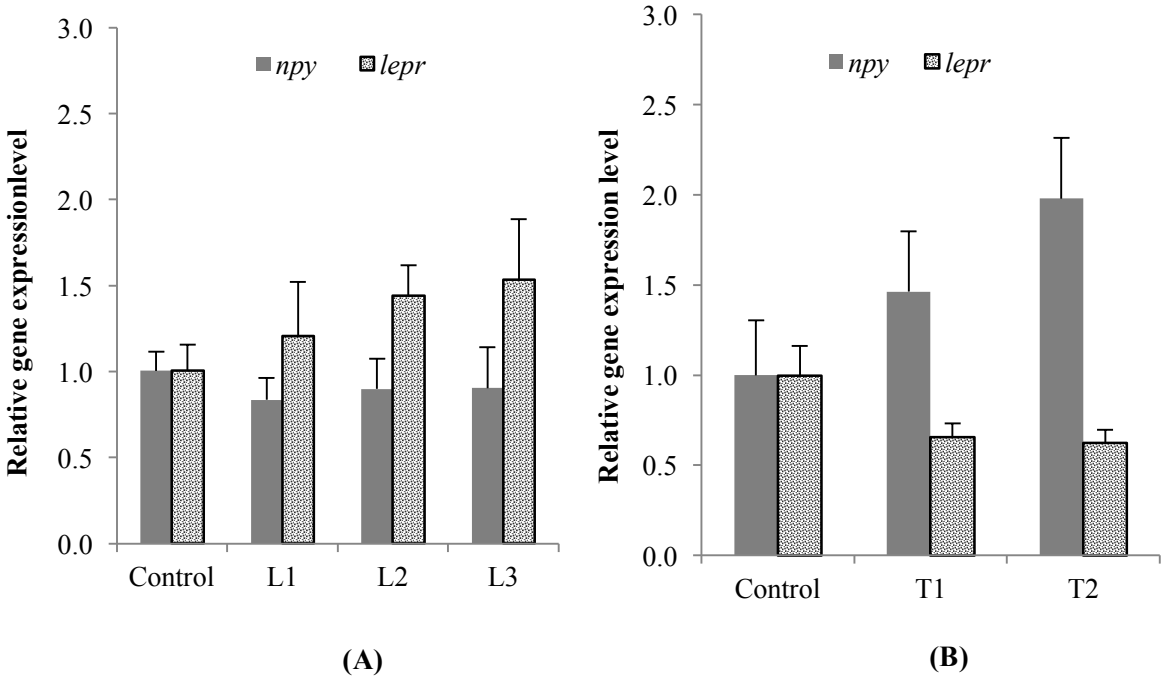

Fig. 7. Expression of pro-neuropeptide y-like (*npy*) and leptin receptor variant XI (*lepr*) genes in the brain of Nile tilapia fed on diets with 0 (control), 200 (L1), 400 (L2) and 600 (L3) ppm limonene (A) and 0 (control), 250 (T1), and 500 (T2) ppm thymol (B). All values are means of treatments ± standard error (N=9).

Tables

Table 1

Proximate composition of the commercial diet used in Experiments I and II.

Analysis	Values
Dry matter (%)	92.3
Moisture (%)	7.7
Crude protein (%)	51.0
Crude fat (%)	20.9
Crude ash (%)	7.5
Crude fibre (%)	1.1
Gross energy (Kj g^{-1})	22.6

Table 2

Nucleotide sequences of primers used for quantitative real-time PCR analysis.

Functional group	Gene symbol	Oligonucleotide sequences (5'- 3')	Size (bp)	Accession number*
Nutrient digestion, absorption and transport	*muc*	F: TGCCCAGGAGGTAGATATGC R: TACAGCATGAGCAGGAATGC	101	XM_005466350.2
	pept1	F: CAAAGCACTGGTGAAGGTCC R: CACTGCGTCAAACATGGTGA	196	XM_013271589
	alp	F: CTTGGAGATGGGATGGGTGT R: TTGGCCTTAACCCCGCATAG	200	XM_005469634.2
	ctra	F: AGTGCCGAGAACATCCAGAC R: GAAGTCTCGGCCACACAAAC	155	XM_003437588.3
	pla2	F: CTCCAAACTCAAAGTGGGCC R: CCGAGCATCACCTTTTCTCG	177	XM_005451846
	glut2	F: TCTAAAGGGGCCGCATGATC R: GAAAGGTGCATCATGAGGGC	153	FJ914656
	ap	F: TTACCACTCCGAACCAGACC R: GAGTAGTTCCCTCCTGCCTC	238	XM_005449270
	p-amy	F: TGGAGGCCCTGGTATCAAAG R: TCCTGTTCCACCACCAGATC	168	XM_003448471.2
Lipid metabolism	*lpl*	F: TGCTAATGTGATTGTGGTGGAC R: GCTGATTTTGTGGTTGGTAAGG	217	NM_001279753.1
	pparα	F: CTGATAAAGCTTCGGGCTTCCA R: CGCTCACACTTATCATACTCCAGCT	106	NM_001290066.1
	srebf1	F: TGCAGCAGAGAGACTGTATCCGA R: ACTGCCCTGAATGTGTTCAGACA	102	XM_005457771.2
	fas	F: TGAAACTGAAGCCTTGTGTGCC R: TCCCTGTGAGCGGAGGTGATTA	144	GU433188
Appetite regulation	*lepr*	F: TCTGTTCCTTTGGGCATAG R: CCCTCCTGATGTCTTTTCCA	143	XM_005460446.1
	npy	F: CCTCTTCCTTACGCATCAGC R: CCCCTCCTCCACTTTACGAT	115	XM_003448854.2
Antioxidant enzyme activity	*gpx*	F: ACAAGTGACATCGAGGCAGA R: CAAACCCAGGCCTGCTATAA	186	NM_001279711.1
	cat	F: TCCTGGAGCCTCAGCCAT R: ACAGTTATCACACAGGTGCATCTTT	79	JF801726
	sod2	F: CTCCAGCCTGCCCTCAA R: TCCAGAAGATGGTGTGGTTAATGTG	58	XM_003449940.3
Somato-tropic axis growthmediation	*gh*	F: TCGGTTGTGTGTTTGGGCGTCTC R: GTGCAGGTGCGTGACTCTGTTGA	90	XM_003442542
	ghr-I	F: ATGGCTCTCTCGCCCTCCTCTAA R: ATGTCGTGTGGTCCCAGTCAGTGA	109	NM_001279601
	igf-I	F: GTCTGTGGAGAGCGAGGCTTT R: CACGTGACCGCCTTGCA	70	NM_001279503
Reference genes	*ef-1α*	F: GCACGCTCTGCTGGCCTTT R: GCGCTCAATCTTCCATCCC	250	NM_001279647
	ß-actin	F: TGGTGGGTATGGGTCAGAAAG R: CTGTTGGCTTTGGGGTTCA	217	XM_003443127.3

muc, mucin-like protein; *pept1*, oligo peptide transporter 1; *alp*, alkaline phosphatase; *ctra*, chymotrypsinogen A-like; *pla2*, phospholipase A2; *glut2*, glucose Transporter 2; *ap*, aminopeptidase N-like; *p-amy*, pancreatic alpha-amylase; lpl, lipoprotein lipase; *pparα*, peroxisome proliferator-activated receptor alpha; *srebf1*, sterol regulatory element binding

transcription factor 1; *fas*, fatty acid synthase; *lepr*, leptin receptor variant X1; *npy*, pro-neuropeptide Y-like; *gpx*, glutathione peroxidase; *cat*, catalase; *sod2*, superoxide dismutase 2; *gh*, growth hormone; *ghr-I*, growth hormone receptor I; *igf-I*, insulin growth factor I; *ef-1α*, elongation factor 1 alpha; *ß-actin,* beta-actin.

*GenBank (http://www.ncbi.nlm.nih.gov/); bp, base pairs

Table 3

Performance and survival rates of Nile tilapia fed on diets with 0 (control), 200 (L1), 400 (L2), and 600 (L3) ppm of limonene after 63 days (Experiment I).

	Experimental diets				
Parameter	**Control**	**L1**	**L2**	**L3**	**P value**
Initial mean weight (g)	1.5 ± 0.0	1.5 ± 0.0	1.5 ± 0.0	1.5 ± 0.0	NS
Final mean weight (g)	40.6 ± 1.4^{a}	44.0 ± 1.4^{ab}	46.4 ± 1.5^{b}	47.2 ± 1.9^{b}	0.012
GR (g d^{-1})	0.6 ± 0.0	0.7 ± 0.0	0.7 ± 0.0	0.7 ± 0.0	NS
% WG	2657.3 ± 44.7^{a}	2917.0 ± 33.5^{ab}	3055.6 ± 38.1^{b}	3133.0 ± 149.1^{b}	0.004
CF	2.0 ± 0.0	2.0 ± 0.0	2.0 ± 0.0	2.0 ± 0.0	NS
% Survival	90.1 ± 5.0	95.5 ± 4.5	96.4 ± 1.8	97.3 ± 0.0	NS
% FI (% body weight d^{-1})	3.2 ± 0.1	3.0 ± 0.1	2.9 ± 0.0	2.8 ± 0.1	NS
FCR	1.1 ± 0.0	1.0 ± 0.0	1.0 ± 0.0	1.0 ± 0.0	NS
PER	1.8 ± 0.1	1.9 ± 0.0	2.0 ± 0.0	2.1 ± 0.1	NS

GR, growth rate; % WG, percentage (%) weight gain; CF, condition factor; % FI, feed intake as percentage of body weight per day; FCR, feed conversion ratio, and PER, protein efficiency ratio.

All values are means of treatments ± standard error. Mean values with different superscript in the same row are significantly different from each other ($P < 0.05$). NS, not significantly different values. For each treatment, N = 111 for initial fish weight; for final fish weight, N = number of alive fish at the end the Experiment; N = 3 replicate tanks for GR, % WG, CF, % survival, % FI, FCR and PER.

Table 4

Two-way ANOVA on the effects of dietary treatment and duration of the experiment on the weight of Nile tilapia fed on limonene supplemented diets for 63 days (Experiment I).

Variable	**Fish weight**
Duration (days)	***
Diet	**
Experiment duration × diet	*

* $P < 0.05$, ** $P < 0.0005$, *** $P < 0.0001$

Table 5

Performance and survival rates of Nile tilapia fed on diets with 0 (Control), 250 (T1), and 500 (T2) ppm of thymol after 63 days (Experiment II).

	Experimental diets			
Parameter	**Control**	**T1**	**T2**	**P value**
Initial mean weight (g)	1.5 ± 0.1	1.5 ± 0.1	1.5 ± 0.1	NS
Final mean weight (g)	37.9 ± 1.3	40.1 ± 1.5	41.0 ± 1.4	NS
GR (g d^{-1})	0.6 ± 0.0	0.6 ± 0.0	0.6 ± 0.2	NS
% WG	2361.0 ± 32.0	2507.4 ± 140.7	2555.8 ± 87.9	NS
CF	1.9 ± 0.0	1.9 ± 0.0	1.9 ± 0.0	NS
% Survival	96.0 ± 2.3	93.3 ± 2.7	90.7 ± 3.5	NS
% FI (% body weight d^{-1})	3.5 ± 0.0	3.2 ± 0.1	3.2 ± 0.01	NS
FCR	1.1 ± 0.0	1.0 ± 0.1	1.0 ± 0.0	NS
PER	1.9 ± 0.0	2.0 ± 0.1	2.0 ± 0.1	NS

GR, growth rate; % WG, percentage (%) weight gain; CF, condition factor; % survival, % FI, feed intake as percentage of body weight per day; FCR, feed conversion ratio; and PER, protein efficiency ratio.

All values are means of treatments ± standard error. NS, not significantly different values ($P > 0.05$). For each treatment, N = 75 for intial fish weight, for final fish weight, N = number of alive fish at end of the Experiment, N = 3 replicates for GR, % WG, CF, % survival, % FI, FCR and PER.

Table 6

Two-way ANOVA on the effects of dietary treatment and duration on the weight of Nile tilapia fed on thymol supplemented diets for 63 days (Experiment II).

Variable	Fish weight
Duration (days)	*
Diet	NS
Experiment duration × diet type	NS

** P < 0.0001, NS, not significantly different (P > 0.05).*