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Nutritional mitigation of winter thermal stress in Nile tilapia by propolis-extract: Associated indicators of nutritional status, physiological responses and transcriptional response of delta-9-desaturase gene

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ABSTRACT

A feeding trial was conducted to investigate the ability of diet supplemented with propolis-extract (winter feed, WF) to assist Nile tilapia, *Oreochromis niloticus* in coping with winter thermal stress. Nile tilapia (average initial weight, 25.40 ± 0.84 g) was fed five isonitrogenous (303.20 g kg^{-1} crude protein) and isocaloric (19.44 MJ kg^{-1} gross energy) diets under thermal winter stress for 60 days. The diets contained five levels of propolis-extract (also known as bee glue): 0 (control), 1, 2, 3 and 4 g kg^{-1} diet. The range of water temperature throughout the experimental period was (16–19 °C). Results indicated that the survival rate was higher in fish fed with the diet supplemented with 4 g kg^{-1} of propolis-extract compared to the remaing diets. Weight gain and specific growth rate were linearly improved with increasing dietary propolis-extract inclusion (linear, $P = .017$; $P = .051$). Feed conversion ratio was significantly improved with the addition of dietary propolis (quadratic, $P = .031$). A linear response in hematocrit (linear, $P = .001$), hemoglobin (linear, P = .031), red blood cells count (linear, $P = .041$) and white blood cells count (linear, $P = .003$) of fish was also observed with the increasing level of propolis-extract. On its turn, significant quadratic decreases in alanine aminotransferase ($P = .001$), aspartate aminotransferase (P = .001), alkaline phosphatase ($P = .002$) and lactate dehydrogenase (P = .002) were detected with increasing dietary propolis-extract levels in the diet. There was a linear response in triglyceride and cholesterol of fish as the level of propolis-extract increased in the diet (linear, $P = .071$ and $P = .003$, respectively). The responses of serum cortisol and glucose in fish under cold stress tended to decrease in response to increasing dietary propolis-extract (respectively, quadratic, $P = .001$ and $P = .012$). The concentration levels of potassium was linearly ($P = .001$), and sodium quadraticly (P = .001) increased with dieatary proplis-extract. The expression of Δ 9D gene of fish under cold stress was upregulated (linearly, P = .001) with increasing dietary propolis-extract levels. To conclude, the optimum dietary inclusion level of propolis was estimated at 4 g kg^{-1} based on the maximization of the growth parameters and survival rate.

1. Introduction

Water temperature is one of the most important environmental factors that determine nearly all physiological processes of fish, including growth, reproduction and metabolism (Azaza et al., 2008). Like other ectotherms, fish may encounter a wide range of daily and seasonal temperature variations in their habitats and deleterious consequences will occur if water temperatures exceed the species-specific thermal tolerance range (Donaldson et al., 2008). It is well known that in the wild fish are exposed to large and rapid changes in environmental temperatures and have developed a variety of physiological responses to cope with these changes (Wu et al., 2019). Nile tilapia have become the most important warm water aquaculture fish group in the world but it is sensitive to cold stress and mass mortality is often caused by winter cold fronts (Zerai et al., 2010). Feed consumption by Nile tilapia starts declining at temperatures below 20 °C, which compromises growth performance (Bhujel et al., 2007). At low temperatures, Nile tilapia are affected by metabolic dysfunctions and immunological disorders,

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making them much more vulnerable to opportunistic infections (Panase et al., 2018). Moreover, high mortalities are related to severe winters with extended cold periods resulting in economic losses (Shi et al., 2015; Nobrega et al., 2017). The tropical/subtropical origins of Nile tilapia are obviously reflected in their thermal preferendum as these fish do not grow well at temperatures below 16 °C and cannot survive for more than a few days below 10 °C (Nobrega et al., 2017).

In order to maximize Nile tilapia yields, juveniles (≈50 g) when transferred to production ponds in the spring will not reach market size fish in the summer, so instead, they should be stocked during the winter season (Charo-Karisa et al., 2005). Thus, the ability to cope with cold stress in winter season is quite important for the survival of fish under farming conditions. Cold stress mitigation is up-regulated by the delta-9-desaturase (Δ 9D) which is widely expressed in many aquatic animals in the process of cold acclimation (Zerai et al., 2010). A major function of Δ 9D is to desaturate membrane lipids in order to sustain membrane fluidity during cold exposure (Polley et al., 2003; Murray et al., 2007).

As information is still scarce, there is an urgent need to develop new technologies to improve the production of Nile tilapia in subtropical regions, where water temperatures have large seasonal fluctuations. Nutritional strategies can help fish to manage stress levels, and functional feed additives are one of the interesting nutritional options. Several strategies have been used to prepare Nile tilapia to endure winter season such as pond coverings or increased depth of pond (Nour et al., 1996; El-Sayed et al., 1996; Abdel-Aal, 2008) and biofloc technology (Crab et al., 2009; Soltan et al., 2015). Studies focused on nutritional approaches for tilapia to mitigate winter stress are limited (Atwood et al., 2003; Nobrega et al., 2017). As for other species, Ibarz et al. (2010) noted an improved condition of seabream fed diets with a high-energy content before the cold season. Also, growth performance and metabolic responses of barramundi exposed to sub-optimal temperature were significantly improved in fish fed with polyunsaturated fatty acid supplemented diet (Williams et al., 2006; Alhazzaa et al., 2013). Tort et al. (2004) and Schrama et al. (2017) observed an improvement of immune status upon cold exposure for seabream fed a winter feed supplemented with vitamin C, vitamin E, choline, inositol, minerals, higher levels of highly unsaturated fatty acids and phospholipids. Richard et al. (2016), recommended that to decrease cold stress in cultured species during winter a diet should strengthen the defense mechanisms of fish through prophylactic administration of immunostimulants. Also, diets rich in poly unsaturated fatty acids such as linseed or sunflower oils could improve the growth of Nile tilapia growth reared at 22 °C (Corrêa et al., 2017, 2018).

One class of promising non-specific immunostimulants, the beehive products, have received increasing attention as they can be easily and cost-effectively incorporated into the diet and elicit low environmental impact (Acar, 2018). Propolis (bee glue) is a mixture of a plant product and secretions by honeybees (*Apis mellifera*), with > 200 bioactive compounds already identified in different samples (Morsy et al., 2015; Acar, 2018). Propolis is known to be active against (gram positive) bacteria, viruses, fungi, parasites, oxidants, inflammation and serve as an immunomodulator. The beneficial characteristics propolis are mainly associated with its phenolic components such as flavonoids (Alencar et al., 2007; de Aguiar et al., 2013; Morsy et al., 2013). Propolis has been supplemented in the diet to enhance the growth performance in Nile tilapia, *Orecochromis niloticus*, at a level of 10 g kg^{-1} (Abd-El-Rhman, 2009) and rainbow trout (*Oncorhynchus mykiss*) at levels of 2 and 4 g kg^{-1} (Kelestemur et al., 2012). Propolis was reported to modulate the non-specific immune response in rainbow trout (Deng et al., 2011). The objectives of the current study aimed to verify to what extent the use of propolis-extract could mitigate winter thermal stress by improving the nutrition, hematological indices, blood biochemical indices and selected gene expression marker (Δ 9D) and survival of juvenile Nile tilapia.

2. Materials and methods

2.1. Propolis-extract

A crude propolis sample was provided by the Honeybee project, Faculty of Agriculture, Benha University, Egypt. Propolis-extract was prepared according to Morsy et al. (2013) with some modification. In brief, 200 ml ethanol (700 ml l^{-1}) were added to 20 g minced crude propolis in bottles which were sealed and continuously shaken in the dark for 1 day at room temperature. The extract was then filtered, twice through a filter paper (Wattman no. 41). The supernatant was transferred to a rotary evaporator (R-1001VN/R-1001LN, Zhengzhou Greatwall Scientific Industrial and Trade Co., Ltd., China) and treated for 30 min at 42 °C to remove the ethanol. The concentrated extract was lyophilized for 3 days to obtain pure propolis extracts in a dry form. A Shimadzu Model GC-2010 Series gas chromatograph, coupled with a Shimadzu series mass-selective detector quadrupole mass spectrometer (model GCMS-QP 2010, Plus Shimadzu, Kyoto, Japan) was used for the analysis of propolis-extract (Table 2) according to the method of Fernández et al. (2008).

2.2. Diets

Five isonitrogenous (300 g kg^{-1} crude protein) and isocaloric (19.44 MJ kg^{-1} gross energy) diets were formulated (Table 3). The five dietary treatments were: a base diet as a control, without propolis-extract supplementation (0 g kg^{-1} diet), and four base diets supplemented with 1, 2, 3 and 4 g kg^{-1} propolis-extract, respectively. All feed ingredients were thoroughly mixed, and then pelletized on a laboratory pellet mill at the National Institute of Oceanography and Fisheries, Cairo Governorate, Egypt (a California Pellet Mill, San Francisco, CA, USA). Pellets were sun-dried (35 °C) for 48 h and stored at 4 °C. Diet proximate composition was analyzed according to AOAC (1995). Dry matter was determined after drying the samples in an oven (105 °C) for 24 h, ash was determined by incineration at 550 °C for 12 h, crude protein was determined by micro-Kjeldhal method, N × 6.25 (using Kjeltech auto analyzer, Model 1030, Tecator, Höganäs, Sweden) and crude fat by Soxhlet extraction with diethyl ether (40–60 °C). Nitrogen-free extract was computed by taking the sum of values for crude protein, crude lipid, crude fiber, ash and moisture then subtracting this sum from 100.

2.3. Fish and experimental design

Healthy fingerlings of Nile tilapia (*O. niloticus*) were obtained from the farm of Aquaculture El-Kanater El-Khayria, Fish Research Station, National Institute of Oceanography and Fisheries (NIOF), Egypt on 15 November 2017. Prior to the start of the experiment, 675 fish were randomly distributed into four fiberglass tanks (1 m^3) for 2 weeks. Fish were acclimated in freshwater under a 12 L / 12D photoperiod regime. During that period fish were fed twice daily at 9:00 and 17:00 with a commercial diet (protein 30%, lipid 6%) until the start of the trial. The study started on 25 December 2017 and lasted until 22 February 2018 (60 days). Fish (initial weight 25.5 ± 0.95 g) were allocated into 15 concrete ponds (0.5 m^3 each and 1.75 m depth; three replicate groups) with a density of 50 Nile tilapia per pond. The diets were fed to experimental groups at 1.5% body weight/day (Dan and Little, 2000), divided in two servings (11:00 and 15:00 h), 6 days per week three hours after sunrise to assure a wormer water temperature. Twenty fish were randomly netted from each pond and weighed to the nearest gram every 2 weeks for track growth and feed conversion. Cumulative mortality rates (%) were also recorded. No aeration was provided, but 20% of the water volume was daily replaced (0.1 m^3 day^{-1} $pond^{-1}$).

During the trial, temperature was monitored daily (Model 30/10FT temperature/salinity meter; Yellow Springs Instrument Company, Yellow Springs, OH, USA). The pH (using an Accumet model 915 pH;

Table 1
Ambient water temperature average during experimental period from 25 December to 24 February.

Treatment periods	Ambient temperature average
25 Dec - 8 Jan (1–2 weeks)	19 ± 0.5 °C
9 Jan – 23 Jan (2–4 weeks)	16 ± 0.3 °C
24 Jan – 9 Feb (4–6 weeks)	17 ± 0.3 °C
10 Feb – 24 Feb (6–8 weeks)	19 ± 0.4 °C

Fisher Scientific, Pittsburgh, PA, USA) ammonia and nitrite according to APHA (1989) and dissolved oxygen (using an Model 58 dissolved oxygen meter; Yellow Springs Instrument Company) were weekly monitored. Water quality characteristics were: water temperature: (Table 1); pH 7.5 ± 0.8; dissolved oxygen 8.5 ± 1.3 mg L^{-1}; ammonia-N 0.2 ± 0.03 mg L^{-1} and nitrite 0.6 ± 0.02 mg L^{-1}.

2.4. Growth performance and feed utilization parameters

Growth performance and feed utilization were measured in terms of final body weight (g), weight gain (WG), specific growth rate (SGR, % day^{-1}) feed conversion ratio (FCR), Protein efficiency ratio (PER) and feed intake. Growth response parameters were calculated as follows: Weight gain (WG) = final body weight (g) – initial body weight (g).

Specific growth rate (SGR) = 100 × ((Ln (W2)-Ln (W1))/T), Where: Ln = the natural log; W1 = initial body weight; W2 = final body weight and T = period of study (60 days).

Feed conversion ratio (FCR) = Feed intake (FI) (g)/WG (g).

Protein efficiency ratio (PER) = WG (g)/Protein intake (g).

2.5. Blood collections and analysis

At the end of the feeding experiment, fish were anaesthetized by immersion in water containing 0.1 ppm tricaine methane sulphonate (MS-222). Ten fish from each replicate (divided into two groups) were sampled for blood collection. Blood samples were collected from the caudal vein of fish. The first portion was collected with anticoagulant 10% ethylenediaminetetraacetate (EDTA) to determine the hematocrit (Htc) and hemoglobin (Hb) according to the standard methods as described by (Rawling et al., 2009). The second portion was allowed to clot overnight at 4 °C and then centrifuged at 3000 rpm for 10 min. The non-hemolysed serum was collected and stored at −20 °C until use. Levels of serum aspartate aminotransferase (AST), alanine aminotransferase (ALT) were estimated according to the method described by Reitman and Frankel (1957). Serum cholesterol, triglycerides, low density lipoprotein-cholesterol (LDL-C), high density lipoprotein-cholesterol (HDL−C) and glucose were measured using standard Kits (Modern Laboratory Kits). Cortisol levels (ng ml^{-1}) were determined by chemiluminescence with an immulite kit.

2.6. Expression of delta-9-desaturase gene

2.6.1. Isolation of total RNA

Total RNA was isolated from liver samples (three fish from each replicate) by the standard Promega RNA Isolation Kit (Cat No. Z3100, USA) and reagent extraction method according to the manufacturer's instructions. The quantity of the RNA was assessed using a Nano-Drop spectrophotometer (NANODROP 1000, Thermo Scientific, USA). The integrity (quality) was checked by denaturing gel electrophoresis (1% agarose gel) and the purity by measuring the OD260/OD280 absorption ratio (> 1.95).

2.6.2. First strand cDNA synthesis

cDNA was generated from 1 μg of total RNA using High Capacity cDNA (Thermo Fisher Scientific, Cat. No 0.436, 8814) reverse transcriptase kit for reverse transcriptase polymerase chain reaction (RT-PCR) following the manufacturer's protocol. The product of the first strand cDNA synthesis was stored at −80 °C until the quantitative RT-PCR (qRT-PCR) runs.

2.6.3. Real-time quantitative RT-PCR

Delta-9-desaturase (Δ 9D) and 18S rRNA gene primers were purchased from Invitrogen, Germany. The primer sequences and calculated efficiency are enlisted in Table 4. Triplicate qPCR reactions were performed on an AriaMx Real-Time PCR System (Agilent Technologies). Reactions containing 5 μl of 5 × diluted cDNA, 10 pmol each of forward and reverse primers, 0.4 μl ROX dye solution (1:500 dilution) and 10 μl SYBR Green PCR MasterMix (Maxima SYBR Green qPCR, Thermo Fisher Scientific, Cat. No # k0251) were performed in a four-step experimental run protocol: a denaturation program (10 min at 95 °C); an amplification and quantification program repeated 40 times (30 s at 95 °C, 50 s at 55 °C and 40 s at 72 °C); a melting curve program (55–95 °C with a heating rate of 0.10 °C/s and a continuous fluorescence measurement) and finally a cooling step. Melt curve analyses of the target genes and reference genes resulted in single products with specific melting temperatures. In addition, “no-template” controls (i.e. with water sample) for each set of genes was also run to ensure no contamination of reagents, no primer–dimer formation. Moreover, 18S rRNA gene was used as an internal standard. The relative mRNA expression levels were calculated by a standard curve method. The expression levels of gene were normalized to the levels of 18S rRNA gene in the same sample. Standard curve was generated by serial dilution of a random mixture of control samples.

2.7. Statistical analysis

Polynomial contrasts were used to detect linear and quadratic effects of various dietary propolis levels on the observed response variables. The level of significance adopted was 5%. A statistical package SAS was used for all statistical analysis response variables. All percentage data were arc-sin transformed prior to analysis (Zar, 1984); however, the data are presented untransformed to facilitate comparisons.

3. Results

3.1. Survival

The survival rate of Nile tilapia fingerlings during 60 days of winter season are presented in (Fig. 1). Survival was significantly lower ($P < .05$) in fish fed the control diet. The higher survival rate was observed in fish fed the diet supplemented with 4 g propolis-extract kg $diet^{-1}$.

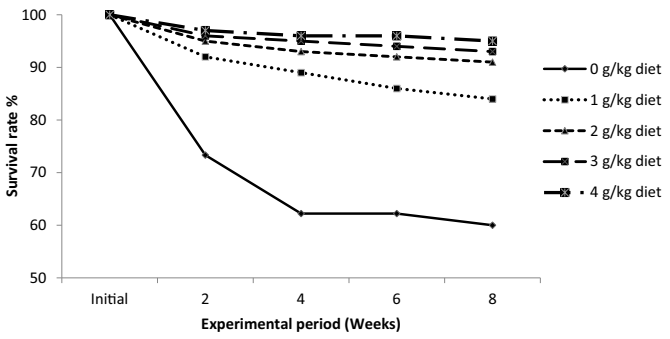

Fig. 1. Effect of different levels of propolis on cummulative survival of Nile tilapia fingerlings during 60 days of winter season.

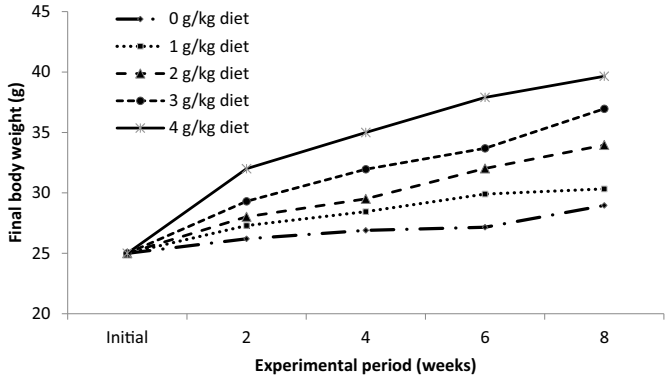

Fig. 2. Effect of different levels of propolis-extract on final body weight of Nile tilapia fingerlings under cold temperature.

3.2. Growth performance and feed utilization

Body weight gain (g) of tilapia is shown in Fig. 2 as affected by different level of propolis-extract. Average WG was significantly higher ($P < .05$) in the supplemented diets compared to the control. Differential growth among the treatments become markedly different from week two onwards, and the lower WG gain was observed in fish fed the control diet (Fig. 3). Final body weight (FBW), weight gain (WG) and specific growth rate (SGR) were linearly improved with the increasing addition of dietary propolis-extract (linear, $P = .021 = 0.017$; $P = .051$), (Table 5). Dietary propolis-extract had a significant improving effect on feed intake (FI) (linear response, $P = .001$) and feed conversion ratio (FCR) (quadratic, $P = .031$).

3.3. Hematological indices

Table 6 shows the effect of propolis-extract on Nile tilapia hematological indices including hematocrit (Htc), hemoglobin (Hb), the red blood cell (RBCs) and white blood cell (WBCs). There was a quadratic response in Htc, Hb and red blood cells count (RBCs) and white blood cells count (WBCs) of fish as the level of propolis-extract increased in the diet (quadratic, $P = .001$, $P = .031$, $P = .041$ and $P = .003$), respectively with the highest values in tilapia fed diet supplemented with 4 g kg^{-1} propolis-extract.

3.4. Blood biochemical indices

Significant quadratic decreases in alanine aminotransferase (ALT) ($P = .001$), aspartate aminotransferase (AST) ($P = .001$), alkaline phosphatase (ALP) ($P = .002$) and lactate dehydrogenase (LDH) ($P = .002$) were observed with the increased levels of propolis-extract

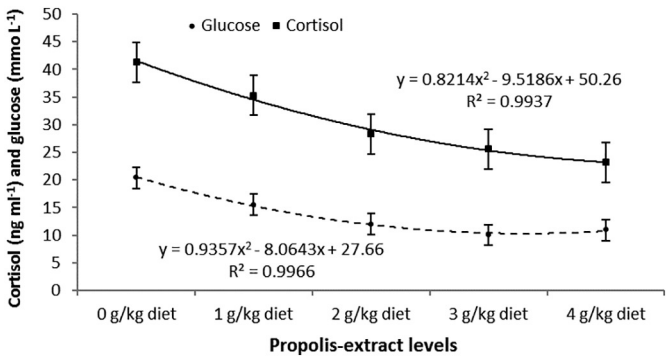

Fig. 3. Effect of different levels of propolis on serum cortisol (quadratic, $P = .001$) and serum glucose concentration (quadratic, $P = .012$) of tilapia fed different levels of propolis-extract in diets under cold temperature.

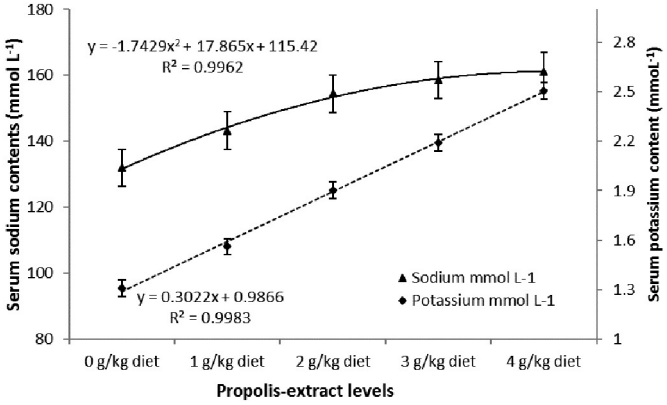

Fig. 4. Effect of different levels of propolis on serum potassium (linear, P = .001) and serum sodium concentration (quadratic, $P = .017$) of tilapia under cold temperature.

in the diets (Table 7). There was a linear response in triglyceride and cholesterol of fish with the increased levels of propolis-extract in the diets (linear, $P = .071$ and P = .003, respectively) (Table 7). There was a quadratic response in low density of lipoprotein cholesterol (LDL-C) (P = .001) and high density of lipoprotein cholesterol (HDL−C) (P = .001) was observed under the same circumstances (Table 7).

Serum cortisol and glucose in the experimental fish evidenced a quadratic response with a decreasing tendency to the increasing level of dietary propolis-extract (Fig. 3). Likewise, both the sodium and potassium concentration levels were respectively, linear (P = .001) and quadratic ($P = .017$) increased with the increase of dietary propolis-extract in the diets with a peak in tilapia fed 4 g kg-1 propolis-extract supplemented diet (Fig. 4).

3.5. Gene expression

Gene expression of delta-9-desaturase (Δ 9D) in livers of Nile tilapia is illustrated in Fig. 5 as influenced by different level of propolis-extract supplementation. The expression of Δ 9D gene of fish under cold stress was linearly upregulated (linear, P = .001) with increasing dietary propolis-extract level.

4. Discussion

While several studies addressed the effect of propolis-extract (bee glue) supplementation in fish nutrition, the influence of propolis extract on tilapia to cope with low temperature has not been reported. The present study examined that effect and it was observed that exposure to low temperatures affects the mortality rate, but fish receiving diets rich in propolis-extract (with an optimum at 4 g kg^{-1} inclusion rate) could overcome the low temperature and increase survival, growth and feed utilization. The survival rate of control fish was low, but similar to Behrends et al. (1990) and Dan and Little (2000) indicating that the immune system of fish was depressed during cooler periods. To our knowledge, there is no available literature about the effect of propolis-extract on survival and growth in fish reared under extended periods of cold temperature to substantiate our findings. In the present study, the improved growth of fish fed a diet supplemented with propolis-extract may be due to the beneficial effect of propolis on the antimicrobial activity of the propolis-extract components (Kujumgiev et al., 1999; Morsy et al., 2013), resulting in better intestinal health and improved digestion and absorption. In addition to its antimicrobial properties, propolis possesses antioxidant biological activities (Orhan et al., 1999). Moreover, propolis contains vitamins (B1, B, C, E) and essential minerals (iron, aluminum, manganese and silicon) which improve the digestive cofactors and enzymatic activity such as succinic dehydrogenase, glucose-6-phosphatase, adenosine triphosphatase, and acid

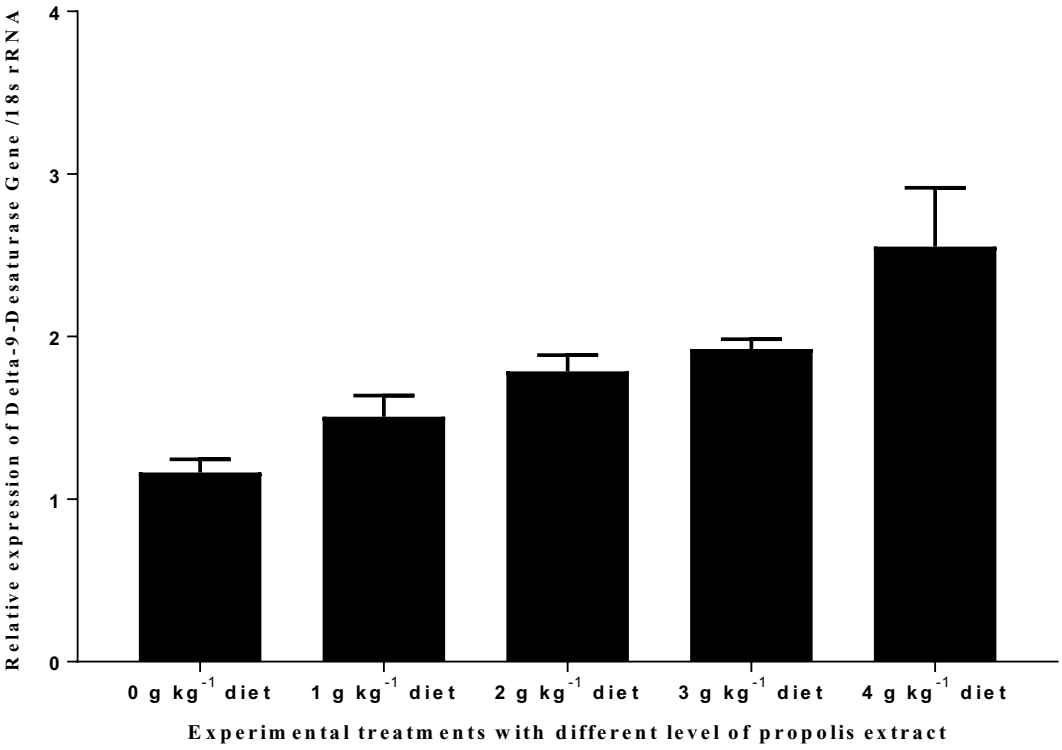

Fig. 5. Response of relative expression of delta-9-desaturase gene (linear, P = .001) of tilapia fed different levels of propolis-extract supplemented in diets under cold temperature.

Table 2
Percentage of compounds identified by gas chromatography/mass spectrometry in ethanol extract of Egyptian propolis (%).

Compounds	Rt (min)	Content (%)
Hexadecanoic acid, trimethylsilyl ester	28.11	24.42
3-(Hydroxymethyl)-1-phenyl-1-heptadecyn-3-ol	33.63	13.85
4-quinolinamine, N,3-diphenyl-2-(phenylmethyl)	36.63	11.03
3,5,7-Tris (trimethylsilyloxy) flavone	39.74	10.15
2-Methyl-1,6-bis[(trimethylsilyl) oxy] anthra-9,10-quinone	39.58	5.95
Trimethylsilyl 3-phenyl-2-propenoate	17.72	3.71
Tetradecanoic acid, trimethylsilyl ester	24.28	3.58
3-Methyl-3-butenyl isoferulate-tms-derivativea	32.66	2.96
Ferulic acid	28.84	2.68
Bis-*O*-trimethylsilyl-palmitinic acid-glycerin-(1)-monoester	37.23	2.65
Octadecanoic acid, trimethylsilyl ester	31.59	2.6
Cinnamic acid, 3,4-dimethoxy-, trimethylsilyl ester	27.90	2.56
1 h-Imidazole-4-carboxamide, 5-amino-, tetrakis (trimethylsilyl) Derivative	35.91	1.94
Hexadecanoic acid, ethyl ester	27.09	1.78
2′,4′,6′-Tris(trimethylsilyloxy)chalcone	36.33	1.73
1 h-Pyrrole, 5-(3-methoxyphenyl)-2,3-diphenyla	39.15	1.61
1,2,4-Tris (*tert* butyldimethylsiloxy) naphthalene	35.36	1.5
Meso-dimethyl-R-3,C-6-bis [(*tert*-butyldimethylsilyl) oxy]-T-4,5-epoxycyclohex-1-ene-1,2-dicarboxylatea	41.03	1.24
Silane, [1-(5-ethenyltetrahydro-5-methyl-2-furanyl)-1-methylethoxy] trimethyl-, cisa	29.16	1.06
Benzoic acid, 2-[(trimethylsilyl)amino]-3-[(trimethylsilyl)oxy]-, methyl ester[a]	37.39	0.96
2-Propen-1-one, 1-(2,6-dihydroxy-4-methoxyphenyl)-3-phenyl-, (E)a	34.44	0.84
Dodecanoic acid, trimethylsilyl ester	20.11	0.69
6,7-Dihydroxycoumarin di-tms	21.95	0.51

Table 3
Composition and proximate analysis of the basal diet (g kg^{-1} dry matter).

Ingredients	Propolis extract (g kg^{-1})				
	0 g kg^{-1}	1 g kg^{-1}	2 g kg^{-1}	3 g kg^{-1}	4 g kg^{-1}
Fish meal	100	100	100	100	100
Soybean meal	460	460	460	460	460
Yellow corn	295	295	295	295	295
Wheat bran	100	99	98	97	96
Soybean oil	30	30	30	30	30
Vitamins and minerals[a]	15	15	15	15	15
Propolis extract	0	1	2	3	4
Proximate analysis (g kg^{-1} dry matter basis)					
Crude protein	300.50		298.80	298.00	297.30
Lipids	56.91		57.20	56.71	57.21
Ash	54.30		54.12	53.81	53.21
Total carbohydrate[b]	588.30		589.88	591.84	592.28
Gross energy (MJ kg^{-1})[c]	19.45		19.44	19.42	19.43

[a] Vitamins and minerals mix: MnSO4, 40 mg; MgO, 10 mg; K2SO4, 40 mg; ZnCO3, 60 mg; KI, 0.4 mg; CuSO4, 12 mg; Ferric citrate, 250 mg; Na2SeO3, 0.24 mg; Co, 0.2 mg; retinol, 40,000 IU; cholecalciferol, 4000 IU; α-tocopherol acetate, 400 mg; menadione, 12 mg; thiamine, 30 mg; riboflavin, 40 mg; pyridoxine, 30 mg; cyanocobalamin, 80 μg;;nicotinic acid, 300 mg; folic acid, 10 mg; biotin, 3 mg; pantothenic acid, 100 mg; inositol, 500 mg; ascorbic acid, 500 mg.

[b] Total carbohydrate = 100 - (crude protein + lipid + ash).

[c] Calculated using gross calorific values of 23.63, 39.52 and 17.15 KJ/ g for protein, fat and carbohydrate, respectively according to (Brett, 1971).

phosphatase. These compounds and enzymes can contribute to improve the digestion and nutrient absorption with a subsequent increase in the fish-weight (Acar, 2018). Some studies have reported the effects of propolis on metabolism and consequently on the growth performance of Nile tilapia (Meurer et al., 2009), rainbow trout, *Oncorhynchus mykiss* (Deng et al., 2011) and *Anguilla japonica* (Bae et al., 2012). Chu (2006) described the effect of propolis addition on the immune system and fish disease resistance of carp (*Carassius auratus gibelio*) and Abd-El-Rhman (2009) in Nile tilapia, both concluding that propolis addition to diets improves growth and stimulate the immune response of these species.

Table 4
List of real time qPCR assays used in this experiment.

Gene	Primers	GenBank no.
18s rRNA	F: GTTGCAAAGCTGAAACTTAAAGG R: TTCCCGTGTTGAGTCAAATTAAGC	AF497908.1
Δ 9D	F: ATCACCACACGTTCCCATATGAC R: CCAGACCCAAGAAACACATGAAG	AY150696

F: Forward primer.
R: Reverse primer.

However, Šegvić-Bubić et al. (2013) observed that the addition of propolis in diets for juvenile European sea bass (*Dicentrachus labrax*) to mitigate the effect of winter thermal stress is limited. Thus, the dose response to the addition of propolis in practical fish diets may be species dependent, so further investigation to establish the immune and growth properties for propolis during winter thermal stress is still required.

Estimation of hematological indices often provides important indicators of physiological responses and nutritional status of the species (Soltan et al., 2017; Hassaan et al., 2018). To the best of our knowledge, there are no studies concerning the effect of propolis-extract and the values of Hematocrit (Htc) and hemoglobin (Hb) in fish exposed to low temperature conditions. In the present study, the addition of dietary propolis-extract (3 and 4 g kg^{-1} of diet) promoted a significant improvement in the Htc and Hb levels compared to control diet. The decrease in the values of Hb in the control group may be the result of erythropoiesis or shrinking of red blood cells, as well as dependency on bound oxygen transport as a result of lower oxygen requirements at low temperature and increased solubility of oxygen in plasma (Atwood et al., 2003). Also, the reduced hematocrit and hemoglobin may indicate that, the fish are not eating well (which may result in anemia) or suffering bacterial infections (Ranzani-Paiva et al., 2004). The increase of Htc and Hb levels could explain the protective effect of propolis-extract supplemented in diet on the health status of the fish. Also, the addition of propolis-extract in Nile tilapia diets may play a role in the increase of erythropoiesis in hematopoietic of fish, because as reported in several studies, the supplementation of direct propolis, and ethanolic extract propolis, in tilapia diets significantly increases ($P < .05$) the hematocrit values (Abd-El-Rhman, 2009; Acar, 2018).

Monitoring the biochemical changes in hepatic enzymes activities such as ALT, AST, LDH and ALP are an indication of liver function (Zhai et al., 2014; Hassaan et al., 2019). The present study noted that the addition of propolis-extract at level 3 and g kg^{-1} decreased the activities of ALT, AST, LDH and ALP of tilapia reared under long-term cold temperature stress. The elevation of liver enzymes activities in fish fed control diet under these conditions may indicate enzyme leakage across damaged plasma membranes and/or increased synthesis of liver enzymes by the action of cold stress (Yang and Chen, 2003). According to Shi et al. (2015), in short-term cold stress (5 days), the profiles of ALT, AST and LDH become prominently elevated in Nile tilapia. Likewise, an increased activity of LDH was reported in milkfish, *Chanos chanos,* and grass carp, *Ctenopharyngodon idella,* under short-term cold shock (Kuo and Hsieh, 2006). In the present study, reduction in ALT, AST, ALP and LDH in Nile tilapia fed propolis-extract supplemented diets demonstrated that the components of propolis (cinnamic acid and tectochrysin and pinocembrin) have a hepatoprotective role. These results are in disagreement with Šegvić-Bubić et al. (2013) who reported that AST in European seabass decreased with increasing levels of dietary propolis supplementation, while after exposure to short cold temperatures stress, AST remained unaffected. Acar (2018) showed that dietary propolis-extract caused decreases in serum ALT, AST, ALP and LDH values of Nile tilapia at levels of 2 and 4 g kg^{-1}. Also, Talas and Gulhan (2009) reported that propolis has a protective effect on liver cells and their enzymes in rainbow trout (*Oncorhynchus mykiss*). On the other hand, Kashkooli et al. (2011) indicated that supplementation with different levels of ethanolic propolis-extract had no effect on ALP of juvenile rainbow trout.

Triglycerides are mainly synthesized in the liver and are monitored to evaluate nutritional status, total lipid metabolism, and high concentrations may occur with nephritic syndrome or glycogen storage disease (Coz-Rakovac et al., 2005). Any change in plasma triglycerides level is used as an indicator of liver dysfunction (Osman et al., 2010). The present study showed that serum triglycerides, cholesterol, LDL-C and HDL-C content tend to increase, but still within normal contents, at a faster rate in fish fed propolis-extract supplemented diets than the control diet. The increase in triglycerides trend, further suggested that the decreasing content of serum triglycerides and cholesterol in the control may disturb lipid mobilization and circulation between liver and tissue under long-term cold stress (Chang et al., 2006). The elevation in the HDL-C indicated that HDL-C was transporting cholesterol from peripheral cells to the liver to reduce the lipid peroxidation damage as a result of cold shock (Fredenrich and Bayer, 2003). Thus, the reduction in serum triglyceride, cholesterol, LDL-C and HDL-C content in this study suggested that propolis-extract plays an important role in the prevention of liver tissue injuries. Previous studies demonstrated that quinic acid derivatives, naturally present in propolis, have strong liver protective effects and promote healing of toxic liver cells (Won Seo et al., 2003).

The effect of tectochrysin was tested on lipid peroxidation, as well as hepatocellular damage in rats, and it was found to have a significant decreasing effect in the liver enzyme activity (Lee et al., 2003). Administration of propolis at a level 0.01 g L^{-1} significantly decreased the triglyceride and cholesterol level of rainbow trout (*Oncorhynchus*

Table 5
Growth performance and feed utilization of Nile tilapia, after feeding diets with different levels of propolis-extract for 8 weeks under cold stress.

	Propolis extract (g kg^{-1})					SEM[g]	*P* values
	0 g kg^{-1}	1 g kg^{-1}	2 g kg^{-1}	3 g kg^{-1}	4 g kg^{-1}		
IBW[a] (g $fish^{-1}$)	25.40	25.36	25.50	25.46	25.50	0.86	0.494
FBW[b] (g $fish^{-1}$)	28.98	30.66	33.98	37.58	39.17	1.52	0.021
WG[c] (g $fish^{-1}$)	3.58	5.29	8.48	12.12	13.76	0.53	0.017
SGR[d] (%, day $fish^{-1}$)	0.22	0.32	0.48	0.65	0.72	0.02	0.051
FI[e] (g $fish^{-1}$)	19.16	20.05	20.96	22.15	23.32	1.12	0.001
FCR[f]	5.36	3.80	2.47	1.83	1.71	0.04	0.031

[a] IBW = Initial body weight.
[b] FBW = Final body weight, $y = 2.73\times + 25.82$; $R^2 = 0.9804$.
[c] WG = Weight gain, $y = 2.719\times + 0.489$; $R^2 = 0.9834$.
[d] SGR = Specific growth rate, $y = 0.133\times + 0.079$; $R^2 = 0.9845$.
[e] FI = Feed intake, $y = 1.042\times + 18.002$; $R^2 = 0.995$.
[f] FCR = Feed conversion ratio, $y = 0.255x^2–2.457\times + 7.6$; $R^2 = 0.9985$.
[g] SEM = Standard error mean.

Table 6
Hematological indices of Nile tilapia, after feeding diets with different levels of propolis-extract for 8 weeks under cold stress.

	Propolis extract (g kg^{-1})					SEM[e]	P values
	0 g kg^{-1}	1 g kg^{-1}	2 g kg^{-1}	3 g kg^{-1}	4 g kg^{-1}		
Htc[a] (%)	10.67	15.57	20.47	25.97	26.07	0.56	0.001
Hb[b] (g dl^{-1})	6.83	7.27	10.03	11.80	12.17	0.49	0.031
RBCs[c] ($\times 10^6$ cmm^{-1})	1.66	1.95	2.17	2.25	2.29	0.22	0.041
WBCs[d] ($\times 10^3$ cmm^{-1})	19.50	27.22	36.00	37.90	39.88	0.65	0.003

[a] Htc = Hematocrit, $y = -0.6429 \times^2 + 7.9771\times + 2.89$; $R^2 = 0.9802$.
[b] Hb = Hemoglobin, $y = -0.0807 \times^2 + 2.0053\times + 4.492$; $R^2 = 0.9392$.
[c] RBCs = Red blood cell count, $y = -0.0457 \times^2 + 0.4303\times + 1.276$; $R^2 = 0.9982$.
[d] WBCs = White blood cell count, $y = -1.3114 \times^2 + 13.013\times + 7.488$; $R^2 = 0.9888$.
[e] SEM = Standard error mean.

Table 7
Blood biochemical indices of Nile tilapia, after feeding diets with different levels of propolis-extract for 8 weeks under cold stress.

	Propolis extract (g kg^{-1})					SEM[g]	P values
	0 g kg^{-1}	1 g kg^{-1}	2 g kg^{-1}	3 g kg^{-1}	4 g kg^{-1}		
[a]ALT UL^{-1}	34.97	29.30	18.30	16.67	16.53	0.93	0.001
[b]AST UL^{-1}	92.30	88.96	66.96	62.90	61.26	1.11	0.001
[c]ALP UL^{-1}	70.43	70.10	67.16	64.07	53.40	1.21	0.002
[d]LDH UL^{-1}	200.367	155.00	115.60	82.63	73.93	1.13	0.002
Triglyceride mmol L^{-1}	1.24	1.03	0.96	0.86	0.67	0.02	0.001
Cholesterol mmol L^{-1}	3.03	2.65	2.33	1.70	1.42	0.05	0.001
[e]LDL-C mmol L^{-1}	1.03	1.28	1.43	1.62	1.64	0.04	0.001
[f]HDL-C mmol L^{-1}	1.64	1.63	1.50	1.27	0.90	0.03	0.001

Triglyceride, $y = -0.131\times + 1.345$; $R^2 = 0.9691$.
Cholesterol, $y = -0.417\times + 3.477$; $R^2 = 0.9861$.
[a] ALT = Alanine aminotransferase, $y = 1.4593x^2–13.707\times + 48.222$; $R^2 = 0.9584$.
[b] AST = Aspartate aminotransferase; $y = 1.5243x^2–17.96\times + 111.59$; $R^2 = 0.9067$.
[c] ALP = Alkaline phosphatase, $y = -1.4814x^2 + 4.8886\times + 66.644$; $R^2 = 0.9797$.
[d] LDH = Lactate dehydrogenase; $y = 5.6964x^2–66.702\times + 262.95$; $R^2 = 0.9966$.
[e] LDL-C = Low density of lipoprotein cholesterol, $y = -0.03x^2 + 0.336\times + 0.722$; $R^2 = 0.9913$.
[f] HDL-C = High density of lipoprotein cholesterol, $y = -0.0586x^2 + 0.1674\times + 1.53$; $R^2 = 0.9988$.
[g] SEM = Standard error mean.

mykiss), and therefore appeared to have the potential to maintain liver health and avoid hyper catabolism at high concentration (0.02 and 0.03 g L^{-1}) in propolis-treated fish. The present results oppose to those found by Šegvić-Bubić et al. (2013) who reported that before and after acute low-temperature stress (24 h) reduction in triglycerides were observed in European sea bass fed propolis supplemented diets. In addition, no significant differences were found in triglycerides of Nile tilapia fed diets supplemented with different levels of propolis-extract (Acar, 2018). Furthermore, a trial with gilthead seam bream, *Sparus aurata,* showed that after the cold stress, serum triglycerides content increased in all groups, indicating elevated energy demands to cope with the processes of restoration, as using triglycerides under cold stress (Kyprianou et al., 2010). The discrepancy of our results with those may be due to differences in duration of exposure to cold stress, water temperature, differences in propolis components and the species itself.

Serum concentrations of glucose are regulated by complex interactions of hormones such as cortisol (Panase et al., 2018). Cortisol can regulate glucose mobilization due to cortisol-dependent enhancement of gluconeogenesis (Vijayan et al., 1997; Mommsen et al., 1999) and cortisol activity in blood is considered an indicator of stress condition (Wendelaar Bonga, 1997). In this study, the highest glucose and cortisol levels were found in fish fed control diet, and the lowest in fish fed 4 g kg^{-1} propolis-extract supplemented diet. In the present study, the increased level of glucose serum in fish fed the control diet and reared under cold stress may be due to the thermal adaption to lower enzymatic reaction rate (Atwood et al., 2003). Also, it might also reflect an increased need for energy to counteract the effects of stress (Sun et al., 2019). A similar trend was observed in juvenile European sea bass reared under temperature variation stress (Varsamos et al., 2006). The effect of propolis on the concentration of serum cortisol and glucose in fish under short-term cold stress has been noted by Šegvić-Bubić et al. (2013) who suggests that diets supplemented with 2.5 g kg^{-1} propolis might inhibit oxidative stress in sea bass exposed to short-term (24 h) cold stress. The protective effect of propolis extract may be related to its antioxidant effect and the ability to control the peroxidation of unsaturated fatty acids, preventing the production of cholesterol (Kitabchi, 1967). Recently, Acar (2018) reported no significant differences ($P > .05$) in glucose concentration in Nile tilapia fed different levels of propolis extract and reared under normal rearing conditions.

In the present study, it was observed a decrease in the sodium and potassium levels in fish fed control diet under cold stress, but levels increased dramatically in fish fed propolis-extract supplemented diets. These stressing agents or disturbances may activate the pituitary-internal axis in the neuroendocrine system and induce the release of stress hormones such as corticosteroid and catecholamines, thereby initiating changes in the concentration of electrolytes (e.g., sodium, and potassium) and osmoregulation.

Acclimation of ectotherms to cold stress occurs by a process of cellular mechanisms called homeoviscous adaptation (Trueman et al., 2000). Δ 9D is widely expressed in many aquatic animals during acclimation to cold, where it introduces double bonds to saturated fatty acids within biological membranes (Zerai et al., 2010). Furthermore, unsaturation supports the conformational flexibility of membrane-bound proteins (Deuticke and Haest, 1987). In the present study, the inclusion of propolis at different levels affected gene expression of Δ 9D (Fig. 5). Fish fed 4 g kg^{-1} supplemented propolis extract diet recorded

the highest gene expression of Δ 9D, while fish fed the control diet recorded the lowest. The increase in the transcription of Δ 9D gene in fish fed propolis may be due to the richness of short chain fatty acids in propolis-extract (Table 2). In this context, Hsieh et al. (2007) reported that the expression of Δ 9D gene was affected by feeding and the types of fatty acids in the diets, and the present results are consistent with Tocher et al. (1996) who reported that the expression of Δ 9D is responsive to different factors like dietary and thermal conditions. In addition, Polley et al. (2003) found that unsaturation of hepatic lipids in rainbow trout in response to diet formulation changes the membrane fluidity to acquire cold protection and help fish to tolerate and adapt to cold during the winter season. In addition, Murray et al. (2007) suggested that changes in membrane fluidity to acquire cold protection exist and dominate the inducible cold tolerance in organisms as these authors observed that cold tolerance is explainable by homeoviscous adaptation or change in lipid saturation.

In conclusion, the supplementation of 3 to 4 g kg^{-1} of propolis-extract in Nile tilapia diets could enhance fish resistance to cold temperature stress during winter season, decrease the mortality, and enhance the physiological status, thus showing a potential for use in aquatic feed industry as a feed additive in winter diets. But as this was the first study to address this issue, further studies are needed to clarify even more the propolis-extract's immunostimulatory and antioxidant beneficial properties and its effective supplementation in Nile tilapia winter diets.

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