Feeding *Glycyrrhiza glabra* (liquorice) and *Astragalus membranaceus* (AM) alters innate immune and physiological responses in yellow perch (*Perca flavescens*).

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ABSTRACT 24

The current work assessed the potential immunomodulatory and growth-promoting effects of *Astragalus membranaceus* (AM) and *Glycyrrhiza glabra* (liquorice) in Yellow perch (*Perca flavescens*). In this regard, fish with an average weight of 31 ± 1.0 g were divided into five groups, and fed daily with an additive-free basal diet (control); 1, 2, and 3% (w/w) *G. glabra*, and the fifth diet was incorporated with a combination of 1% *G. glabra*-AM for a four-week period. Immunological, biochemical and growth parameters were measured; and sub-groups of fish were exposed to 1-week starvation. The results showed that incorporating AM and liquorice in the diet significantly improved Immunological [superoxide dismutase (SOD), glutathione peroxidase (GPx), catalase (CAT), Lipid peroxidase (LPx) and lysozyme activities], biochemical [Aspartate Aminotransferase (AST) and Alanine Transaminase (ALT) activities; and glucose and cortisol concentrations] and growth performance parameters [body mass gain (BMG), specific growth rate (SGR), length, condition factor (K) and feed conversion ratio (FCR)]. In addition, markedly up-regulated the expression of related genes [Insulin-Like Growth Factor-1 (IGF-1), Serum amyloid A (SAA), Complement Component C3 (CCC3), Alpha 2 Macroglobulin (A2M), SOD and GPx] in treated fish groups compared to the control. Conclusively, feeding AM and liquorice diets significantly increased ($P < 0.05$) growth performance, antioxidant and immune response profiles throughout the entire experiment, suggesting their beneficial rule as natural anti-stress agents.

Keywords: 45

Astragalus membranaceus; *Glycyrrhiza glabra*; Immune response; Growth performance; Gene expression; Starvation; yellow perch. 46
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1. Introduction

Yellow perch (*P. flavescens*) is one of the most valuable fish species in North America, particularly in the Midwest [1]. Various husbandry practices can result in physiological stress response such as elevated rearing densities, starvation, handling, anesthesia, and transportation stressors [2-5]. Starvation enhances reactive oxygen species generation that will lead to oxidative stress [6]. Oxidative stress results when the antioxidant defenses are overcome by pro-oxidant forces and reactive oxygen species are not adequately removed [7].

Most phytochemicals are considered natural anti-oxidants and redox active molecules similar to that of superoxide dismutase (SOD) and xanthine oxidase inhibitors. Their activity can be attributed to many types of active components that they contain [8, 9]. In this study, attention focused on *Glycyrrhiza glabra* (liquorice) and *Astragalus membranaceus* (AM). *G. glabra* is one of the oldest and most frequently used herbal medicinal plants [10]; it belongs to family *Fabaceae* [11]. A large number of active components have been isolated from Liquorice, with glycyrrhizic acid normally being considered to be the main biologically active component [12, 13]. It has been reported to possess immunomodulatory, hepatoprotective and antioxidative attributes [14] and growth-promoting effects in fish [15]. Dried root of *A. membranaceus* is a traditional Chinese medicinal herb [16] that contains more than 126 different components [17], such as: *Astragalus* polysaccharides (APS), organic acids, alkaloids, glucosides, and volatile oil as major components [18, 19].

To the best of our knowledge, no studies for the effect of the two medicinal plants on the expression of immune related genes (SAA, CCC3, A2M, SOD and GPx)

and growth related gene (IGF-1) have been reported in fish. The purpose of the present study was to evaluate the effects of dietary supplementation of *G. glabra* and a mixture of *G. glabra* -AM on the immune response and growth performance of yellow perch in response to starvation stress exposure.

2. Materials and methods

2.1. Fish

P. flavescens of average weight 31 ± 1.0 g were procured from the Aquaculture Research Center Hatchery at Ohio State University South Centers, Piketon, Ohio, USA and kept in a 2,200 l fiberglass aerated tank filled with disinfected de-chlorinated well water. The health status was examined for evidence of diseases according to the methods of Austin et al. [20]. Fish were acclimated for ten days by feeding a basal diet. Temperature was maintained at $15 \pm 0.36^{\circ}\text{C}$ and dissolved oxygen concentration at 5 ± 0.89 mg l⁻¹. Water quality parameters were monitored daily throughout the experiment.

This study and all experimental procedures involving animals were performed according to the protocol approved by the Ohio State University Institutional Animal Care and Use Committee.

2.2. Herbal plants

Both *G. glabra* and *A. membranaceus* were commercial products in powder form provided by Oregon's wild harvest, Sandy, Oregon, USA.

2.3. Experimental diets

Five experimental diets were prepared. Briefly, fine commercial powdered basal diet *Aquamax® Fingerling starter 300*, *PMI Nutritional International, LLC., Brentwood, MO, USA; Lot # 5D03* (Crude Protein Minimum 50.0%, Crude Fat Minimum 16.0%, Crude Fiber Maximum 3.0%, Calcium Minimum 2.0%, Phosphorous (P) Minimum 1.3%, Sodium (NA) Minimum 0.1% and Ash Maximum 12.0%) was divided into five portions, the first three portions were incorporated with 10, 20, and 30 g *G. glabra* /kg feed to obtain the desired concentrations of 1, 2, and 3%/kg, and the fourth portion was incorporated with a combination of 10 *G. glabra* (Liquorice): 10 *A. membranaceus* (AM) g/kg feed to obtain the desired concentration of 1%/kg and the last portion was kept free of any additives as a control. All components were then combined and mixed for 15 min. After that, each blend was adjusted to a desired pre-extrusion moisture content of ~45% by adding adequate amounts of water, then mixed again for 15 min. After extrusion, the pelletized feed blends were dried in a laboratory oven (Thelco Precision, Jovan, Winchester, Virginia, USA) at 50°C for 24 h. After drying, the diets were broken up and sieved into proper pellet size (1.5 × 3.0 mm and 2.5×5.0 mm), then were packed in clean dry plastic containers and stored at –15 °C until use.

2.4. Experimental design

The experiment was designed to include two phases (I and II).

2.4.1. Phase I (Pre-exposure)

The 450 fish were evenly assigned into five experimental groups in five rectangle fiberglass tanks (240 × 60 × 35 cm), each having three chambers or replicates (30 fish per replicate), in a flow-through system. The control group was fed a basal diet without any herbal plants incorporated, and the four treated groups

received *G. glabra* at 1, 2, 3% /kg, and a mixture of 1% *G. glabra*-AM (mix diet). 117

Fish were fed to satiation in two equal parts twice a day at 9:00 a.m. and 4:00 p.m. for 118

four weeks. Water quality was monitored throughout the experiment and adjusted at 119

$17 \pm 1.2^{\circ}\text{C}$ [21-24] and dissolved oxygen (DO) at 5 ± 0.89 mg/L. Temperature and 120

DO were measured and recorded in the morning and afternoon daily for each 121

tank/treatment. All chambers were siphoned daily to remove excess feed and fecal 122

matter to maintain good water quality. Water flow was adjusted across all tanks to 123

maintain the targeted temperature for each tank. The number of dead fish was 124

recorded daily in all groups during the experimental period. 125

2.4.2. Phase II (Starvation exposure) 126

Experimental diets were withheld from all experimental tanks, except for the 127

control group that received the control basal diet. Three fish were sampled from each 128

replicate after one week of food deprivation. 129

2.5. Sampling 130

2. 5.1. Blood sampling 131

Sampling included two sampling points: (1) after feeding on experimental 132

diets for four weeks and before exposure to starvation stress (pre-exposure; at the end 133

of phase I) and (2) after exposure to 1-week starvation stress (post-exposure; at the 134

end of phase II). Three fish per replicate (9 fish per group) were carefully netted, and 135

were euthanized by tricaine methanesulfonate (MS222) at 250 ppm in water 136

(Syndel Laboratories Ltd., Vancouver, British Columbia) for blood and tissue 137

sampling. Blood was drawn from caudal vessels using 1 cc U-100 heparinized 138

syringes (Becton Dickinson, Franklin Lakes, NJ, USA) in heparinized tubes and kept 139

on crushed ice until centrifugation at 3600 rpm for 5 minutes at 4°C to obtain plasma 140

samples. Separated plasma was stored in clean centrifuge tubes at -80°C until used for
assaying Glucose, cortisol, ALT, and Lysozyme activities values at both sampling
points. Sedimented RBCs from the same blood samples were lysed in four times its
volume of ice-cold HPLC grade water (Sigma, USA), centrifuged at 5000 g for 15
min. at 4°C, then the supernatant (erythrocyte lysate) was collected and stored at -
80°C for determination of SOD, GPx, and CAT activities at both sampling points.

2. 5.2. Tissue sampling

After blood sampling, the fish were carefully dissected to isolate liver samples,
which were then divided into two parts. The first part was used for assaying different
antioxidant indices and the second part was stored in RNAlater (Ambion, USA) and
kept at -20°C for studying gene expression. The first part was rinsed with phosphate
buffered saline (PBS), pH 7.4, to remove any red blood cells and clots and transported
on ice and then each sample was divided into 3 portions, one portion was
homogenized in AST kit's assay buffer for Assaying AST, the second portion was
homogenized in 50 mM PBS containing 1 Mm EDTA (pH 7.4) for assaying SOD,
CAT, GPx, and ALT, and the final portion was homogenized on ice in
Malondialdehyde (MDA) Lysis Buffer for assaying LPx. Then, it was centrifuged at
15,000 × g for 10 minutes at 4°C to remove insoluble materials and supernatant was
separated and stored in clean centrifuge tubes at -80 °C until assayed.

2.6. Immunological and Biochemical parameters

2.6.1. Immunological parameters

2. 6.1.1. Lysozyme activity

Lysozyme activity was measured using a Microplate Spectrophotometer (BioTek's Epoch™, USA), according to Lysozyme Detection kit's manufacture's protocol (Sigma-Aldrich, USA) at 450 nm and using the following formula:

Lysozyme activity (U/ml) = $[(\Delta A_{450} / \text{min Test} - \Delta A_{450} / \text{min Blank}) \times (\text{df})] / [(0.001) \times (0.03)]$. Where: df is dilution factor, 0.001 = ΔA_{450} as per the Unit Definition and 0.03 is Volume (in milliliters) of enzyme solution.

One unit of enzyme activity was defined as the amount of enzyme causing a decrease in absorbance of 0.001 per minute at pH 6.24 and 25°C, using a suspension of *Micrococcus lysodeikticus* as substrate.

2.6.1.2. Antioxidant enzymes assays

Superoxide Dismutase (SOD), Catalase (CAT) and Glutathione Peroxidase (GPx) activities were measured according to each assay kit's manufacture's protocol (Cayman Chemical, USA) as previously described by Elabd et al. [25]. In addition, Lipid Peroxidase (LPx) activity was assayed according to the manufacture's protocol (Biovision, USA). All were measured using a Microplate Spectrophotometer (BioTek's Epoch™, USA) at 532 nm (LPx) according to the following formula:

LPx activity (nmol/mg) = $[(A / (\text{mg})) \times 4 \times D]$. Where: A is sample MDA amount from the standard curve (in nmol), mg is original tissue amount used, 4 is correction for using 200 µl of the 800 µl reaction mix and D is dilution factor.

2.6.2. Biochemical parameters

Glucose and cortisol concentrations; and Aspartate Aminotransferase (AST) and Alanine Transaminase (ALT) activities were assayed according to the Kits manufacture's protocol, using methods and formulas mentioned by Elabd et al. [25].

2.7. Growth performance parameters 187

All fish were weighed at the start of the experiment, at end of phase I (pre- 188
exposure) and after exposure to starvation stress to calculate growth performance 189
parameters as follows: 190

Body Mass Gain (BMG) (%) = $100 \times [\text{final body mass (g)} - \text{initial body mass}$ 191
(g)]/initial body mass (g)]. 192

Specific Growth Rate (SGR) (% day⁻¹) = $[(\ln \text{ final body mass in g}) - \ln \text{ initial body}$ 193
mass in g)/number of trial days] X 100. 194

Length gain rate (%) = $100 \times [\text{Average terminal body length (cm)} - \text{Average initial}$ 195
body length (cm)]/Average initial body length (cm). 196

Condition Factor (CF) = $100 \times [\text{Weight, g}]/(\text{Length, cm})^3$. 197

Feed Conversion Ratio (FCR) = $F/(W_f - W_i)$; where F is the weight of feed offered to 198
fish, W_f is the final weight of fish and W_i is the weight of fish at stocking. 199

2.8. Gene Expression 200

2.8.1. RNA Extraction and reverse transcription 201

Total RNA was isolated from liver samples using the Trizol method 202
(Invitrogen, Carlsbad, USA) according to the manufacturer's instructions. The 203
extracted RNA samples were subjected to DNA-free (DNase) treatment to avoid 204
genomic DNA contamination. The quantity of the RNA was evaluated by using 205
Nano-Drop spectrophotometry (Thermo Scientific, USA). The integrity (quality) was 206
checked by denaturing gel electrophoresis (1% agarose gel) and purity by 207
OD₂₆₀/OD₂₈₀ nm absorption ratio 1.80: 2.00. 208

Reverse transcription was performed using a high capacity cDNA reverse 209
transcription kit (Invitrogen, USA) following manufacturer's instructions for 20-μL 210

total volume of cDNA. Briefly, the total volume of 10 RT μ L master mix was prepared per reaction on ice by adding 2 μ L (10 $\times$ RT Buffer), 0.8 μ L (25 $\times$ dNTP Mix (100 mM)), 2 μ L (10 $\times$ RT Random Primers), 1 μ L (RNase Inhibitor), 3.2 μ L (Nuclease free H₂O) and 1 μ L (MultiScribe™ Reverse Transcriptase) into a microcentrifuge tube. Then, 10 μ L of 2 $\times$ RT master mix was added to 10 μ L the of RNA sample in each tube and mixed by pipetting, centrifugation and the thermal cycler (Biorad, USA) was adjusted following manufacturer's instructions, and the resulting cDNA stored at -20°C.

2. 8.2. *Primer design*

Primers for IGF-1, SAA, CCC3, A2M, SOD, GPx and β -actin genes were designed using Primer-BLAST from NCBI. Insulin-Like Growth Factor-1 (IGF-I) is very important for growth regulation [26, 27]. Serum amyloid A (SAA) is one of the major positive acute phase proteins that is involved in inflammatory cells chemotactic recruitment to inflammation sites and lipid metabolism [28]. Complement Component C3 (CCC3) is the most abundant component of complement protein and plays an important role in the complement cascades and participates in chemotactic factors generation, opsonization, modification of B and T cell responses, and elimination of immune complexes [29]. Alpha 2 Macroglobulin (A2M) is the most widely studied protease inhibitor that mainly function to maintain body fluid homeostasis and are involved in acute phase reactions and defense against pathogens that secrete proteolytic enzymes [30]. All primers were designed so that amplification covered at least 1 intron, which ensures that cDNA products would be distinct from any potential genomic DNA contamination. Primers sequences are presented in Table 1. For each gene, two or more primer pairs were evaluated and primer pairs with the best

performance were selected for use. Primers were manufactured by IDT (Coralville, IA, USA).

2. 8.3. Real-time PCR

PCR amplification was performed with 7500 Real-Time PCR System (Applied Biosystems®, USA) using 2 µL of cDNA and 18 µL of SYBR select Master Mix (Applied Biosystems, USA), which was prepared by adding 10 µL of 2x SYBR green Master Mix, 6 µL of ddH₂O, and 1 µL each of forward and reverse primer. The real-time analysis program consisted of 1 cycle of 95°C for 15 min, and 45 cycles of 95°C for 15s, 56°C for 15s, and 72°C for 10s. On each plate, for every sample, the target gene (gene of interest) and endogenous control (normalize gene: β-actin) were tested in triplicate. Then expression levels of the genes of interest were normalized to β-actin, and the fluorescence threshold cycle (CT) determined and the relative expression of each gene was calculated.

The relative expression level of the target gene in the test sample relative to the calibrator sample was carried out following protocol described by Livak and Schmittgen [31].

2.9. Statistical analysis

The data were analyzed by One-Way Analysis of Variance (ANOVA) and Duncan's multiple range tests were used to determine significant difference between groups using the Statistical Package for the Social Sciences (SPSS) software (version 22.0). Values are expressed as means ± standard error and a value of P < 0.05 was considered significant.

3. Results

<i>3.1. Immunological and Biochemical parameters</i>	258
<i>3.1.1. Immunological parameters</i>	259
<i>3.1.1.1. Lysozyme activity</i>	260
Liquorice supplementation showed the most significant increase ($P < 0.05$) in	261
lysozyme activity for 2% (w/v) <i>G. glabra</i> group before exposure to any stress and for	262
the 1% (w/v) <i>G. glabra</i> treated groups after exposure to one week starvation stress (P	263
< 0.05) compared to the control (Fig. 1 (B)).	264
<i>3.1.1.2. Antioxidant enzymes assays</i>	265
<i>Superoxide Dismutase (SOD) activity</i>	266
SOD activity was significantly high ($P < 0.05$) in the liver samples in the 2	267
and 3% /kg <i>G. glabra</i> groups (Fig. 2 (A)), and in the 3% /kg <i>G. glabra</i> group in	268
erythrocyte lysate (Fig. 2 (B)) compared with the control at four weeks from the start	269
of the experiment and before exposure to starvation. Exposure to starvation stress	270
resulted in significant decrease in SOD activity in control groups. Groups fed with 1%	271
/kg (Liquorice-AM mixture) at post-starvation exposure (in both liver and erythrocyte	272
lysate) showed the most significant increase ($P < 0.05$) in SOD activity compared to	273
the control (Fig. 2 (A and B)).	274
<i>Glutathione Peroxidase (GPx) activity</i>	275
Groups fed with 1% /kg <i>G. glabra</i> gave the highest significant increase ($P <$	276
0.05) in GPx activity in both liver and erythrocyte lysate at four weeks from the start	277
of the experiment and before exposure to starvation (Fig. 3 (A and B)). Exposure to	278
starvation resulted in a significant decrease ($P < 0.05$) in GPx activity for 2% /kg <i>G.</i>	279

glabra treated groups after exposure to 1-week starvation stress in both liver and erythrocyte lysate compared to the control (Fig. 3 (A and B)).

Catalase (CAT) activity

CAT activity had the highest significant increase ($P < 0.05$) in the 2 % /kg *G. glabra* group in liver (Fig. 4 (A 5)), and in the 1 % /kg *G. glabra* group in erythrocyte lysate (Fig. 4 (B)), compared with the control at four weeks from the start of the experiment and before exposure to starvation. Exposure to starvation caused a significant decrease in CAT activity in control groups, with the highest significant increase ($P < 0.05$) for 1% /kg *G. glabra* treated groups after exposure to 1-week starvation stress in both liver and erythrocyte lysate compared to the control (Fig. 4 (A and B)).

Lipid Peroxidase (LPx) activity

Feeding yellow perch with 1 and 2% /kg *G. glabra* incorporated diets gave the highest significant increase ($P < 0.05$) in LPx activity at 4 weeks from the start of the experiment and before exposure to starvation compared to the control (Fig. 1 (A)). Starvation exposure caused a significant increase in LPx activity in control groups. 3% /kg *G. glabra* treated groups after exposure to 1-week starvation stress showed the highest significant decrease ($P < 0.05$) in LPx activity when compared with the control (Fig. 1 (A)).

3.1.2. Biochemical assays

Adding 2% /kg liquorice diets to yellow perch gave the highest significant decrease ($P < 0.05$) in glucose and cortisol levels at four weeks prior to starvation exposure (Table 3). Incorporating the diets with 1% /kg *G. glabra* gave the most

significant decrease ($P < 0.05$) in glucose and cortisol levels after 1-week starvation stress compared to the control (Table 3).

ALT activity showed the maximum significant decrease ($P < 0.05$) in 1% /kg (Liquorice-AM mixture) treated groups in both liver and plasma at pre- and post-exposure to starvation compared to the control groups (Fig. 5 (A and B)). The most significant decrease ($P < 0.05$) in AST activity was for groups receiving 3% /kg *G. glabra* diet given at pre-exposure and for the 1% /kg *G. glabra* group post-exposure to 1-week starvation stress (Fig. 5 (C)).

3.2. Growth performance

Growth performance parameters of yellow perch are presented in Table 2. Incorporating yellow perch diets with *G. glabra* and 1% *G. glabra*-*A. membranaceus* mixture significantly enhanced the growth performance parameters (SGR, BMG, LGR, and condition factor) throughout the experiment, and the highest significant increase ($P < 0.05$) was for group fed with 1% (w/w) (Liquorice-AM mixture) followed by those fed with 3, 2, and 1% (w/w) liquorice diets, respectively after four weeks feeding on experimental diets at pre-exposure and post-exposure to 1-week starvation stress (Table 2). The highest significant decrease in feed conversion ratio (FCR) at pre-exposure was also for groups fed with 1% (w/w) (Liquorice-AM mixture) diets compared with the control and for the 2% (w/w) liquorice group at post-exposure to 1-week starvation stress (Table 2). Groups receiving 1 and 2% (w/w) liquorice diets did not show a significant difference in the condition factor at pre and post-exposure to 1-week starvation stress (Table 2).

3.3. Gene Expression

3.3.1. Expression of selected genes pre-exposure to starvation 326

Groups fed with 1% (w/w) (Liquorice-AM mixture) incorporated diets showed 327
significant high ($P < 0.05$) expression of Insulin-Like Growth Factor-1 (IGF-1), 328
Serum amyloid A (SAA) and Complement Component C3 (CCC3) genes than the 329
control group (Fig. 6 (A-C)). The group fed with 1 % (w/w) *G. glabra* incorporated 330
diets gave the highest significant increase ($P < 0.05$) in A2M gene expression 331
compared to the control (Fig. 6 (D)). 332

3.3.2. Expression of selected genes post-starvation stress exposure 333

After exposure to 1-week starvation stress, yellow perch groups fed with 1% 334
/kg (Liquorice-AM mixture) incorporated diets gave the highest significant increase in 335
($P < 0.05$) expression of A2M, SOD and GPx genes than the control group (Fig. 7 (B- 336
D)). The group fed with 3 % (w/w) *G. glabra* incorporated diets gave significant 337
increase ($P < 0.05$) in SAA gene expression compared to the control (Fig. 7 (E)). 338
Experimental diets did not show a significant difference in expression of IGF-1 and 339
CCC3 genes after exposure to 1-week starvation stress (Fig. 7 (A and F)). 340

4. Discussion 341

Fish in intensive culture are affected by various environmental stressful 342
conditions which can lead to suppressing the immune response and predispose for 343
infection [32]. Starvation is one of those stressors that enhances reactive oxygen 344
species generation that will lead to oxidative stress [6]. Most phytochemicals are 345
considered as natural anti-oxidants and redox active molecules through inhibiting the 346
generation of oxygen anions and scavenging free radicals [8, 9]. The present study 347
focuses on *G. glabra* (liquorice) and *A. membranaceus* (AM), which are well known 348

herbal medicinal plants with many beneficial properties [10, 16]; of natural origin; 349
lack of drug residues and very low side effects as reviewed by Tian and Feng [33]. 350

Lysozyme is one of the humoral elements in the innate immune system. Here, 351
liquorice and 1% *G. glabra*-*A. membranaceus* mixture led to significant increase ($P <$ 352
0.05) in lysozyme activity. Similar results have been reported by Yan et al. [17], 353
Zahran et al. [34], Chen et al. [35] and Jian & Wu [36]. These findings can be 354
attributed to the immunostimulating effects of *A. membranaceus* and *G. glabra* [14, 355
37, 38]. In contrast, there were no significant differences in the lysozyme activity in 356
carp; fed two different levels of herbal mixture [39]. 357

SOD, GPx, CAT and LPx are important antioxidant enzymes [40-42] that can 358
improve the imbalance in biological systems [41; 43-45]. In this study, although 359
exposure to starvation significantly altered antioxidants activities, incorporated diets 360
significantly mitigated those alterations and returned it to the control levels. SOD and 361
CAT activities were significantly decreased, while GPx and LPx activities were 362
significantly elevated in response to 1-week starvation. Similar results have been 363
reported by Zahran et al. [34] who showed that dietary supplementation of 364
Oreochromis niloticus with APS upregulated SOD and GPx activities. This may be 365
attributed to active components of both liquorice and AM. Liquorice has been 366
reported to possess immunostimulating, hepatoprotective and antioxidative activities 367
in fish [14, 18, 38]. 368

Plasma cortisol and glucose levels are often used as environmental stress 369
indicators in fish [46] and plasma cortisol is considered one of the fish primary 370
responses to different kinds of stressors [47]. In the present study, glucose and cortisol 371
levels showed significant decrease in experimental groups compared to the control. 372

This can be possibly explained with that liquorice and AM to active components might have stimulated the insulin activity and reduced the glucose level as recorded in previous studies [48-51], ensuring the hepatoprotective activities of tested diets in fish [14, 18].

The results obtained in this study reflected those of Guojun Yin [14], Zhang et al. [37] and Jia et al. [38]. Liquorice and AM were effective in decreasing ALT and AST activities at pre-exposure and post-exposure to starvation compared to the control. These findings can be attributed to the hepatoprotective effects of *A. membranaceus* and *G. glabra*. Our results come in agreement with Zaki et al. [52] who reported that lowest AST and ALT activities were obtained by *O. niloticus* group fed with 1% fenugreek sprouts meal .

The findings from this study highlights the benefits of *G. glabra* and 1% *G. glabra*-*A. membranaceus* mixture as feed additives for yellow perch, that was noticed by significantly increased ($P < 0.05$) growth performance parameters of *P. flavescens*. Previous studies demonstrated the effectiveness of dietary incorporation of *G. glabra* , *Astragalus* and other herbals in increasing BW and other growth performance parameters of fish [15; 25; 34; 50; 53-58] for treated groups over the control. Perhaps, the growth promoting activities of liquorice and the liquorice-AM mixture may be attributed to the active ingredients that those herbs contain as AM contains *Astragalus* polysaccharides (APS), which have been shown to enhance digestion [18, 37]. In addition, it can markedly improves growth in fish through its content of monosaccharides, organic acids, alkaloids, glucosides, and volatile oil as major active components together with are essential micronutrients of choline, betaine, folic acid, various amino acids, mucoitin, gum, cellulose, and fourteen trace minerals, including

selenium, zinc, and iron [18]. At the same time, liquorice growth promoting effect
may be related to its content of active components including mainly flavonoids and
pentacyclic triterpene saponin and glycyrrhizin [12, 13].

On molecular level, IGF-I is very important for growth regulation [26, 27]. In
the present study, IGF-I expression was positively correlated with growth
performance, indicating that IGF-I gene expression can be used as a molecular
biomarker for growth in Yellow perch and that the above-mentioned incorporated
diets effectively improved growth performance of yellow perch. These results came in
agreement with Shaheen et al. [22]. Furthermore, Immune related genes including,
SAA, CCC3, A2M, SOD, and GPx were enhanced by *G. glabra* and the *G. glabra*-
AM mixture incorporation, indicating their beneficial role. Similarly, expression ratio
of target genes (IL-8, IL-1 β and TGF- β) of the head kidney of rainbow trout
Oncorhynchus mykiss fed with 1% and 2% lupin, mango and stinging showed up-
regulation of target genes as compared to the control [59]. Also, head kidney
leucocytes from trout cultured with lipopolysaccharide (LPS) showed an increase in
IL-1 β expression [60]. This can be attributed to active components of both liquorice
and AM and their hepatoprotective, immunostimulating and antioxidative activities in
fish [14, 18, 37, 38, 61]. Conclusively, the present study revealed that *G. glabra* and
A. membranaceus dietary supplementation enhanced growth performance, immune
response Yellow perch and thus can be used as natural anti-stress agents.

Acknowledgements

This study was supported by the National Institute of Food and Agriculture
(NIFA), U.S. Department of Agriculture, under Agreement No 2010-38879-20946,
and Cultural Affairs and Missions Sector, Ministry of Higher Education and Scientific

Research, Egypt. Salaries and research support were provided by state and federal 421
funds appropriated to The Ohio State University, Ohio Agricultural Research and 422
Development Center. We thank Kun-Qian Zhu, Dean Rapp, and Paul O'Bryant for 423
their assistance throughout the experiment, and Joy Bauman for her comments on the 424
manuscript. 425

Conflicts of Interest 426

The authors declare no conflict of interest. 427

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Table 1

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Primers sequences for the expression study of selected genes in Yellow perch
P. flavescens.

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Gene of interest	Forward primer sequence (5'-3')	Reverse primer sequence (5'-3')	Function
β-actin	GCCTCTCTGTCCACCTTCCA	GGGCCGGACTCATCGTACT	Internal standard
IGF-1	AGTACCGCAGGGCACAAAGT	CTGGCTGCTGTGCTGTCCTA	Growth
SOD	GCATGTAGGAGACTTGGGCAAT	CCGTGATTTCTATCTTGGCAACA	Detoxification/oxidative stress
GPx	GTCTTGGGTAACCCACCAG	GACACTTGGATGCCACCTCA	
SAA	ACCATGCTCGTTTGCCTTCT	TGTGGCGAGCATACAGTGAT	
CCC3	GCACAGGAGAAGCAACAGTG	AGGAGCTGCACTGACAAGTTA	Immune related
(α 2-M)	TACAGGAGCACCAAGTGCAG	GACTGACCACACGCTCTTCA	

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Table 2

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Growth performance parameters of *P. flavescens* after feeding with different
levels of *G. glabra* and *G. glabra* - *A. membranaceus* mixture incorporated diets at
pre and post- exposure to 1-week starvation.

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Liquorice and Liquorice-AM mixture %/kg feed		SGR (%)	BMG (%)	LGR (%)	K	FCR
Pre-exposure						
0	Liquorice	0.1 ± 0.0	5.3 ± 0.16	4.5 ± 0.0	1.2 ± 0.0	5.4 ± 0.0
1		0.4 ± 0.0	31.1 ± 0.13	12.3 ± 0.0	1.2 ± 0.0	1.0 ± 0.0
2		0.4 ± 0.0	34.0 ± 0.01	12.9 ± 0.0	1.3 ± 0.0*	0.9 ± 0.0
3		0.5 ± 0.0	39.1 ± 0.08	13.6 ± 0.0*	1.3 ± 0.0*	0.8 ± 0.0
1 mix.		0.5 ± 0.0*	44.7 ± 0.12*	13.6 ± 0.0*	1.3 ± 0.0*	0.7 ± 0.0*
Post-Starvation exposure						
0	Liquorice	19.2	0.02 ± 0.0	3.0 ± 0.01	1.18 ± 0.03	18.5 ± 0.06
1		13.5	0.37 ± 0.0	11.6 ± 0.06	1.20 ± 0.03	1.0 ± 0.01
2		16.0	0.37 ± 0.0	11.1 ± 0.06	1.27 ± 0.01	0.9 ± 0.03
3		26.8	0.42 ± 0.0*	10.5 ± 0.06	1.26 ± 0.03	0.9 ± 0.02*
1 mix.		20.9	0.43 ± 0.0*	11.5 ± 0.06*	1.30 ± 0.00*	0.9 ± 0.02*

Values are mean (n = 30) ± SEM. Mean values with asterisk (*) are different
significantly (P < 0.05).

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SGR = Specific growth rate, BMG = Body Mass Gain, LGR = Length Gain Rate, K =
Condition Factor, FCR = Feed Conversion Ratio.

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Table 3

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Effect of *G. glabra* (Liquorice) and *G. glabra* (Liquorice)-*A. membranaceus* (AM) incorporated diets on glucose and cortisol levels of yellow perch (*P. flavescens*) at pre and post- exposure to 1-week starvation.

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Liquorice and Liquorice-AM mixture %		Glucose (Mg/dl)	Cortisol (pg/ml)
Pre-exposure			
0	Liquorice	31.9 ± 0.5	3851.8 ± 0.0
1		35.3 ± 0.5	3863.0 ± 0.2
2		21.3 ± 0.4 [*]	3827.5 ± 0.2 [*]
3		51.8 ± 0.4	3858.6 ± 0.6
1 mix.		45.8 ± 0.6	3856.2 ± 0.3
Post-Starvation exposure			
0	Liquorice	19.2 ± 0.5	3855.2 ± 0.5
1		13.5 ± 0.1 [*]	3806.6 ± 0.6 [*]
2		16.0 ± 0.6	3833.4 ± 0.2
3		26.8 ± 0.2	3850.8 ± 0.4
1 mix.		20.9 ± 0.6	3844.4 ± 0.6

Values are mean (n = 9) ± SEM. Mean values with asterisk (*) are different significantly (P < 0.05).

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Figures Legend

Fig. 1. LPx activity in liver (A) and Lysozyme activity in plasma (B) of yellow perch (*P. flavescens*) after feeding with different levels of *G. glabra* (Liquorice) and *G. glabra* (Liquorice)-*A. membranaceus* (AM) incorporated diets pre and post- exposure to 1-week starvation. Values are mean ($n = 9$) $\pm$ SEM. Mean values with asterisk (*) are different significantly ($P < 0.05$).

Fig. 2. SOD activity in liver (A) and erythrocyte lysate (B) of yellow perch (*P. flavescens*) after feeding with different levels of *G. glabra* (Liquorice) and *G. glabra* (Liquorice)-*A. membranaceus* (AM) incorporated diets pre and post- exposure to 1-week starvation. Values are mean ($n = 9$) $\pm$ SEM. Mean values with asterisk (*) are different significantly ($P < 0.05$).

Fig. 3. GPx activity in liver (A) and erythrocyte lysate (B) of yellow perch (*P. flavescens*) after feeding with different levels of *G. glabra* (Liquorice) and *G. glabra* (Liquorice)-*A. membranaceus* (AM) incorporated diets pre and post- exposure to 1-week starvation. Values are mean ($n = 9$) $\pm$ SEM. Mean values with asterisk (*) are different significantly ($P < 0.05$).

Fig. 4. CAT activity in liver (A) and erythrocyte lysate (B) of yellow perch (*P. flavescens*) after feeding with different levels of *G. glabra* (Liquorice) and *G. glabra* (Liquorice)-*A. membranaceus* (AM) incorporated diets pre and post- exposure to 1-week starvation. Values are mean ($n = 9$) $\pm$ SEM. Mean values with asterisk (*) are different significantly ($P < 0.05$).

Fig. 5. Effect of *G. glabra* (Liquorice) and *G. glabra* (Liquorice)-*A. membranaceus* (AM) incorporated diets on ALT in liver (A) and plasma (B); and AST in liver (C) of yellow perch (*P. flavescens*) at pre and post- exposure to 1-week starvation. Values are mean ($n = 9$) $\pm$ SEM. Mean values with asterisk (*) are different significantly ($P < 0.05$).

Fig. 6. IGF (A), SAA (B), CCC3 (C) and A2M (D) genes expression in yellow perch pre- exposure to starvation. Values are mean ($n = 9$) $\pm$ standard error. Mean values with asterisk (*) are significantly ($P < 0.05$) different.

Fig. 7. IGF (A), SOD (B), GPx (C), A2M (D), SAA (E) and CCC3 (F) genes 691
expression in yellow perch post-starvation stress exposure. Values are mean (n = 9) ± 692
standard error. Mean values with asterisk (*) are significantly (P < 0.05) different. 693

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Fig. 1

Fig. 2

Fig. 3

Fig. 4

■ Pre-exposure ■ Post-Starvationexposure

Fig. 5

Fig. 6

Fig. 7