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Effects of resveratrol on growth and skeletal muscle physiology of juvenile southern flounder

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ABSTRACT

Resveratrol is a naturally occurring antioxidant that has been widely studied in mammals due to its potential to extend lifespan. However, antioxidants may also limit protein damage and therefore reduce rates of protein degradation, providing a potential avenue for enhancing growth in an aquaculture setting. The present study tested the hypotheses that in Southern flounder, *Paralichthys lethostigma*, resveratrol would decrease protein carbonylation and 4-HNE (indicators of protein and lipid oxidative damage, respectively), levels of ubiquitinylation and LC3 (indicators of non-lysosomal and lysosomal protein degradation, respectively), while having no effect on S6K activation (indicator of protein synthesis). These effects were predicted to increase growth rate. Mitochondrial volume density was also examined since resveratrol may lead to the proliferation of mitochondria, which are the principal source of reactive oxygen species (ROS) that cause oxidative damage. Juvenile fish (n = 142) were fed a control diet or a diet supplemented with 600 μg resveratrol per g of food for 16 weeks. Fish treated with resveratrol had a 9% greater length and 33% greater body mass than control fish after 16 weeks. Additionally, there was lower protein carbonylation and lipid 4-HNE within the muscle tissues of treated fish, indicating decreased oxidative damage, and reduced protein ubiquitinylation in the resveratrol fed flounder, indicating less protein degradation. However, there was not a significant difference in LC3, S6K activation, or mitochondrial volume density. These results suggest that resveratrol has positive effects on growth due to its antioxidant properties that reduce non-lysosomal protein degradation.

1. Introduction

Muscle growth and atrophy are dependent on the net balance between protein synthesis and degradation (Millward et al., 1975; Goldspink, 1991; Houlihan, 1991; McCarthy et al., 1994; Mente et al., 2011). In aquaculture, there is considerable emphasis on maximizing net protein synthesis and growth, particularly in muscle, and this can be achieved by increasing rates of protein synthesis and/or decreasing rates of protein degradation. Protein synthesis in muscle is primarily regulated by the mechanistic target of rapamycin (mTOR) (Hemmings and Restuccia, 2012), and an increase in the activation of mTOR and its downstream targets is typically used as an indicator of enhanced rates of protein synthesis (Hemmings and Restuccia, 2012). Protein degradation in muscle occurs primarily through the non-lysosomal ubiquitin–proteasome and the lysosomal autophagy pathways (Bonaldo and Sandri, 2013). Non-lysosomal protein degradation begins when damaged or unnecessary proteins are tagged by multiple ubiquitin molecules. These marked proteins are then recognized and broken down by the proteasome complex (Bonaldo and Sandri, 2013). Lysosomes are acidic organelles that receive material to be degraded via endocytosis, phagocytosis or, in the case of muscle protein turnover, autophagy (Dell'Angelica et al., 2000; Saftig and Klumperman, 2009; Bao et al., 2010). In autophagy, lysosomes fuse with autophagosomes to form autolysosomes, where degradation ensues (Saftig and Klumperman, 2009), and various lysosomal hydrolyses and lysosomal membrane proteins target specific substrates for degradation (Conus and Simon, 2008; Saftig and Klumperman, 2009).

Reactive oxygen species (ROS) are produced naturally during electron transport, phagocytosis and by specific enzymes such as oxidases (Rice-Evans and Burdon, 1993). ROS can cause oxidation of proteins, peroxidation of lipids, and breaks in DNA (Latruffe and Rifler, 2013). In most cells, ROS are primarily generated within the mitochondria where oxygen is converted into the superoxide anion, which occurs

Abbreviations: LC3, microtubule-associated protein 1A/1B-light chain 3; LDL, low-density lipoprotein; mTOR, mechanistic target of rapamycin; PBS, phosphate buffered saline; P-S6K, phosphorylated S6K; ROS, reactive oxygen species; S6K, p70S6 kinase; TEM, transmission electron microscopy; TTBS, tris buffered saline with 0.1% Tween; 4-HNE, 4-hydroxynonenal

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when dioxygen is reduced by one electron (Latruffe and Rifler, 2013). Cells have mechanisms to manage ROS levels, such as antioxidant enzymes (Nyström, 2005), and dietary antioxidant molecules such as vitamins E and C (Hamre et al., 2004). However, an oxidative challenge can occur if the formation of ROS exceeds the organism's antioxidant defenses, and at least some oxidative damage is an unavoidable consequence of respiration (Rice-Evans and Burdon, 1993; Pandey and Rizvi, 2011; Latruffe and Rifler, 2013). For example, protein carbonylation is an irreversible modification that occurs fairly rapidly during oxidative stress and is a commonly measured indicator of ROS induced protein damage (Dalle-Donne et al., 2003; Nyström, 2005). Oxidation of proteins can lead to unfolding, which may increase the exposure of hydrophobic amino acid residues to the aqueous environment (Grune et al., 2004; Nyström, 2005). This conformational change makes the protein more vulnerable to ubiquitin tagging, and thus proteosomal degradation (Dukan et al., 2000; Bota et al., 2002; Grune et al., 2003, 2004; Nyström, 2005), suggesting that excessive protein carbonylation may reduce net protein synthesis and limit growth.

Resveratrol is an antioxidant compound in the stilbene group of polyphenols that can be found in the skins of red grapes as well as other plants such as peanuts (Latruffe and Rifler, 2013). Resveratrol has been implicated in protection against aging, obesity, and development of insulin resistance in rodents (Baur et al., 2006; Lagouge et al., 2006; Park et al., 2012). Resveratrol scavenges ROS directly, and also increases the level of mitochondrial antioxidant enzymes and modifies cell signaling pathways and kinase activities (Kairisalo et al., 2011; Xia et al., 2011; Giovannini and Masella, 2012). Thus, resveratrol's antioxidant properties might be expected to reduce protein degradation and potentially enhance growth rates.

As in mammals, fish tissues generate small quantities of ROS during normal metabolism (Rice-Evans and Burdon, 1993; Hamre et al., 2004) and dietary supplementation with antioxidants has been shown to enhance growth in fishes (Tocher et al., 2002; Wang et al., 2003; Lee and Dabrowski, 2004; Gao et al., 2012). However, while resveratrol has been found to increase the longevity of a short-lived seasonal fish *Nothobranchius furzeri* (Valenzano et al., 2006), the effect of resveratrol on growth in fishes has not been investigated. The Southern flounder *Paralichthys lethostigma* is a marine flatfish inhabiting estuarine and shelf waters of the south Atlantic and Gulf coasts of the U.S. High commercial value, declining natural populations, and wide temperature and salinity tolerances make the southern flounder a versatile species for aquaculture (Daniels et al., 2010). Additionally, spawning and larval rearing techniques for this species have been documented (Watanabe et al., 2006; Daniels et al., 2010). The present study tested the hypothesis that resveratrol supplementation in juvenile Southern flounder would decrease markers of muscle oxidative damage and protein degradation, and therefore lead to an increase in growth.

2. Materials and methods

2.1. Animal maintenance

Adult southern flounder broodstock held in photothermally controlled tanks were induced to spawn by using luteinizing hormone-releasing hormone analog (Watanabe et al., 2006) at the University of North Carolina Wilmington Aquaculture Facility (Wrightsville Beach, NC). Embryos were hatched and larvae reared according to published protocols (Daniels and Watanabe, 2003). Early juveniles were raised in 1000-L recirculating tanks and fed a commercially prepared diet (50% protein and 15% lipid) (Skretting, Vancouver, British Columbia) until the experiment was started.

The experimental system consisted of 6 circular, 680-L tanks at the University of North Carolina Wilmington. Temperature was maintained at 21 ± 1 °C and salinity was 33 ± 1 ppt. Levels of ammonia (0 to 0.1 ppm), nitrate (10 to 25 ppm), nitrite (0 to 0.1 ppm), dissolved oxygen (6.5 to 7.8 mg/L), as well as pH (7.7 to 8.0) were measured throughout the experiment, and these parameters were maintained within the optimum levels of southern flounder culture as described by Daniels and Watanabe (2003). Uniform water quality and temperature were maintained in all the tanks, as they were supported by a recirculating aquaculture system, including mechanical and biological filters and a UV sterilizer. Total system volume, including the six experimental tanks was 3800 L. A single cohort of 144 juvenile southern flounder were used in the experiments (mean initial weight: 28.55 ± 1.59 g; mean initial total body length: 13.62 ± 0.28 cm). Light conditions were set to 12 h of light and 12 h of darkness. Fish were held for two weeks to adjust to new tank conditions before trials began. During this holding period, fish were fed a standard aquaculture diet (47% protein, 12% lipid, Table 1: control diet). After two weeks, the control diet and resveratrol diets were added to appropriate tanks and food consumption, as well as length measurements were recorded (body mass was measured at three sub-sampling periods throughout the experiment, further detailed below). Fish were maintained and processed according to the UNCW Institutional Animal Care and Use Committee standards.

2.2. Diets and growth

Two isonitrogenous (47%) and isolipidic (12%) control and resveratrol-based (0.06%) diets were prepared (Table 1). The formulation of the basal diet was according to recent published information on the nutrient requirements of southern flounder (Alam et al., 2009, 2011). All ingredients were the same in both diets except α-cellulose was added in the control diet as a filler instead of resveratrol. Resveratrol was supplemented in the test diet at a dose of 600 μg/g of diet. This concentration was chosen because it had been used previously by Valenzano et al. (2006) to demonstrate resveratrol's impacts on longevity in turquoise killifish. Control and resveratrol diets were made at the UNC Wilmington aquaculture facility (Table 1) as described by Alam et al. (2011). Three replicate tanks were assigned to both the treatment and control groups, with 24 fish per tank. Flounder were fed twice a day at 10:00 am and 5:00 pm until satiation and food consumption was recorded for each tank. Fish were considered satiated once aggressive pursuit of food slowed and food remained untouched for several minutes.

Table 1
Composition (g/100 g diets) of diets for control and resveratrol treated fish.

Ingredients	Control diet	Resveratrol diet
	% in diet	% in diet
Solvent-extracted soybean meal [a]	20	20
Menhaden fish meal [b]	37	37
Poultry meal [c]	20	20
Wheat starch	6.94	6.94
Wheat gluten (binder) [d]	4	4
Menhaden fish oil [e]	5	5
Soybean lecithin [f]	1	1
Vitamin premix [g]	3	3
Mineral premix [g]	3	3
Resveratrol [h]	0	0.06
Cellulose	0.06	0
Total	100	100
Proximate composition		
Protein (%)	47	47
Lipid (%)	12	12
Energy (kj/g diet) [i]	16.9	16.9

[a] Southern States, Wallace, NC, USA (solvent extracted, crude protein 47.5%).
[b] Omega protein, Houston, TX, USA (crude protein 60%, lipid 10%).
[c] Mallick Aquiafeed, Wallace, NC, USA (crude protein 60%, lipid 10%).
[d] Sigma-Aldrich, St. Louis, MO, USA (crude protein 80%).
[e] Virginia Prime Silver, Omega Protein, Hammond, LA, USA.
[f] DM, IL,USA.
[g] Tomita Pharmaceutical Company, Kagoshima, Japan as Alam et al. (2011).
[h] Mega Resveratrol, Danbury, CT, USA (certified 99% pure trans-resveratrol).
[i] Calculated based on carbohydrates, proteins and lipids are 17.2, 23.6, and 39.5 kJ g^{-1}, respectively (Blaxter, 1989).

At day 0, three fish from each tank were sampled. Sub-sampling of body mass, total body length and tissues occurred at weeks 0, 8, and 16. Additionally, each week total body length was measured via photography and image analysis (ImageJ, National Institutes of Health, //rsb.info.nih.gov). This method was used to minimize stress throughout the experiment. All photographs were taken 8.5 ft above the floor and each tank had a 15.24 cm piece of PVC for image scaling.

2.3. Tissue collection

At week 16, fish were sampled from each tank (9–11 fish sampled per tank). Before sampling, photographs were taken for analysis. Sampling began prior to that day's routine feeding time to ensure that feeding responses would not confound results. Fish were then chosen at random and quickly transported to a bucket containing tricaine methanesulfonate (MS-222; Argent Laboratories, Redmond, WA, USA) diluted in filtered seawater to 500 mg/l (euthanizing dose). This process was carried out as quickly as possible to minimize stress of the animal. Once euthanized, body mass, total body length and body width were recorded. Scales and skin were removed from a region above the anteroposterior line to avoid contamination from the abdominal cavity. White epaxial muscle was then extracted in rectangular sections parallel to the direction of the fibers. One sample to be used for transmission electron microscopy (TEM) was placed immediately in 2.5% gluteraldehyde in 0.133 M phosphate buffered saline at pH of 7.4 (PBS) and stored at 4 °C for >24 h. Two additional muscle tissue samples to be used for biochemical analysis were immediately placed in cryovials, frozen in liquid nitrogen, and stored at −80 °C.

2.4. Dot blotting and western blotting

A total of 36 muscle samples were randomly selected from the samples that were flash frozen after 16 weeks. Samples were selected so that the mean body masses of control and resveratrol samples used for all biochemical assays were not significantly different from one another (Student's *t*-test, $p > 0.05$). Samples were chosen in this way to ensure that any differences seen in biochemical assays were not due to differences in the size of the fish. Muscle tissue was thoroughly homogenized in 1x RIPA buffer and sonicated on ice for 30 s. Samples were centrifuged at 14,000 rpm (16,000 × *g*) for 20 min at room temperature. Supernatant was transferred to a fresh tube and a standard Bradford assay was used to determine the amounts of protein within each sample.

For dot blotting, PVDF membrane was labeled with pencil and then soaked in 100% methanol for 5 min followed by tris buffered saline with 0.1% Tween (TTBS) for 5 min. Samples were thawed at room temperature, and once the membrane dried completely, samples were spotted on the membrane and allowed to dry for 30 min. The membrane was then blocked for 1 h at room temperature using 5% caseine in TTBS, and incubated overnight at 4 °C in primary antibody diluted in 5% caseine/TTBS. The membrane was then washed in TTBS 3× for 10 min and incubated in secondary antibody conjugated to horseradish peroxidase (HRP) diluted in 5% caseine/TTBS. The membrane was washed in TTBS 3× for 10 min and incubated in ECL detection solution (50% luminol/50% peroxide (Biorad)) for 5 min. Excess ECL was drained off and the membrane was laminated in clear plastic wrap. The membrane was then exposed to X-ray film in a dark room and developed. The electronic image of film was analyzed with ImageJ software.

For western blotting, SDS sample buffer (1X Laemmli) was added to each sample and samples were heated at 100 °C for 5 min. 30 μg of protein/sample were loaded into each gel lane and the gels were run at 100 V for approximately 70 min or until migration completed. PVDF membranes were soaked in 100% methanol for 5 min and then in transfer buffer for 10 min. Fiber pads and filter paper were soaked in transfer buffer for 5 min. The stacking gel was removed and gels were placed in transfer buffer for approximately 5 min. Transfer took 70 min at 400 mA and 4°°C, stirring constantly. Once transfer was completed, PVDF membranes were washed 5 times with TTBS for 5 min/wash. Membranes were incubated overnight in primary antibody in 2%BSA/TTBS Membranes were then washed 8 times in TTBS for 5 min/wash, and incubated in secondary antibody conjugated to horseradish peroxidase (HRP) in 2%BSA/TTBS for 1 h at room temperature. Membranes were washed 8 times in TTBS for 5 min/wash. ECL detection was carried out with BioRad ECL detection kit (peroxide/luminol mixture 1:1). The membrane was laminated with cling wrap and exposed to X-ray film in a dark room for approximately 10 min and analyzed with ImageJ.

2.5. Protein carbonylation and lipid peroxidation

Protein carbonylation and lipid peroxidation were examined to assess the extent of oxidative damage to proteins and lipids, respectively. Protein carbonylation was analyzed using dot blotting methods described above and using a protocol adapted from Robinson et al. (1999). A concentration of 100 mg/ml 2,4-dinitrophenylhydrazine (DNPH, Aldrich Chemistry: 101028457) was used as the first incubation. A primary antibody solution consisting of a 1:2500 dilution of the anti-2,4-dinitrophenol antibody (anti-DNP, Invitrogen Molecular Probes: A11097) and a 1:5000 dilution of the secondary antibody (goat-anti-rabbit, Cell Signaling: 7074) were carried out. 0.1% Coomassie Brilliant Blue R-250 dye was used to label total protein, which was used to normalize the carbonylation dot blots.

Standard Western blotting procedures, as detailed above, were used to assess the amount of 4-hydroxynonenal (4-HNE), a natural byproduct of lipid peroxidation. Lipid peroxides are indicators of oxidative stress and are unstable, breaking down to form reactive compounds such as 4-HNE. A primary antibody (Cell Biolabs: STA-035) with a dilution of 1:1000 and a secondary antibody (goat-anti-rabbit IgG, Cell Signaling: 7074) with a dilution of 1:2000 were used for 4-HNE western blotting. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was labeled with a primary antibody (Cell Signaling: 2118) dilution of 1:2000 and a secondary antibody (goat-anti-rabbit IgG, Cell Signaling: 7074) dilution of 1:2000 and was used to normalize 4-HNE levels.

2.6. Ubiquitinylation and LC3

Ubiquitin and microtubule-associated protein 1A/1B-light chain 3 (LC3) were measured to assess indicators of the amount of protein degradation occurring. Ubiquitin acts as a marker for proteosomal (non-lysosomal) protein degradation. A standard dot blot was used to measure the amount of ubiquitin present within the control and resveratrol groups. A primary antibody (Enzo Life Sciences: BML-PW8810-0100) dilution of 1:500 and a secondary antibody (goat-anti-mouse, Bio Rad Laboratories: 170-5047) dilution of 1:5000 were used for the dot blot. 0.1% Coomassie Brilliant Blue R-250 dye was used to stain the blot for total protein, which was used for normalization. A western blot was conducted to measure LC3 content, which is an indicator of lysosomal protein degradation. This protein associates with the autophagosome membrane and plays a role in autophagy. LC3-II is a post-translationally modified form of LC3-I, and is actively associated with autophagosome formation. A primary antibody (anti-LC3, Novus Biologicals: NB-100-2331) dilution of 1:1000 and a secondary antibody (goat-anti-rabbit IgG, Cell Signaling: 7074) dilution of 1:2000 were used for the Western blot. The average ratio of type I to type II LC3 present muscle tissue of juvenile southern flounder after 16 weeks of treatment with resveratrol and control treatments were analyzed. Type I LC3 appears directly above the band for type II LC3.

2.7. S6K activation

p70S6 kinase (S6K) is activated through the mTOR pathway, and elevation of the active phosphorylated form of S6K (P-S6K) is a commonly used indicator of enhanced rates of protein synthesis. Western blotting

was used to determine the activation state of S6K. A primary antibody (Santa Cruz Biological Technologies: sc11759-R) dilution of 1:1000 and a secondary antibody (goat-anti-rabbit IgG, Cell Signaling: 7074) dilution of 1:2000 were used for the Western blot, and GAPDH was used as a normalization standard as described above.

2.8. Mitochondrial volume density

Since resveratrol may activate pathways that lead to mitochondrial proliferation and therefore enhance ROS production, transmission electron microscopy (TEM) was used to determine mitochondrial volume density of muscle tissues. 12 samples were chosen at random and prepared for TEM. Samples were first washed in 200 mM PBS, and then placed in secondary fixative (1% osmium tetroxide, 0.8% potassium ferricyanide, 0.2 M cacodylate buffer, pH 7.40). Samples were rinsed in PBS as well as distilled water, and then dehydrated using an ascending series of ethanol (50%, 70%, 95%, 100%, 100%). A 1:1 mixture of Spurr epoxy resin and ethanol was used to infiltrate samples for 1 h, after which they were embedded in 100% Spurr's epoxy resin and rotated overnight. Tissues were then removed from resin and placed into aluminum weigh boats, fresh 100% Spurr epoxy resin was added and samples were baked at 70 °C for 8 h. After removal from the oven, the blocks were trimmed and sectioned using an UltraCutE ultramicrotome (Reichert Jung). Sections were collected at a thickness of 90 nm on 200 mesh copper grids coated in 0.25% formvar dissolved in ethylene dichloride. A systematic random sampling method was used where one micron of tissue was skipped between each section that was collected to ensure new regions of mitochondria were sampled, as we have described previously (Hardy et al., 2009). Grids were then stained with uranyl acetate (2% solution mixed in 50% ethanol with 1:10 glacial acetic acid) and Reynold's lead citrate. Images were collected as montages with individual images at 4800× magnification. Images were analyzed using Image Pro Plus software (Media Cybernetics Inc. 2012) using point-counting stereology on a total of 15 images per sample (3 images per montage, 1 montage per grid, total of 5 grids used per sample). Thus, each sample had 5 sections of 90 nm thickness, spaced 1 μm apart, which is adequate to capture variation in mitochondrial density that occurs on the scale of sarcomere length (approximately 2 μm), and allows determination of mitochondrial volume density (Hardy et al., 2009).

3. Statistics

Body mass was estimated for the entire sampling period from length data using a 3rd order polynomial fit. Flounder feeding and growth data were analyzed in JMP 10.0 (SAS Institute Inc. 2014) using a random effect nested ANOVA design with tanks nested within the treatments with a univariate repeated measures specification. Student's t-tests were used to compare the control and treatment groups for all of the western and dot blot data, as well as for mitochondrial volume density data. All data are presented as means ± SEM.

4. Results

4.1. Diets and growth

Resveratrol had an effect on growth but not feed consumption during the 16 week experimental period (Fig. 1). The inset in Fig. 1a represents the relationship between body mass and total body length, and the data were derived from the 3 subsampling time points where both variables were measured (see Materials and methods section). A 3rd order polynomial was fit to these data and used to predict body mass for all of the fish using the total length data that was collected weekly through image analysis. The data for both the control and resveratrol fish were combined here, since there was no significant interaction of treatment and total length on body mass (DF: 1, F ratio: 3.33, $p = 0.07$). As expected, there was a significant effect of week (time) on both total length (DF: 1, F ratio: 27,985.80, $p < 0.0001$) and predicted body mass (DF: 1, F ratio: 20,945.48, $p < 0.0001$). There was also a significant interaction of treatment group and time on both total length (DF: 1, F ratio: 606.13, $p < 0.0001$) and predicted body mass (DF: 1, F ratio: 712.44, $p < 0.0001$), where the resveratrol treatment group grew larger than the control group over the course of 16 weeks (Fig. 1a). However, the differences in growth were not due to differences in food intake, as there was no significant interaction of treatment and time whether the data were normalized to total length (DF: 1, F: 2.651 $p = 0.10$) or predicted body mass (Fig. 1b; DF: 1, F ratio: 3.070, $p = 0.080$).

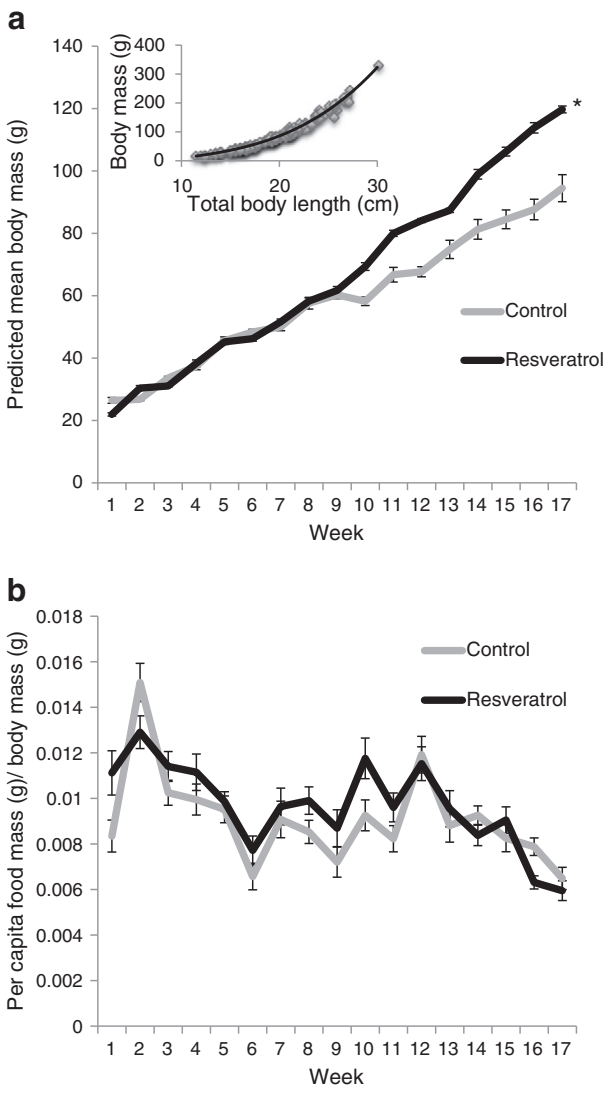

Fig. 1. a: Predicted body mass for both the resveratrol and control groups. Body mass was estimated based on its dependence on total length using a 3rd order polynomial fit where $BM = -161.67 + 12.37 \times \text{total body length} + 0.76 \times (\text{total body length} - 19.36)^2 + 0.02 \times (\text{total body length} - 19.36)^3$ ($r^2 = 0.982$; $p < 0.0001$; see inset). There was an effect of week on body mass, and an interaction of week and treatment on body mass (*) (This interaction term was also significant for total body length; data not shown) (see Results). a: Per capita food mass consumed per predicted body mass, for both the control and resveratrol groups. Due to subsampling initially and at 8 weeks, the N values changed during the experiment. Each of the 6 tanks (3 control, 3 resveratrol) had 24 fish for the initial measurements, 21 fish for the first 8 weeks, and 18 fish for the final 8 weeks.

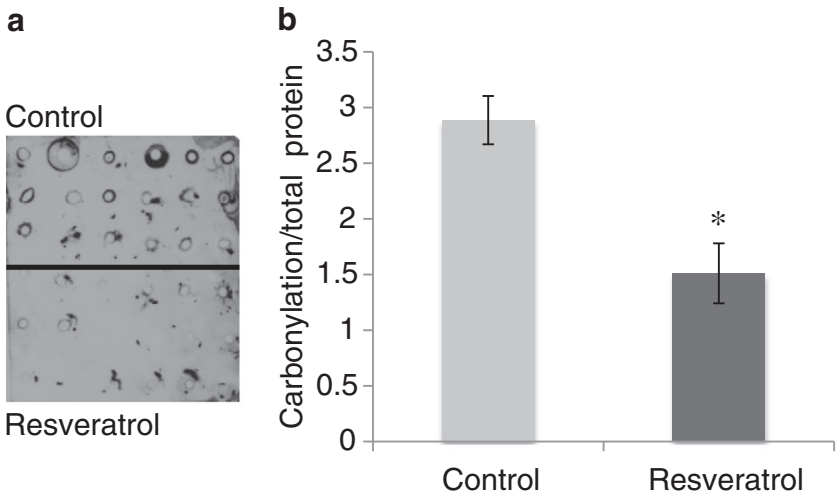

Fig. 2. a: Dot blot of protein carbonylation for control and resveratrol groups of juvenile southern flounder muscle tissue after 16 weeks of treatment. b: Mean protein carbonylation in muscle tissue of juvenile southern flounder after 16 weeks of treatment. The asterisk indicates that the resveratrol treatment (n = 18) was significantly lower than the control treatment (n = 18) (see Results section).

4.2. Protein carbonylation and lipid peroxidation

There was significantly less protein carbonylation within the muscle tissue of the resveratrol treatment than within the control at the end of the 16-week experimental treatment (Fig. 2, Student's *t*-test, $p < 0.0001$). Additionally, there was significantly less 4-HNE present within the muscle tissue of the resveratrol treatment than within the control at 16 weeks (Fig. 3, $p < 0.0008$). These results are consistent with reduced oxidative damage to proteins and lipids, respectively, in muscle tissue from the resveratrol treatment group.

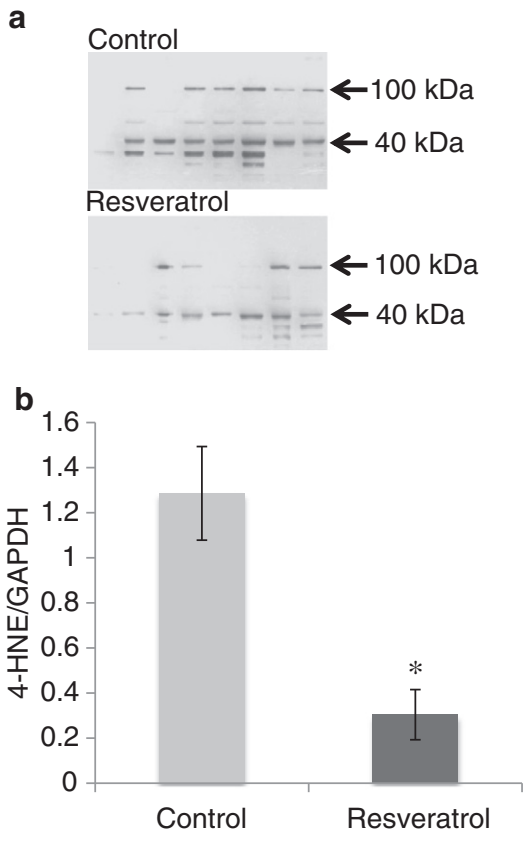

Fig. 3. a: Image of western blot conducted for control and resveratrol treatments of muscle tissue after 16 weeks of experiment. b: Average 4-HNE present in muscle tissue of juvenile southern flounder in resveratrol (n = 8) and control treatments (n = 8). The asterisk indicates that the resveratrol treatment is significantly lower than the control treatment (see Results section).

4.3. Ubiquitinylation and LC3

As expected, there was significantly less ubiquitin within the resveratrol treatment than within the control group for muscle tissue samples collected at the 16 week time point (Fig. 4, $p < 0.0001$). However, there was no difference in the ratio of type I to type II LC3 between the two treatments in muscle tissue (Fig. 5, $p = 0.48$). These results suggest that non-lysosomal, but not lysosomal, protein degradation is reduced in muscle from the resveratrol treated group.

4.4. S6K activation

There was no difference in P-S6K levels between the resveratrol and control treatments (Fig. 6, $p = 0.10$) in muscle tissue samples. This suggests that while non-lysosomal protein degradation appears to be reduced in the resveratrol treated groups, protein synthesis was unaffected.

4.5. Mitochondrial volume density

There was no significant difference in muscle mitochondrial volume density between controls and resveratrol treatments after 16 weeks (Fig. 7, $p = 0.19$). Thus, the capacity for mitochondrial ROS production appears to be unaffected by resveratrol treatment.

5. Discussion

Resveratrol has many demonstrated antioxidant effects in mammals, but there is relatively little known about its impacts in fishes. This study showed that resveratrol appears to exert antioxidant effects in muscle, and likely other tissues, when fed to juvenile southern flounder, leading to reduced protein and lipid oxidative damage, less protein degradation and enhanced growth.

Prior studies have examined the effects of antioxidants on growth in fishes to assess the practical application of these supplements in aquaculture. Antioxidants such as vitamins E, C, and D, astaxanthin, iron, copper, manganese, and ascorbic acid have all been the focus of recent studies (Hamre et al., 2004; Darias et al., 2011; Gao et al., 2012).

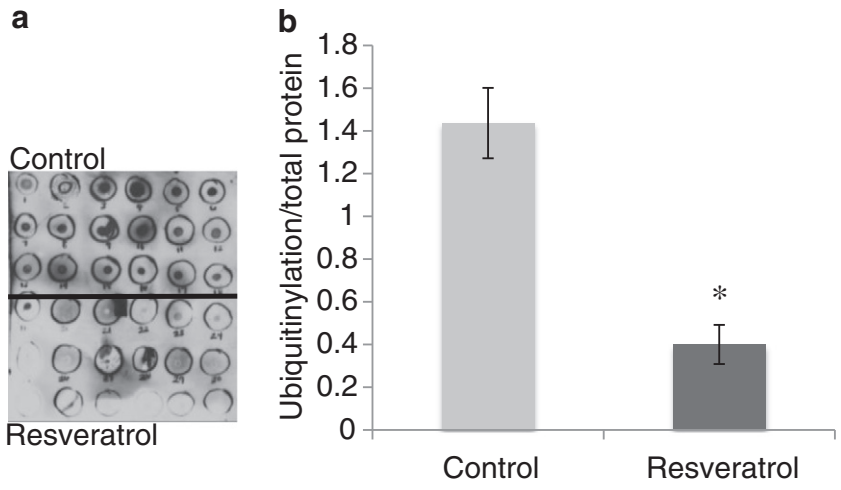

Fig. 4. a: Image of dot blot conducted for control and resveratrol treatments of muscle tissue after 16 weeks of experiment. b: Mean ubiquitinylated protein present in muscle tissue of juvenile southern flounder in resveratrol (n = 18) and control treatments (n = 18). The asterisk indicates that the resveratrol treatment is significantly lower than the control treatment (see Results section).

Hamre et al. (2004) found that variation in micronutrients such as iron, copper and manganese, had minor effects on growth in Atlantic salmon, although vitamin E was found to protect against iron ascorbate- stimulated oxidation (Hamre et al., 2004). Gao et al. (2012) found that juvenile red sea bream fed vitamin E grew significantly larger than fish without vitamin E in the diet. Additionally, dietary vitamin E led to a lower thiobarbituric acid reactive substances value (TBARS), which is an indicator of lipid peroxidation (Gao et al., 2012), and vitamin E also prevented lipid peroxidation within liver and muscle tissue of juvenile turbot, halibut and sea bream (Tocher et al., 2002). Dietary vitamin C supplementation has also been found to enhance growth in fishes such as yellow perch (Lee and Dabrowski, 2004), sea bass (Phromkunthong et al., 1997), and parrotfish (Wang et al., 2003), although there was no effect on growth in juvenile angelfish (Blom and Dabrowski, 2000). These studies and the present work indicate that antioxidant supplementation in fishes has the potential to enhance growth, but the effects are species specific and dose dependent (Darias et al., 2011). This view is also consistent with studies that examined the effects of oxidized products within the diet of fishes. Dietary supplementation of oxidized fish oil has been shown to increase oxidative stress, leading to decreased growth of fish such as juvenile red sea bream (Gao et al., 2012), whereas supplementation of vitamin E in conjunction with oxidized fish oil has been shown to offset some of these effects and improve the health of some species (Gao et al., 2012).

A current emphasis in fish aquaculture is the development of more affordable, plant-based dietary protein supplements, such as sweet

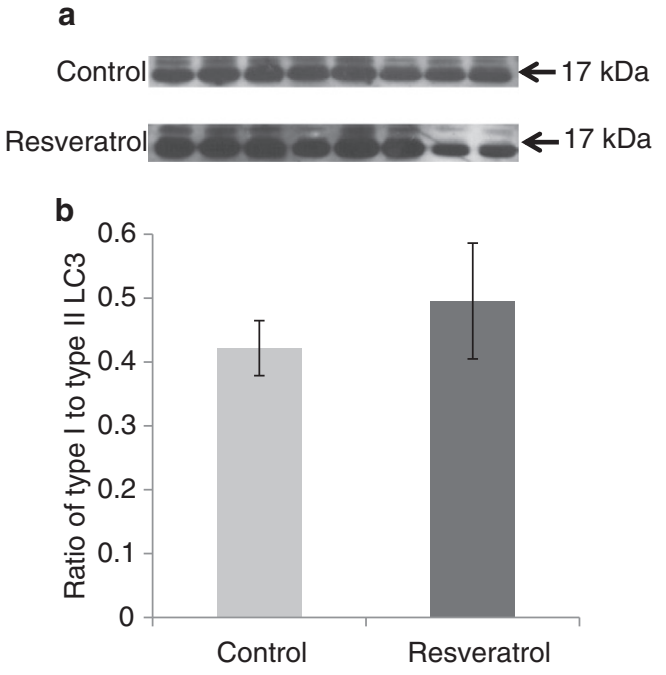

Fig. 5. a: Images of western blots conducted for control and resveratrol treatments of muscle tissue after 16 weeks of experiment. The less dense top band in each blot is LC3 I and the more intense bottom band is LC3 II. b: Mean ratio of type I to type II LC3 present in muscle tissue of juvenile southern flounder in resveratrol (n = 8) and control treatments (n = 8) (see Results section).

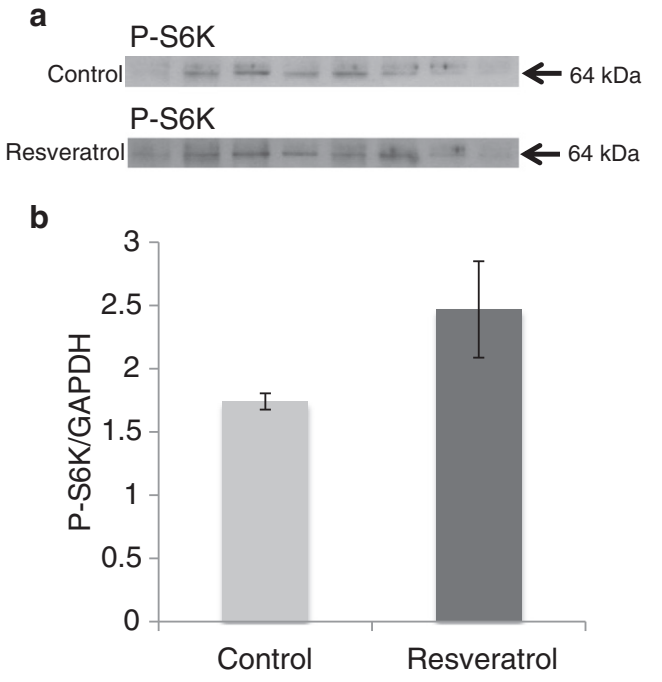

Fig. 6. a: Images of western blots conducted for control and resveratrol treatments of muscle tissue after 16 weeks of experiment probed for P-S6K. P-S6K data were normalized to GAPDH. b: Mean P-S6K present in muscle tissue of juvenile southern flounder in resveratrol (n = 8) and control treatments (n = 8) were not significantly different (see Results section).

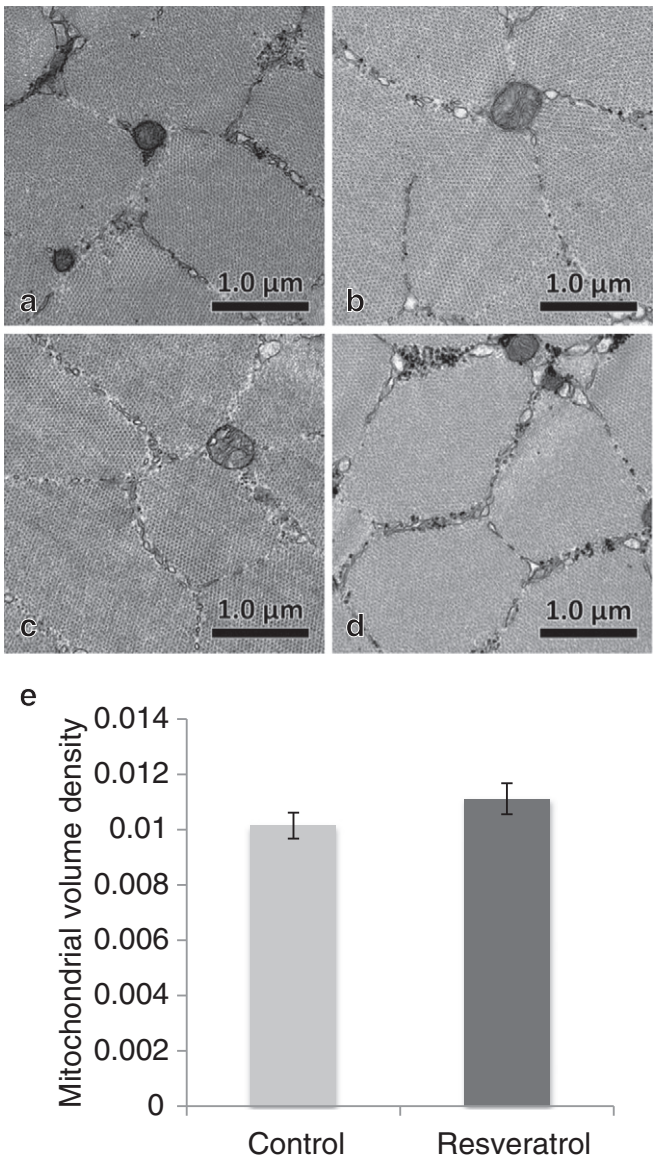

Fig. 7. TEM images of muscle tissue samples for both control (a, c) and resveratrol (b, d) treatments after 16 weeks. Mitochondria are the circular, darkly stained organelles. e: Mitochondrial volume density for control and resveratrol treatments in muscle tissue of juvenile southern flounder were not significantly different (see Results section).

potato leaf meal or soybean meal, which also contain polyphenols that may lead to enhanced antioxidant activity (Alam et al., 2011; Lochmann et al., 2013). However, Lochmann et al. (2013) studied the effects of dietary sweet potato leaf meal on juvenile channel catfish and found that there were no significant effects on weight gain, feed conversion ratio or survival. In this case, the polyphenol content of the control and treatment diets were similar and there may not have been a large enough difference to elicit significant effects (Lochmann et al., 2013). In a study of rainbow trout, a dietary supplement of carotenoids from vegetable crops enhanced specific growth rate and weight gain of rainbow trout (Sheikhzadeh, 2013). Carotenoids are organic pigments produced by plants that can act as antioxidants and function in cell growth regulation, modulation of gene expression, and immune response (Sheikhzadeh, 2013). Carotenoids have been found to scavenge ROS and protect against oxidative damage, increasing the proliferation of cells and lysozyme levels (Amar et al., 2001; Sheikhzadeh, 2013). Therefore, there is evidence that antioxidants naturally present in plants may offer additional health and growth benefits in fishes.

In the current study, the resveratrol treatment led to significantly less carbonylation at the end of the 16 week trial indicating decreased ROS induced oxidation of proteins in the white epaxial muscle. In muscle of cod, oxidative stress also led to higher levels of protein carbonylation, specifically of structural proteins (actin and myosin), and fish muscle appears to be highly susceptible to protein carbonylation (Pazos et al., 2011). Hegazi et al. (2010) found that in juvenile Nile tilapia liver and white muscle tissues, chronic exposure to ammonia led to greater protein carbonylation, and this treatment has been shown to reduce growth rate (El-Shafai et al., 2004; Hegazi and Hasanein, 2010; Hegazi et al., 2010). These results are consistent with the findings from the present study that higher protein oxidative stress in tissues from fishes causes an increase in carbonylation and slower growth. Data from non-fish model organisms indicate, like the present study, that protein carbonylation leads to enhanced protein degradation, and antioxidants can offset this effect (Dargelos et al., 2009; Baraibar et al., 2011). For instance, Pandey and Rizvi (2011) found that resveratrol protects against protein carbonylation (and lipid peroxidation) in human erythrocytes. Olesen et al. (2013), also found that resveratrol leads to lower levels of protein carbonylation in mouse skeletal muscle. Additionally, more indicators of protein degradation were found in diabetic rat bladder tissue exhibiting high oxidative stress (Kanika et al., 2011).

Lipids are also highly susceptible to oxidation and damage from ROS. Fish tissues and diets typically contain high levels of polyunsaturated fatty acids that are vulnerable to lipid peroxidation (Tocher et al., 2002; Sargent et al., 1999). The present study found lower levels of 4-HNE, a bi-product of lipid peroxidation, indicating that resveratrol decreased the amount of lipid peroxidation in the muscle tissue. Tocher et al. (2002) found that antioxidant supplementation, in the form of vitamin E, also decreased the lipid peroxidation within juvenile turbot, halibut and sea bream. Additionally, Chen et al. (2012) found that dietary supplementation of oxidized fish oil enhanced lipid peroxidation and this effect could be mitigated by vitamin E and Se supplementation in juvenile largemouth bass. In young grass carp, dietary copper supplementation was found to increase growth, digestion, and absorption ability while decreasing lipid peroxidation and protein oxidation (Tang et al., 2013). This effect was due in part to enhanced antioxidant defense in the hepatopancreas and intestine (Tang et al., 2013). Juvenile grouper fed diets low in copper exhibit higher levels of hepatic lipid peroxidation (Lin et al., 2008; Tang et al., 2013). Findings from mammalian studies have also demonstrated that resveratrol protects against lipid peroxidation and DNA damage caused by ROS (Leonard et al., 2003; Marchal et al., 2013). Thus, the lower levels of both protein carbonylation and lipid peroxidation within the resveratrol treatment of the present study, as well as data from other studies, provide strong evidence that dietary resveratrol supplementation limits oxidative damage in fish muscle.

In resveratrol fed fishes, there was no difference in LC3, a marker of lysosomal degradation, although ubiquitin, a marker of non-lysosomal degradation was significantly reduced. While it is generally accepted that lysosomal degradation is the primary mechanism by which most cells enhance protein degradation, in muscle this is not the case (Furuno and Goldberg, 1986; Furuno et al., 1990; Medina et al., 1995; Taillandier et al., 1996). Myofibrillar proteins typically comprise about 60% of the muscle protein and myofibrillar components are largely degraded by non-lysosomal processes, while lysosomal degradation accounts for much of the non-myofibrillar protein (Lowell et al., 1986). Additionally, in denervation (Furuno et al., 1990; Medina et al., 1995), starvation (Medina et al., 1995), disuse (Taillandier et al., 1996), Ca^{2+} and injury related atrophy (Furuno and Goldberg, 1986), the ubiquitin-proteosomal system has been found to account for the majority of the protein degradation. Thus, the ubiquitin-proteosomal system is the most active in skeletal muscle and would likely be the most sensitive to stress or environmental conditions. The reduced ubiquitinylation and lack of change in LC3 observed with resveratrol

supplementation in the present study are therefore consistent with this view, as is the increase in growth.

To our knowledge, there is only one other study that examined the effect of resveratrol in a fish species (Valenzano et al., 2006). These authors found that resveratrol supplementation in turquoise killifish extended lifespan, but had no effect on growth, although growth measurement was not the primary objective of the study. However the turquoise killifish is a short-lived species (9 weeks), and treatment occurred over the lifetime of the animal. Thus, the animals may have reached the same maximal body size because net growth had slowed or stopped near the end of life. In contrast, Southern flounder have a predicted maximum lifespan of 20 years (Reagan and Wingo, 1985), and enhancing growth during the juvenile stages as demonstrated in the present study has the greatest application to aquaculture.

In summary, the present study found that resveratrol enhanced growth in juvenile Southern flounder, and this presumably arises from its antioxidant protective effects that limit protein and lipid damage, leading to reduced protein (and possibly lipid) turnover. As a naturally occurring compound, resveratrol therefore has the potential for application to finfish aquaculture.

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